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The Search for Modifiers of the Maze Gametophyte Factor Ga1-s and Quantitative Trait Polymorphisms Emerging from Doubled-Haploid Maize Lines

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THE SEARCH FOR MODIFIERS OF THE MAIZE GAMETOPHYTE FACTOR *Ga1-s*

AND

QUANTITATIVE TRAIT POLYMORPHISMS EMERGING FROM DOUBLED-HAPLOID MAIZE LINES

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree in Biological Sciences specialization in Biology and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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The project was designed to conduct two independent projects. The first project was to identify the genomic localization of the modifiers of the maize gametophyte factor (*Ga1-s*) and the second project was to establish and identify heritable polymorphic lines that have descended from a single doubled-haploid B73 plant. The objectives were (1) to search for modifier genes and loci on the maize chromosomes for the trait and determine genetic effects of them using QTL mapping; (2) to demonstrate the heritable polymorphism of the quantitative traits emerged from the descendants of a single doubled-haploid maize plant.

Regarding the QTL mapping of the modifiers of the maize gametophyte factor; two maize lines had been identified that are polymorphic to B73 relative to *Ga1-s* resistance to *ga1* pollen: Ky21 shows less resistance and M162w shows greater resistance. The recombinant inbred lines (RILs) of both Ky21 and M162w have already been developed and genotyped for both of these with B73. 200 RILs each of Ky21 and M162w were sown in summer of 2014 and crossed with pollen from plants homozygous for *Ga1-s*. The strength of *Ga1-s* in each F1 line was evaluated by pollinating with *Rscm2*

ga1 pollen the first day and allowing open pollination on the second day. A strong effect of *Ga1-s* is indicated if the resulting ear has few or no blue kernels and a weak effect is ear being heavily contaminated with blue kernels. A standardized scale of contamination was established to score ears. Composite interval mapping method was conducted for the QTL analysis. RILs (B73 X Ky21) show QTLs on chromosome 1S and 4S while RILs (B73 and M162w) shows QTLs on 5L and 10L.

On doubled-haploid maize, the source material was provided by James A. Birchler, University of Missouri. One kernel from this ear was used as a source germplasm for this project and was designated as S0. The diploid progeny resulting from self-pollination of the S0 plant was designated as S1. Similarly, one diploid progeny from self-pollination of an S1 plant was designated as S2 and so on to the S3 generation. From the S3 generation, ten random seeds from a uniform good-looking ear were selected to become the source of separate descent lineages. Each lineage was maintained through selfing and one progeny was selected for advancement to the next generation. In the summer of 2014, we planted the seed for two sequential generations from each of ten lineages. These were planted in triplicate in a randomized complete block design. The resulting plants were evaluated for 15 quantitative traits (plant height, number of tassel branches, 100 grains weight, etc.). A partial replication of experiment of 2014 was conducted in 2015. A heritable polymorphism for any particular trait is indicated if there is no significant difference between the two generations of a lineage but the lineage is significantly different from other lineages. Number of tassel branches, total number of kernels per ear, days to pollen shed and days to silk emergence has demonstrated heritable polymorphism.

Chapter 1 General Introduction and Literature Review (*Ga1-s* Project)

Thesis Organization

This thesis includes two independent projects. The chapters are written in a manuscript format to submit for publication. Chapter one is a general introduction and literature review on maize gametophyte factor (*Ga1-s*). Chapter two elaborates the genomic localization of the modifiers of the *Ga1-s* factor and Chapter three is the general introduction and literature review on quantitative trait polymorphism emerged from doubled-haploid maize lines. Chapter four describes the process of re-establishing of the heritable polymorphic lines that descend from a single double-haploid plant. The last chapter is a summary and the future prospects of both research projects.

General Introduction and Literature Review

Importance, Evolution and Genome structure

Maize is a monoecious plant that belongs to family Poaceae. It is the most widely grown grain crop in the United States covering a total of over 35 million hectares (FAO, 2015). Maize is a major caloric source for humans and farm animals and has grown in popularity as a fuel source. Maize is a member of the grass family, Poaceae. Its wild ancestor is a grass called teosinte (*Zea mays* ssp. *parviglumis*), grows in several areas of Mexico and Central America. Early isozyme studies indicate that central Balas teosinte is the closest form to the progenitor of maize (Doebley, 1990). Further, the molecular advancements of the previous decade have shown significant evidence that teosinte is the progenitor of maize (Wilkes, 2004). Teosinte and maize are able to cross-breed to form maize-teosinte hybrids that are fully fertile. It is widely understood that maize is a

domesticated form of teosinte and their morphological differences were the result of human selection (Doebley, 1990).

Maize genome is diploid and consists of ten chromosomes. The genome size ranges from 2.3 to 2.7 GB. This is similar to the human genome size and is considered as intermediate in size among the grass family crops (Schnable et al., 2009). The maize genome consists highly of non-genic, repetitive low-copy DNA which harbors genes or small groups of genes (Llaca et al., 2011). The genome of maize has undergone many rounds of duplication (Schnable et al., 2009). A duplication event about 5 to 12 million years ago distinguishes maize from its close relative, *Sorghum bicolor* (Schnable et *al.*, 2009*)*.

Other mechanisms that have had an effect on the evolution of the maize genome are DNA transposition and retrotransposition, capture and translocation of gene segments or genes by transposons, recombination, and gene conversion events, and single base mutations and expansion/contraction of simple sequence repeats (Llaca et al., 2011). The genome of inbred line B73 was sequenced in 2009 (Schnable et al., 2009).

Maize Biology and Fertilization

Similar to another angiosperm, maize follows alternation of generations: sporophytic and gametophytic generations. Sporophytic generation consists of diploid phase. The sporophyte is diploid whereas the gametophyte is haploid. The male sporophyte is tassel and the female sporophyte is the silk whereas the male gametophyte is the pollen grain and the female gametophyte is the embryo sac.

Maize, being a monoecious plant, develops both male and female flowers in physically separated parts of the plant. The staminate flowers arise from the shoot apical meristem on a structure called a tassel, whereas, the pistillate flowers originate from the axillary bud apices that develop into ears. The tassel bears the male inflorescence that produces anthers that produce pollen grains. It is located at the apex of the main stem. It consists of a central spike (rachis) and about 10-50 lateral branches. Within each male flower spikelet, there are usually two functional florets. Each floret contains a pair of lemma and palea, three anthers, two lodicules and rudimentary pistil. Pollen grains per anther have been reported to range from 2000 to 7500 (Kiesselbach, 1999) .

The ear bears the female inflorescence. One or several axillary buds terminate in an ear that produces mature kernels. Each of these axillary buds is covered with about 8-14 modified leaves called husks, and a prophyll. The ear branch, or shank, consists of several nodes and short internodes. The ear does not have lateral branches but has thick axis called the cob, similar to the central spike in the tassel in that it produces multiple rows of paired spikelet. The silk emerging from each ovary are the elongated stigmas. Maize is generally protandrous, i.e. the male flower matures earlier than the female flowers.

Gametogenesis and Fertilization

The embryo sac is the gametophyte within each female flower. The single megasporocyte in the ovule undergoes meiosis resulting in the production of four megaspores, three of which degenerate. The megaspore undergoes three successive mitotic divisions to produce an embryo sac containing eight haploid nuclei (Maheshwari, 1950).

In microsporogenesis, the microspore mother cell undergoes two successive meiotic division and produce four microspores. Each microspore undergoes a mitotic cell division resulting the formation of generative and tube nucleus. The generative nucleus further undergoes the mitotic division to produce two sperm cells (Bedinger and Russell, 1994).

Fertilization occurs between 16 and 24 hours after pollination, depending on temperature and silk length. The pollen shed is not a continuous process in maize. It generally begins two to three days prior to silk emergence and lasts for five to eight days. Pollen shed stops when the tassel is too wet or too dry and begins again when temperature conditions are favorable. The silks consist of fine sticky hairs that anchor the pollen grains. Under suitable conditions, pollen grain remains viable for only 18 to 24 hours. Cool temperatures and high humidity favor pollen longevity. The pollen grains from the anther are carried by wind or insects and when landed on stigmas of female i.e. silk, germination takes place.

Fusion of one sperm cell with the egg cell results in the formation of the diploid zygote. Fusion of the other sperm cell with the two polar nuclei in the central cell results in the formation of triploid endosperm cell (Randolph, 1936) and eventually results in the formation of the kernel. Kernels in maize ear are the seeds of the maize.

Maize gametophyte factor (*ga1*)

Maize is a cross-pollinated crop and wind is an important factor. Organic corn needs to be certified and should be able to provide sufficient evidence that the grain is free from the genetic modifications. Organic certification is difficult to achieve if a genetically modified (GM) corn is nearby. Temporal and spatial strategy have employed

in the past to avoid the contamination. A study by (Halsey et al., 2005) suggests that a distance of at least 750 m, as well as temporal separation of at least two weeks, is good enough for avoiding the contamination. However, it is always not easy to achieve temporal and spatial isolation. A gene-based incompatibility will be more effective and efficient to avoid the contamination and the gametophyte factor (*Ga1*) offers a solution.

Fertilization is achieved through the interaction between the male gametophyte (pollen) and the female sporophyte (silk). There are three known alleles of *ga1*:

ga1 (Cross-fertile): This allele is recessive and accepts pollen from all allele types but cannot pollinate *Ga1-s* silk. The recessive *ga1* lacks pollen-blocking ability in female (Mangelsdorf and Jones, 1926, Nelson Jr, 1952).

Ga1-s (Cross Incompatible): is partially dominant or semi-dominant and accepts *Ga1-s* and *Ga1-m* pollen but not *ga1* (Demerec, 1929, Schwartz, 1950).

Ga1-m (Cross neutral): is dominant and able to pollinate both *ga1* and *Ga1-s* and also accepts the pollen from both alleles (Mangelsdorf and Jones, 1926, Nelson Jr, 1952).

Most of the North American maize lines lacks *Ga1-s* and hence can be used for reproduction isolation between organic and GM maize (Nelson Jr, 1952). *Ga1-s*, (s is strong allele) when homozygous, is strong and completely blocks the *ga1* pollen. However, heterozygous *Ga1-s/ga1-s* is incomplete in blocking the *ga1* pollen and therefore the gene action may be additive or incomplete dominant (Kermicle and Evans, 2005).

Gametophyte factor: From Past to the Present

The discovery of the gametophyte factor was due to segregation distortion of few of the genes which are linked to the gametophyte factor (Emerson, 1934). Sugary 1 (*su1*) in the short arm of chromosome four, the recessive allele of *su1* is characterized by translucent and wrinkled endosperm when dry (Neuffer et al., 1997).

Cross of inbred lines with sugary and starchy endosperm (*su1/su1 x Su1/Su1*) normally segregate 25% sugary in the F_2. Correns and Correns (1924) observed 16% sugary kernels in an F_2 of a cross between sugary x White Rice popcorn. Further, the reciprocal backcrosses of F_1 with the parental line carrying sugary gene segregate 50% sugary whereas reciprocal backcrosses with the starchy parent did not segregate. The results of this study were further confirmed by Jones (1924). He reported that when certain varieties of popcorn (*Zea mays everta*) type are crossed with sweet corn, a deficiency in the number of recessive *su1* segregates is commonly observed in F2 generation. In the study, F1s from a cross between Rice Popcorn having *Ga1-m* and a hybrid sweet corn was developed. When these F1s were used as male parents to backcross to Rice Popcorn as female, unexpected segregation ratios were observed. He confirmed that plants having the dominant *Su1,* inherited from the Rice Popcorn, and had a better compatibility to those also with *Su1* compared to the compatibility of *su1* with *Su1*. Jones (1924) stated that pollen carrying the dominant factor is better able to accomplish fertilization than the pollen carrying its recessive allelomorph. The findings were hypothesized as a result of differential pollen-tube growth rate where gametes with the *Su1* allele are more competitive than gametes with the *su1* allele in silks of genotype *Su1/_*.

A study by Mangelsdorf and Jones (1926) observed the distorted segregation ratios in the inheritance of the defective kernel gene *de1*. Defective seeds may be described as lethal (*de1*) characters in which the endosperm and embryo are greatly reduced in size or almost completely lacking. In contrast with F_2 of sugary x White Rice where a deficiency of sugary seeds was observed, the cross *De/De x de/de* resulted in excess of defective in F_2 generation. They reported that an excess of the defective kernels was due to the differential pollen tube growth caused by *Ga* (later found to be *Ga1-m*), a gene linked to *de1*. This gives *Ga1-m* a competitive advantage in pollination success over *ga1*. They also put forward their views on the study done by Jones in 1924 that the distorted segregation ratios were probably the result of linked *Ga1* with *Su1* rather than *Su1* itself.

Further investigation on the *Ga1* was carried out. Demerec (1929) studied about the reciprocal cross between the white Rice popcorn and non-popcorn cultivar. When Rice popcorn was used as the female, none of the crosses were successful, however, when used as a male, seed set was perfect. There was a complete exclusion of the non-popcorn pollen on white Rice popcorn silks. These results differ from Jones (1924) and Mangelsdorf and Jones (1926) where they did not observe the complete exclusion but rather selective fertilization. Demerec (1929) also divides the silks of Ga carrying ears and pollinated those of one side with Ga and those of the other side with ga pollen and concluded that differential fertilization is due to the cross-sterility of ga pollen on Ga silks.

Emerson (1934) reported that the abnormal segregation of sugary in F2 of *Ga1 Su1/ga1 su1* and defective kernels in F_2 of *Ga1 de/ga1 De* is due to linkage of *Ga1* with

Su1 and *de*, respectively. Later on, it was concluded that Demerec and Mangelsdorf actually had seen different pollination behaviors due to the allelic difference between *Ga1-s* and *Ga1-m*.

Schwartz (1950) proposed a new allele type at the *ga1* locus, $Ga1^s$. It was actually found by Dr. M. M. Rhoades who gave it over to Schwartz for further analysis. Based on the linkage with the *su1* gene, he reported this as an allele of the *ga1* locus rather than a separate gene. He studied the three allele types *ga1*, *Ga1* (currently *Ga1-m*) and $Ga1^s$ (currently *Ga1-s*). He was the first one to cite their interaction on pollination. His study showed that *ga1*was unable to pollinate on silks of homozygous $Ga1^s$ but slightly successful on pollinating heterozygous *Ga1s* silks. *Ga1* and $Ga1^s$ could successfully pollinate the silks of all three allele types. He concluded that the cross sterility was the result of the lack of ability of the *ga1* pollen tubes to fertilize the embryo sac of the *Ga1-s* silks. His studies reported that when the female parent is homozygous recessive for *ga1*, *ga1* pollen can compete successfully against *Ga1* and able to fertilize half of the ovule. However, if the female plant is heterozygous or homozygous for *Ga1*, the *ga1* pollen becomes poor competitor and achieves fertilization in only 0-4 % of the ovules.

Nelson Jr (1952) found the same three allele's types of *ga1* locus as found by Schwartz (1950). His study showed that the majority of the popcorn inbred possessed *Ga1-s*. White Rice popcorn studied by Jones and Mangelsdorf in 1926 was the only cross neutral popcorn consisting of *Ga1-m*. Nelson also determined that most of the North American field corn do not possess either *Ga1-s* or *Ga1-m*, but rather *ga1*. Hence, *Ga1-s*

can be used as a genetic barrier in the field or organic corn to avoid unwanted pollination. This will definitely help in reproductive isolation of two corn cultivars.

Whiteley et al. (1957) used backcrossing of *Ga1-s* into popcorn inbred in order to minimize the cross pollination from the field corn. He found that some popcorn fail to set seed when pollinated by other popcorns or by dent corn, however, they are cross-compatible when used as pollen parents. His hypothesis was that upon transferring the genetic factors for the cross-incompatibility with dent corn, to compatible popcorn inbreds through successive backcrossing, the problem of dent contamination can be overcome.

Kermicle and Evans (2005) showed that *Ga1-s* allele controls nonreciprocal crossability with *ga1* allele by means of allele-specific congruence rather than active rejection. They use the hetero-allele pollen *Ga1-s/ga1* as a male parent to pollinate *Ga1-s/ Ga1-s* and *ga1/ga1* silks. The active rejection indicates that the hetero-allele pollen will not be able to fertilize and will not be accepted. However, if matching allele is required for the cross to be successful, pollen would be accepted and in congruence with respect to the matching allele. They observed that all plants were successfully crossed with hetero-allele pollen suggesting the requirement of matching allele in both pollen and pistil (specific congruence) rather than active rejection.

Introgression of *Ga1-s* to different genetic backgrounds and the variable expression of the *Ga1-s* in different genetic backgrounds led the researcher to understand the *Ga1* in various genotypes and environment. Ashman (1975) noticed the variable strength of *Ga1-s* when it was introgressed to the different genetic background. He reported that this variability might be due to the different genetic backgrounds and

indicated the possibility of modifiers genes. His ideas were verified by González et al. (2012) in studying Genotype x Environment (GxE) effects.

There are a few recent investigations on the mapping of the *Ga1-s* factor. Zhang et al. (2012) fine mapped the *Ga1-s* locus to a 1.5 cM region of the short arm of chromosome 4 and also developed eight tightly linked markers. Bloom (2012) also mapped this region and their study was consistent with Zhang et al (2012), with the interval containing *ga1* among their study overlapped in a 2.2 Mbp interval on chromosome 4 which contains 13 predicted genes in the B73 reference sequence.

Pollen-Pistil Interaction:

Lausser et al. (2009) studied the sporophytic control of pollen tube growth and guidance in maize. He studied the inter- and intra-specific crossing barriers in maize and its close relative *Tripsacum dactyloides* and described progamic pollen tube development in maize. *T. dactyloides* pollen shows high relative germination efficiencies on both self and alien species, whereas germination efficiency of maize pollen is reduced on *T. dactyloides* silks. When *Ga1-s/Ga1-s* silks were pollinated with pollen from maize genotype *ga1/ga1* as well as pollen from *T. dactyloides, the* growth of pollen tubes were arrested within the first 0 to 4 cm of the pollination site. Pollen tube of *ga1* stopped their growth more frequently and after a shorter distance than pollen tube of *T. dactyloides.* They did not find any striking difference between pollen tubes originating from *Ga1-s/Ga1-s* and *Ga1-s/ga1-s* plants on *Ga1-s/Ga1-s* silks. Silk of heterozygous *Ga1-s/ga1-* plants shows intermediate pollen tube growth length behavior when pollinated with *ga1* pollen. Pollen from all three genotypes of maize grew normally on *ga1/ga1* silks.

Lausser et al. (2009) revealed the occurrence of the inter-specific crossing barrier at various levels in maize. Pollen capture, hydration, and germination do not seem to represent the essential crossing barriers. It was found that pollen tube guidance signals in the ovary cavity are exclusively controlled by the maternal sporophytic tissues of the ovule.

Zhang et al. (2012) also performed pollen tube growth study on both compatible and incompatible reaction of *Ga1-s*. They used the popcorn line SDGa25 as a source for homozygous *Ga1-s*. Regarding the pollen tube growth study, the following pollen-pistil combinations were used: W22 pollen onto SDGa25 pistils (incompatible reaction), SDGa25 pollen onto SDGa25 pistils (compatible reaction), and SDGa25 pollen onto W22 pistils (compatible reaction). Silks were fixed and stained with aniline blue at 0.15, 0.5, 1, 2, 5, 10, and 20-hour intervals after pollination. Thirty silks from three plants at each time interval were averaged. Pollen tubes germinated and entered the transmitting tract in all cases, but once in the silk, there were significant differences in tube growth. Significant differences in growth were seen two hours after pollination. Pollen tubes in compatible reactions grew at a rate of 10 mm h^{-1} whereas the incompatible reactions that grew only 2.8 mm h^{-1}. After 20 hours of growth, pollen tubes in compatible reactions grew completely and reached the ovary, however, in incompatible reactions, pollen tube growth arrested 5.5 cm distal to the ovule and fertilization never occurred. Investigation on pollen-pistil mechanism has increased in recent years. However, the mechanism of pollen abortion is still unclear. The molecular mechanism regarding the pollen-pistil interaction as well as deciphering the mystery of cross-incompatibility of *ga1* pollen on *Ga1-s* silk remain a topic of great interest.

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Chapter 2 The Search for Modifiers of the Maize Gametophyte Factor (*Ga1s*)

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Abbreviations: *ga1*, gametophyte factor1; *Ga1-s*, gametophyte factor 1-strong allele; RILs, recombinant inbred lines, QTL; quantitative trait loci; LOD; logarithm of odds

ABSTRACT

The maize gametophyte factor (*Ga1-s*) has the potential to avoid cross-contamination of organic corn, sweet corn and landraces with that of GM corn and hybrids. *Ga1-s* (*s* refers to the strong allele) confers cross-incompatibility because it blocks the ability of pollen without this factor (*ga1*) to fertilize a plant that possesses this factor. We found that in some genetic backgrounds, heterozygous *Ga1-s* / *ga1* offers stronger resistance to *ga1* pollen than in others. A study was conducted to map and identify the modifier genes that alter the strength of the *Ga1-s.* Two maize lines have been identified that are polymorphic to B73 relative to *Ga1-s* resistance to *ga1* pollen: Ky21 shows less resistance and M162w shows greater resistance. The recombinant inbred lines (RILs) of both Ky21 and M162w have already been developed and genotyped for both of these with B73. 200 RILs each of Ky21 and M162w were sown in summer of 2014 and crossed with pollen from plants homozygous for *Ga1-s*. The strength of *Ga1-s* in each F1 line was evaluated by pollinating with *Rscm2 ga1* pollen the first day and allowing open pollination on the second day. A strong effect of *Ga1-s* is

indicated if the resulting ear has few or no blue kernels and a weak effect is ear being heavily contaminated with blue kernels. A standardized scale of contamination was established to score ears. RILs (B73 X Ky21) show QTLs on chromosome 1S and 4S while RILs (B73 and M162w) shows QTLs on 5L and 10L.

Introduction

More than 80 % of the corn in the US is genetically modified (USDA, National Agricultural Statistics Service, and June Agricultural Survey for the years 2000-15). Hybrids are flourishing in developing countries. On the other hand, there is increasing demand for organic corn; imports to the U.S. of Romanian corn rose to $11.6 million in 2014 from $545,000 the year before (Bjerga, 2015). Cross contamination of organic maize and landraces is a major concern as hybrids are selected for their production capabilities; leading to the loss of genetic diversity and quality traits possessed by the landraces. Maize being a cross-pollinated crop and wind is an important factor for cross-pollination. Cross pollination in maize is so high that it is evident that cross-fertilization was detected at a distance of 650 m from the pollen source and maize pollen could travel up to several kilometers (Henry et al., 2003, Kozjak et al., 2011). Certain types of maize such as sweet corn, organic corn, and waxy maize, are required to be genetically pure and free from foreign pollen due to the xenia effect. The effect is defined as the effect of pollen on the development and characteristics of seed or fruits. To avoid the cross contamination of the maize neighboring fields, strategies such as physical barriers, spatial and temporal isolation were adopted. For instance, a four to five days planting shift led to a 25 % reduction in the cross-fertilization rate whereas it's 50 % reduction in case of 6 days shift (Della Porta et al., 2008) and 70 % reduction when the planting shift was over 10 days (Kozjak et al., 2011). However, the above-mentioned strategies are not always reliable and feasible in every situation. Hence, finding genetic factor as a reproductive barrier will be far more effective compared to above-mentioned strategies in avoiding

cross contamination. It is a challenge for maize geneticists and breeders to address the issue of cross-contamination.

The maize gametophyte factor (*ga1*) offers a solution to this problem. There are numerous gametophyte factors in the maize. The gametophyte factors are mostly found in the popcorns and acts as a pollen barrier to dent and flint maize strains, however, the reciprocal crosses are successful (Nelson Jr, 1952). The discovery of the gametophyte factor was due to the segregation distortion to the Mendelian inheritance of few of the genes such as *su1* linked to gametophyte factor (Correns and Correns, 1924, Jones, 1924). In the gametophyte factor, *Ga* pollen can pollinate *Ga/Ga, Ga/ga and ga/ga* pistils, however, *ga* pollen cannot able to fertilize *Ga/Ga* pistil limiting the gene flow (Kermicle, 2006). Six *Ga* loci (*Ga1, Ga2, Ga3, Ga4, Ga6 and Ga10)* distributed over four chromosomes behave in a similar manner to *Ga1* (Nelson, 1993). Numerous gametophyte factors have been reported on maize chromosome 1, 2, 3, 4, 5, 6, 7 and 9.

Ga1 was detected by Correns and Correns (1924) by the aberrant F2 ratios for sugary-starchy. Discovery of *Ga* was in the early 1920s, however, no *Ga* genes have been molecularly isolated yet. *Ga1-s* is the strongest allele of *Ga1* and *ga1* pollen are found to be dysfunction on the silks of the homozygous *Ga1-s* (Schwartz, 1950). *Ga1* was mapped to chromosome 4 by classical linkage method, approximately 23.2 cM from *Su1* (Mangelsdorf and Jones, 1926). In recent, *Ga1* has been mapped to 1.5 cM regions on chromosome 4S in a cross between Chinese popcorn and dent cultivars (Zhang et al., 2012).

Although *Maize gametophyte factor* (*Ga1***)** offers an answer to this question, we have found that the expression of *Ga1-s* into the different genetic background differs

significantly. It appears that even the strong allele (*Ga1-S*) is modified by other factors in the genome. In some genetic backgrounds, heterozygous *Ga1-S* / *ga1* offers strong resistance to *ga1* pollen while in other backgrounds there is a lot of pollen contamination. Our hypothesis is that there are certain modifiers genes that affect the strength of the *Ga1-s* in the different genetic background. *Gal-s* allele along with the modifier genes will be useful for isolating one category of commercial varieties from another. The identification of the genomic positions of the modifiers of the *Ga1-s* along with the published marker information can be utilized for marker-assisted introgression of *Ga1-s* into sweet corns, popcorns and organic corn varieties that essentially needs isolation from the transgenic maize pollen. Studies have been done to identify the genomic localization of the *gametophyte factor* (Bloom, 2012, Liu et al., 2014, Mangelsdorf and Jones, 1926, Zhang et al., 2012). The objective of the research is to identify the genomic location of the modifiers of the *Ga1-s* and eventually find and cloned those modifiers genes. I believe this is the first paper studying the mapping of the **modifiers** of the maize *gametophyte factor*

Materials and methods

In order to know the strength of introgressed *Ga1-s* in the genetic background that is homozygous for *ga1,* we came out with a protocol that will help us to know the strength or weakness of the introgressed *Ga1-s*. The strength of *Ga1-s* in each F1 line was evaluated by pollinating with *Rscm2 ga1* pollen (*Rscm2* is a color marker that makes both the endosperm and embryo of the kernel blue) the first day and allowing open pollination on the second day. A strong effect of *Ga1-s* is indicated if the resulting ear has few or no

blue kernels and a weak effect is ear being heavily contaminated with blue kernels (Figure 2.1).

We used the progenitors of the Nested Association Mapping (NAM) lines to identify the lines that are polymorphic to B73 for *Ga1-s* resistance to *ga1* pollen. Two maize lines have been identified that are polymorphic to B73 relative to *Ga1-s* resistance to *ga1* pollen: Ky21 shows less resistance and M162w shows greater resistance (Figure 2.2). Ky21 and M162w being the progenitors of NAM populations, recombinant inbred lines (RILs) have already been developed and genotyped for both of these with B73. The first population is a set of 200 recombinant inbred lines (RILs) derived from the cross between inbred B73 and inbred Ky21. The lines were derived from the 5^{th} generation repeated selfing. These lines have been genotyped by 1106 polymorphic single nucleotide polymorphism (SNP) markers (McMullen et al., 2009). Genotype data are publicly available at www.panzea.org. Seeds for this population are available at USDA Maize Genetic Cooperation Stock Center. The second set of RILs was derived from crosses between the inbred M162w and inbred B73. Similarly, 200 RILs was obtained through single seed descent method through selfing F2 up to five generations. These 200 RILs each of Ky21 and M162w were sown in summer of 2014 and resulting plants were crossed with pollen from plants homozygous for *Ga1-s*. Some of the lines did not germinate. We created 171 F1s of ((RILs B73 X Ky21) X *Ga1-s/Ga1-s*) and 115 F1s ((RILs B73 X M162w) X *Ga1-s/Ga1-s*).

Mapping the Modifier of *Ga1-s* in ((B73 X Ky21 RILs) X *Ga1-s*) F1s:

The 171 F1s derived from the cross of ((RIL B73 X Ky21) X homozygous *Ga1-s)* were planted in rows each containing 13 plants in SDSU Experimental station in the

summer of 2015. Few rows of the *Rscm2-ga1* pollen donor were planted in a separate location but close to the recipient RILs and seeds were hands planted on three different dates over a period of three weeks to provide a consistent supply of *Rscm2-ga1* pollen during the time that RILs flowered. The strength of *Ga1-s* in each F1 line was evaluated by pollinating five plants from each F1s with *Rscm2 ga1* pollen the first day and allowing open pollination on the second day as described in figure 2.1.

Phenotyping

A standardized scale of contamination was established to score the matured ears. Five ears from each line of F1s ((RILs B73 X Ky21) x *Ga1-s*) were scored from 0-5 (Figure 2.3) where 0 = no colored kernels, 1= up to 4 % colored kernels, 2 = up to 8 % colored kernels, 3 = up to 16 % colored kernels, 4 = up to 32 % colored kernels and 5 = > 32 % colored kernels. We used the above-mentioned scoring system to simulate the effect of change observed in nature rather than using any specific interval such as 0-25 %, 26-50% and so on.

Genotyping

The genetic map has been already generated through Nested Association Mapping project (Yu et al., 2008).The genetic map and the genotype data were collected from www.Panzea.org . The RILs have genotyped by1106 polymorphic SNP markers from Illumina (McMullen et al., 2009). The genotype dataset was taken from the imputed dataset that was used for QTL analysis for the maize flowering time by Ed Buckler (Buckler et al., 2009).

Mapping the Modifier of *Ga1-s* in ((B73 X M162w RILs) X *Ga1-s)* F1s:

Similarly, the 115 F1s derived from the cross of ((B73 X M162w RILs) X homozygous *Ga1-s)* were planted in rows containing 13 plants each in SDSU Experimental station in the summer of 2015. *Ga1-s* efficacy test was followed in the similar manner discussed in Figure 2.1 and the matured ears were scored by the same protocol described in Figure 2.3.

QTL Analysis

QTL for the modifiers of *Ga1-s* was mapped using QTL cartographer 2.5 (Wang et al., 2007). Genome-wide significance thresholds (LOD) were estimated with 1000 permutations for each analysis with an alpha level of significance at 0.05. Composite interval mapping using Kosambi map function was used for the analysis. The additive QTL effects and coefficient of determination (R^2) were estimated. Results are made based on maximum LOD score, LOD marker interval and percentage of variation explained (R^2).

Result and Discussion

QTL Analysis for (B73 X Ky21 RILs)

QTL mapping of the modifiers of the maize gametophyte factor was conducted using composite interval mapping (CIM) (Jansen and Stam, 1994) and the Kosambi mapping function. The genotypic data were retrieved from www.panzea.org. QTLs were based on the LOD threshold after 1000 permutation and the threshold was set up at 3.0. Two QTL locations were obtained: one on the short arm of chromosome 4 and the other on the short arm of chromosome 1 (Figure 2.4). These QTL explained the phenotypic variation from 5.78 to 18.75 % (Table 2.1). Marker *PZA00975.1* located at 25.9 cM on

chromosome 4 shows the highest LOD peak and explained the highest phenotypic variation of 18.75 %. Marker *PZA01476.1* at chromosome 1 located at 72.5 cM shows the highest LOD peak and explained the highest phenotypic variation of 5.78 %. In Chromosome 4S, the 1-LOD support interval spanned the genetic map at 21.3 – 40.4 cM and it encompasses the *Ga1* locus. The QTL from the Ky21 shows the positive additive effect whereas the QTL from B73 shows the negative additive effect (Table 2.1).

Same QTL region was found at chromosome 4S when a mapping study of *Ga1-s* was done by Bloom (2012) in the B73 X Hp301 NAM RILs. They observed the markers showing the most severe segregation distortion located from 19.4 to 33.9 cM on the map, delimiting the position of *ga1* to this region, and the same marker PZA00975.1 located at 25.9 cM showed the highest segregation distortion. They compared the *ga1* position mapped by Zhang et al. (2012) and combine their information for fine mapping the region and refined the region containing 13 predicted genes, listed in table 2.2. One of the genes, GRMZM2G039983, had homology to WDL1 of Arabidopsis that regulates anisotropic cell growth and might be involved in pollen tube growth (Yuen et al., 2003).

QTL Analysis for (B73 X M162w RILs)

Two QTL locations were obtained: one on chromosome 5L and the other on chromosome 10L (Figure 2.5). These QTL explained the phenotypic variation from 9 to 12.74 % (Table 2.1). Marker *PHM18513.156* located at 63 cM on chromosome 10 shows the highest LOD peak of 4.4 and explained the highest phenotypic variation of 12.74 % whereas marker *PZA02390.1* located at 138 cM on chromosome 5 shows the highest LOD peak of 3.6 and explained the highest phenotypic variation of 12 %. The QTL from

the B73 shows the positive additive effect whereas the QTL from M162w shows the negative additive effect.

The QTL at 4S encompasses the *Ga1* locus and indicates that the modifiers gene might be the different version of the same gene (*Ga1-s*). Interaction of the modifiers alleles from a different genetic background with *Ga1-s* has a significant effect on resisting the *ga1* pollen. Results in RILs (B73 X Ky21) has shown that *Ga1-s* work effectively in resisting the *ga1* pollen in combination with the B73 allele but not in combination with Ky21 allele while in another RIL population (B73 X M162w), *Ga1-s* has shown to work much more effectively in combination with M162w. Understanding the interaction of various modifiers genes relative to *Ga1-s* will definitely helpful in introgression of *Ga1-s* in that background that shows weak expression of resisting *ga1* pollen.

Kermicle and Evans (2005) showed that *Ga1-s allele* controls nonreciprocal crossability with *ga1* allele by means of allele-specific congruence rather than active rejection. They use the hetero-allele pollen *Ga1-s/ga1* as a male parent to pollinate *Ga1-s/ Ga1-s* and *ga1/ga1* silks. They observed that all plants were successfully crossed with hetero-allele pollen suggesting the requirement of matching alleles between pollen and pistil (specific congruence) rather than active rejection. Introgression of *Ga1-s* to the different genetic background and the variable expression of the *Ga1-s* in different genetic background led the researcher to understand the *Ga1* in various genotypes and environment. A study conducted by Ashman (1975) noticed the variable strength of *Ga1-s* when it was introgressed to the different genetic background. He reported that this variability might be due to the different genetic background and indicated the possibility

of involvement of modifiers genes. His ideas were verified by González et al. (2012) in studying Genotype x Environment (GxE) interaction in populations possessing *Ga1-s* and *ga1* allele. He reported that environment and GxE effects were not significant in his experiment indicating that incompatibility may be selected effectively over different environment using the *Ga1-s* system.

In summary, quantitative trait loci mapping approach for mapping modifiers of the *Ga1-s* was described in this paper. This approach is useful in replicating the experiment in other populations. RILs (B73 X Ky21) show QTLs on chromosome 1S and 4S while RILs (B73 and M162w) shows QTLs on 5L and 10L. Fine mapping of 4S QTL shows 13 predicted genes. Mapping of the modifier genes will definitively useful, not only for practical reasons but also to aid in understanding the molecular principles of reproductive isolation. The future direction of the project is to fine map the other QTLs intervals, identify the candidate genes, clone, and introgression the *Ga1-s* along with the modifier genes into those elite lines that weakly express *Ga1-s*.

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Table 2.1 Result in the summary of the QTL analysis. Showing chromosome number, position, LOD score and phenotypic variation (R2)

RILs	**Population**	**Chromosome**	**Marker**	**peak position (cM) †**	**LOD Score**	**LOD interval (cm)**	**Additive effect**	**R^2-value (%) ‡**
(B73 X Ky21)	171	4S	PZA00975.1	25.9	7.9	21.3 - 40.4	0.57	18.75
		1S	PZA01476.1	72.5	3		-0.32	5.78
(B73 X M162w)	115	10L	PHM18513.156	63	4.4	58.4 - 69.2	0.41	12.74
		5L	PZA02390.1	138	3.6	138 - 153.3	-0.39	12

† Position of likelihood peak (highest LOD score)
‡ R^2 (Coefficient of Determination) – percentage of phenotypic variation explained by the QTL.

Table 2.2 Predicted genes from fine mapping the 4S interval overlapping regions containing *ga1* (Adopted from Bloom and Holland, 2012)

Gene ID	Transcript start	Transcript end	Conserved domains
GRMZM2GO12821	7616846	7618466	F-box domain cyclin-like kinesin motor domain nodulin-like
GRMZM2G424553	7653177	7691914	
GRMZM2G135056	7780877	7782970	
GRMZM2G181073	8078275	8079905	
GRMZM2G029496	8305887	8308705	
GRMZM5G835418	8899536	8900563	
AC196002.2_FG002	8901387	8901950	
AC201986.3_FG002	9183034	9183546	
GRMZM2G702344	9259652	9260731	
GRMZM5G817995	9325329	9325631	
GRMZM2G419836	9351020	9354236	
GRMZM2G027021	9485207	9494351	
GRMZM2G039983	9589010	9592389	thioredoxin-like fold GTP-binding protein hflX Xklp2 targeting protein, WDL1

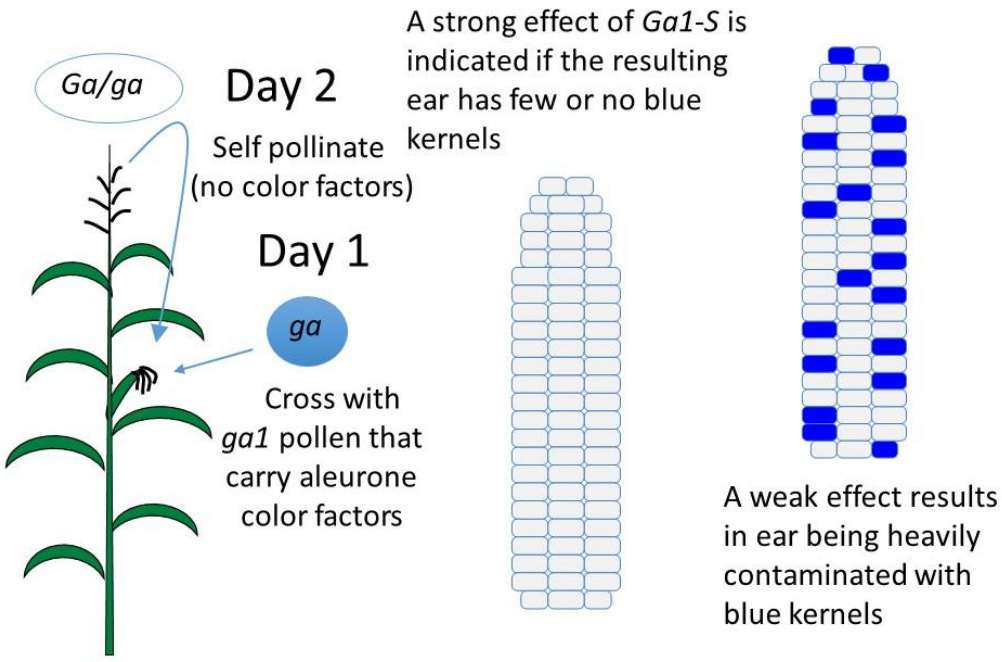


Figure 2.1 Test of the strength of Ga1-s efficacy in the heterogeneous background.

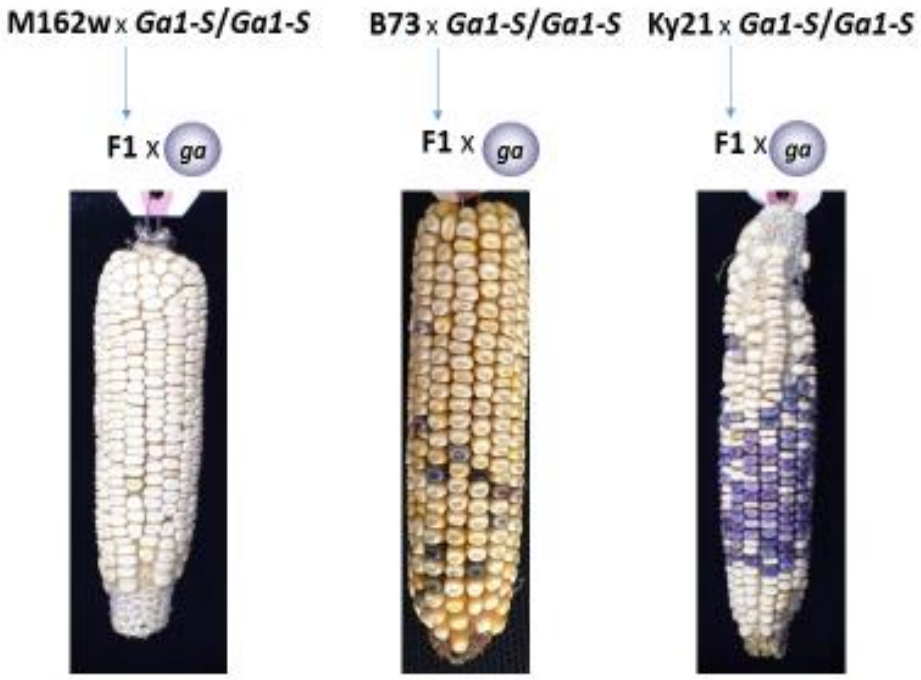
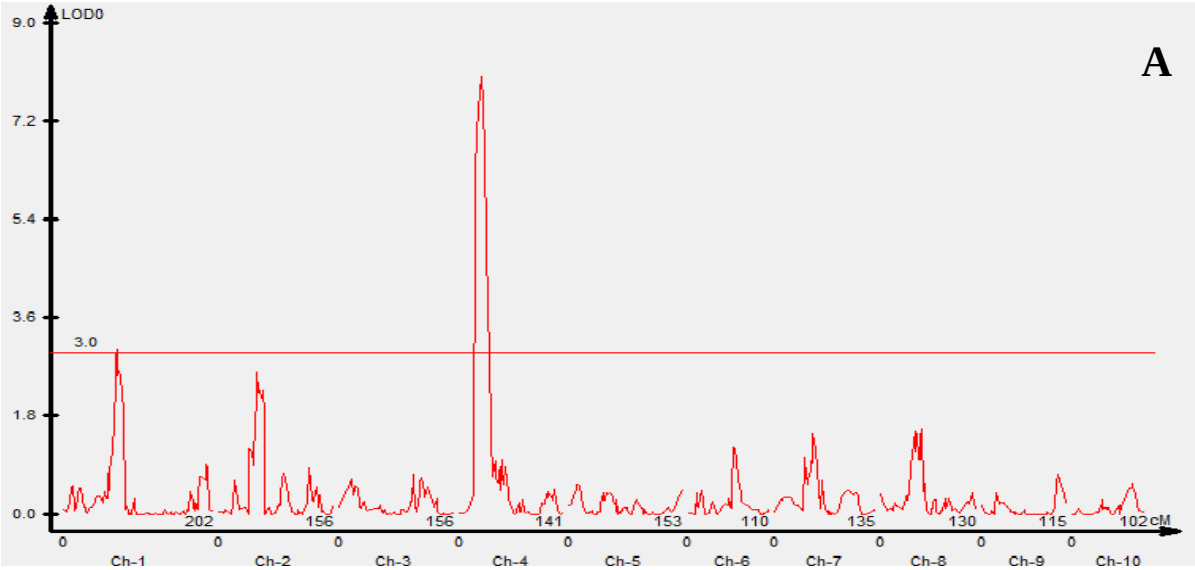


Figure 2.2 M162w and Ky21 are found to be polymorphic relative to B73 and are a candidate for QTL analysis.

Figure 2.3 Phenotyping of the matured ears using standardized scale: 0 = no colored kernels, 1= up to 4 % colored kernels, 2 = up to 8 % colored kernels, 3 = up to 16 % colored kernels, 4 = up to 32 % colored kernels and 5 = > 32 % colored kernels.



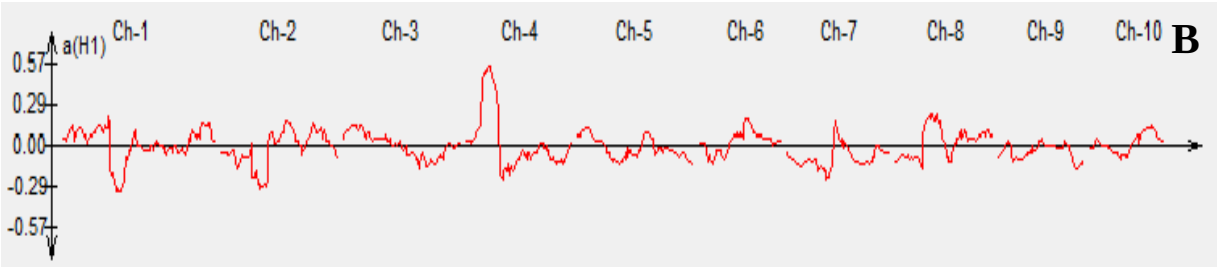


Figure 2.4 Composite Interval Mapping done using QTL cartographer 2.5 (NCSU, Dept. of Statistics and Bioinformatics) using 1000 permutations. (A) Using RILs (B73 X Ky21) QTL for modifiers of Ga1-s was confirmed at 4S and 1S. Permutation test was carried out to calculate the 95% confidence threshold and the LOD threshold is shown with a solid horizontal red line. (B) The additive effects are estimated across the whole genome.

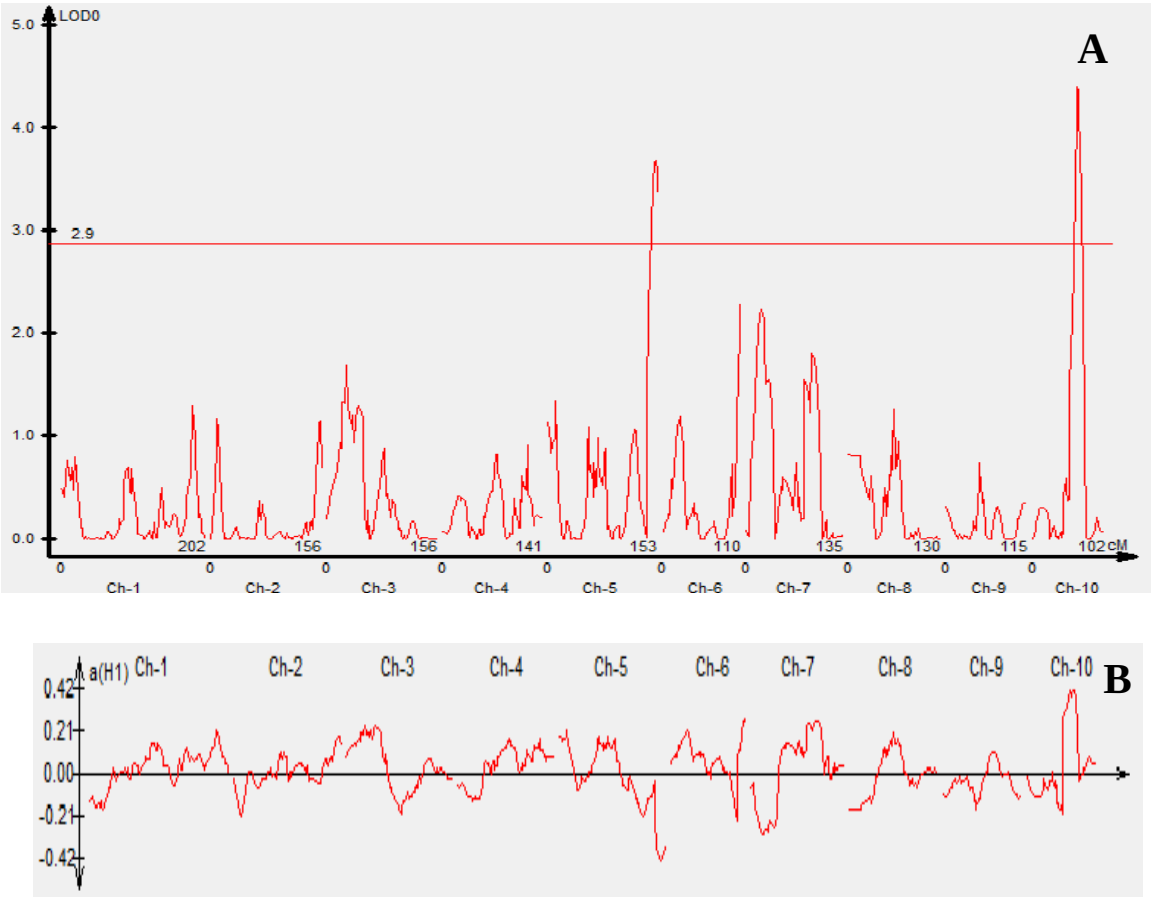


Figure 2.5 (A) Using RILs (B73 X M162w) QTL for modifiers of Ga1-s was confirmed at 5L and 10L. Permutation test was carried out to calculate the 95% confidence threshold and the LOD threshold is shown with a solid horizontal red line. (B) The additive effects are estimated across the whole genome.

Chapter 3 Doubled-haploid Project Introduction and Literature Review

General Background:

Plants exhibit alternation of the generation having diploid (2n) sporophytic generation and haploid (n) gametophytic generation. A haploid derived from a diploid is called monoploid and polyhaploid if derived from polyploid species. In maize, haploid and monoploid are the same, having n=10. Haploids are produced in nature spontaneously or artificially induced by in vitro culture of immature male (anther or pollen) and female (ovary) gametophytes or in vivo such as inter and intraspecific hybridization and centromere-mediated hybridization. The first natural sporophytic haploids were reported in Jimson weed (*Datura stramonium L.*; Blakeslee et al. (1924). Later on, Chase (1947) and (1949) isolated a few monoploids from maize. A few important advancements in haploid induction revolutionized doubled-haploid technology. A doubled-haploid is a true breeding genotype formed by either natural or artificial doubling of haploid chromosome complement. The main merit of doubled-haploid application is that it takes just one generation to develop a completely homozygous inbred line whereas for conventional methods it requires six to ten generations of selfing to gain sufficient homozygosity. Today, doubled-haploid inbred lines have become instrumental, potentially serving as the backbone of the hybrid maize industry in the future. The importance of doubled-haploids increased when Guha and Maheshwari (1964) produced haploids from anther culture in Datura. Similarly, Kasha and Kao (1970) produced haploids in barley (*Hordeum vulgare* L.) through interspecific crosses followed by embryo culture. Haploids plants are smaller and less vigorous compared to their corresponding homozygous diploid (Auger et al., 2004, Chase and Gowen, 1952) and most of the haploids lack male fertility (Chase and Gowen, 1952). Haploids were

invariably small, narrow and erect leaves, tassels and ears are completely sterile, anther much smaller than normal, zebra-striping in leaves and linear sectors of white tissue are common (Coe, 1959)

Spontaneous haploids were observed by Chase and Gowen (1952) in US corn-belt germplasm at a rate about 0.1%, which was too low for the commercial application of haploids. Later Coe (1959) found that Stock 6, an inbred line, with an induction rate of 1 to 2 %. This line became the progenitor of all subsequently developed inducer lines.

One of the common haploid inducers is the colored crown or Navajo kernel trait encoded by the dominant allele R1scm2 or *R1-nj* of the color gene *R1*. *R1scm2* kernels have pigmentation of the in the crown region of the aleurone layer of the endosperm as well as the scutellar region of the embryo in the presence of the other dominant pigmentation genes. Another effective inducer line is RWS (Rober et al., 2005), obtained from the cross between an inbred line originating from the Russian inducer synthetic KEMS (Shatskaya et al., 1994) and the French inducer line WS14 (Lashermes and Beckert, 1988).

Quantitative trait polymorphism study in maize: From the past to present

Doubled-haploids are expected to be completely homozygous and the progeny of these doubled haploids are expected to be genetically homogeneous and, except for rare mutations, should show no genetic diversity. Even so, over 50 years ago Sprague et al. (1960) demonstrated that heritable variations in quantitative traits quickly emerged among the progeny of doubled haploids maize. The rate of variation was greater than the rate of spontaneous mutations. They felt that double-haploid stocks (due to their genotypic and phenotypic uniformity) would provide an excellent source for the

mutations (if any) affecting the quantitative traits in maize. They took four generations (S3 to S6) of seeds derived from the single seed descent using monoploid plant designated as S0 as the source material. Experiment was conducted and the material for the study was assigned on the basis of S2 origin using bifurcation method. For instance, the experiment consisted of progenies from the parental S2, the two S3, the four S4, and the eight S5 ears all derived from to a single S2 plant. Analysis of variance was calculated on the plot means. Significant differences between means were observed for nine of the quantitative traits. The traits measured were plant height, leaf width, no. of tassel branches, no. of kernel rows, ear length, ear diameter, weight per 100 kernels, and weight of shelled grain per plant and date of silking. Genetic changes were considered only, when change resulting in significant differences between parent and progeny or between siblings within a given generation. Those significant differences were interpreted as some sorts of mutational change. Unfortunately, due to the lack of sophisticated techniques such as sequencing at the time, Sprague was unable to point the real cause of mutation. The observed rate of mutation that he observed was 4.5 mutations per attribute per 100 gametes tested.

Another stability study was performed by Russell et al. (1963) where they estimated the mutation rate in long-time inbred maize. They took six inbred lines that had inbred for at least of ten generations, hence, it was expected that any heterozygous loci present would be mainly due to recent mutations and not due to residual heterozygosity. These lines were maintained continuously through selfing in ear-to-row progenies. Nine traits were studied. Ear and grain traits were not found to have significant differences. They found an increasing number of significant differences with successive generations that

indicate of greater genetic variability among the siblings. The previous study by Sprague et al. (1960) hypothesized that the monoploid method of developing inbred lines as a possible cause of high mutation rates observed. However, this study also came out with large mutation rates although slightly lower than Sprague's study. They estimated the rate of mutation as 2.8 mutations per attribute per 100 gametes tested.

Similar research was done by Legg and Collins (1968). They investigated the stability of six doubled haploid stocks of an autogamous, amphidiploid, tobacco *Nicotiana tabacum* L. They evaluated ten quantitative traits and observed significant differences among lines within five of the stocks. They found that the different stocks show different rates of mutation. Sprague et al. (1960) found similar results among the monoploid derived stocks of maize. One of the families showed mutations in only two of nine traits and a total of three mutations for all traits whereas another family showed mutations in all nine traits and a total of twenty-two mutations. Legg and Collins (1968) findings suggest that different haploids differ in degree of stability indicating the haploid-derived lines should be carefully evaluated before they are used for the constant controls in long-term experiments or in studies where minimum plant-to-plant variation is required.

Russell and Vega (1973) studied the genetic stability of eleven long-time inbred lines of maize and evaluated ten traits in successive generations reproduced through selfing in ear-to-row progenies. The lines under study were inbred more than ten generations before the start of the actual project. The plant ear and seed traits were analyzed. Results showed that the inbred lines of maize were not genetically stable. Further, the instability appeared more on plant traits rather than ear and seed traits. They

found that genetic changes occurred continuously. Their findings showed that the most of the significant variations observed in an inbred line were due to gene mutations rather than residual heterozygosity.

Studies on genetic variation were also done in plants derived from tissue culture. Bregitzer and Poulson (1995) studied on determining the ability for recovering cultivars derived from tissue culture without somaclonal variation. For this, they studied agronomic performance of 30 families of tissue cultured derived lines from six barley cultivars. Each family was derived from a single regenerated plant that in turn was derived from an immature embryo produced in a callus culture. They found an important source of variation of the families within cultivars and concluded that the level of genetic stability in tissue culture derived families varies with cultivar. Hence, they suggested that selection of tissue culture derived lines without somaclonal variation is cultivar dependent and therefore require screening regenerated plants with large populations.

A similar study done by Kaeppler et al. (2000) highlighted the epigenetic aspects of somaclonal variation in plants. He elaborated on the causes of such variations, which includes cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, gene activation and silencing. The study reveals that DNA methylation patterns are highly variable among the regenerated plants and their progeny. This indicates that DNA modifications are less stable in culture grown plants compared to seed grown plants. Brown et al. (1991) first reported on a variation in methylation patterns among regenerated plants and their progeny in maize.

An investigation on the epigenetic variation on complex traits was done by Johannes et al. (2009). They reported that the loss or gain of DNA methylation affects gene

expression and these changes can sometimes transmit across the generations, indicating a possible source of heritable polymorphism in the absence of DNA sequence change. They used epiRILS (epigenetic Recombinant Inbred Lines) where two parents having little DNA sequence polymorphism, but significant methylation profiles differences in *Arabidopsis thaliana*. Results showed that epiRILS showing variation and high heritability for flowering time and plant height and stable inheritance of multiple parental DNA methylation variants over at least eight generations.

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Chapter 4 Quantitative Trait Polymorphisms Emerging from Double-haploid Maize Lines

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ABSTRACT

Doubled-haploids are useful in plant breeding and genetics. Because they are expected to be completely homozygous, the progeny of these doubled haploids is expected to be genetically homogeneous and, except for rare mutations, should show no genetic diversity. Even so, over 50 years ago George Sprague and his associates demonstrated that heritable variation in quantitative traits quickly emerged among the progeny of doubled haploids maize. Sprague demonstrated that the rate of variation was greater than the rate of spontaneous mutations, but he did not have the means to determine the source of that variation. We believe that, with new technologies, the means now exist. We are establishing and identifying heritable polymorphic lines that have descended from a single doubled-haploid B73 plant. In the summer of 2014, we planted the seed for two sequential generations from each of ten lineages. These were planted in triplicate in a randomized complete block design. The resulting plants were evaluated for 15 quantitative traits (plant height, number of tassel branches, 100 grains weight, etc.). A partial replication of experiment of 2014 was conducted in 2015. A heritable polymorphism for any particular trait is indicated if there is no significant difference between the two generations of a lineage but the lineage is significantly different from

other lineages. Number of tassel branches, total number of kernels per ear, days to pollen shed and days to silk emergence has demonstrated heritable polymorphism.

Introduction:

Inbred that possess desirable trait are selected to combine desirable traits for the production of hybrids in maize. Further, inbreds are popular as an experimental material due to their supposed uniformity and stability. Inbreds generally maintain the same genotype from generation to generations. However, the methods of production and maintenance of the inbred lines differ among breeders. Several rounds of selfing lead to homozygosity and it is expected that most plant in the line will be homozygous for most of the loci. Researchers have found polymorphisms in both qualitative and quantitative traits of inbreds maize lines. Mutation and residual heterozygosity have been frequently proposed as the cause of heritable variation.

Creating the inbred lines using double-haploid technique has been widespread. Unlike inbreeding, using double-haploid technique will eliminate the residual heterozygosity from the genome and homozygosity can be achieved in just one generation. Quantitative traits are governed by multiple genes where each contributes an incremental effect. These traits are much affected by the environment. Hence, germplasm used to study the heritable polymorphism need to be less affected by environments so that the genetic variance could be easily detectable and could be distinguished from the uncontrollable errors. The detection of mutations that affect quantitative traits requires a substantial genetic uniformity of both the experimental materials as well as the experimental techniques used. Therefore, doubled-haploids offer an ideal source of experimental materials to study mutations affecting the quantitative traits in maize

(Sprague, 1960). The stable changes observed among the families of the doubled-haploid lines are more probably due to genetic changes than from segregation of pre-existing alleles. Much of the research estimating the mutation rates has been done using qualitative traits rather than quantitative traits. Crow (1948) suggested that the average mutation rate in maize is 1×10^{-5} per locus or less. However Sprague et al. (1960) reported a much higher mutation rate for quantitative traits. They estimated 4.5 mutations per attribute per 100 gametes tested in a study of eleven double monoploid maize stocks. Similarly, a study conducted on a long time maize inbred by Russell et al. (1963) estimated mutation rate was 2.8 mutations per attribute per 100 gametes tested. However, the ability to determine the cause of the variation at the DNA level was not available when these studies were done, but we believe that, with new technologies, the means now exist. Hence, finding the cause of heritable polymorphism in the descendants of double-haploids maize lines will open up the channels to control the process of making hybrids. Researcher in the future can exploit the observed genetic changes to improve the hybrids. The objective of this project is to re-establish heritable polymorphic lines that have descended from a single doubled-haploid plant. Once we demonstrate this, it will encourage us to pursue molecular analysis to study the variation at the molecular level.

Materials and Methods

Germplasm

James A. Birchler, University of Missouri, provided the source material for this project. A haploid kernel was generated by crossing inbred B73 with pollen from a haploid inducer line Stock 6 Coe (1959). The haploid kernel was germinated and the resulting seedling was treated with nitrous oxide (N_20) by Kato and Geiger (2002) in

order to induce sectors of double-haploid tissue that would support meiosis. The resulting plant was selfed to produce a partially fertile ear (Figure 4.1). One kernel from this ear was used as a source germplasm for this project and was designated as S0. The diploid progeny resulting from self-pollination of the S0 plant was designated as S1. Similarly, one diploid progeny from self-pollination of an S1 plant was designated as S2 and so on to the S3 generation.

From the S3 generation, ten random seeds from a uniform good-looking ear were selected to become the source of separate descent lineages (Figure 4.2). Each lineage was maintained through selfing and one progeny was selected for advancement to the next generation. Failure to produce a useable ear in any generation would cause a failure in advancement and at the time of the assessment, the ten lineages varied from S6 to S10.

Experimental Design:

In order to identify heritable polymorphisms, seeds from sequential generations from each lineage were sown in the summer of 2014 in a randomized complete block design (RCBD). There were ten lineages with two generations each and in some case three generation nested together in each block. The same experimental material was replicated in three blocks. In each block, there were 23 rows and in each row, 13 seeds were sown. Partial replication of the experiment from 2014 was conducted in the summer of 2015 for those traits that were found to be significant in 2014. Families that were found to be significantly different from each other for those traits were given the priority. Also, advance generation obtained from the selfing of each significant family was included. There were five lineages with two generations each and in some case four

generation nested together in each block. The same experimental material was replicated in three blocks. In each block, there were 12 rows and in each row, 13 seeds were sown.

Data collection:

The resulting plants were evaluated for 15 quantitative traits in 2014. The traits were categorized as eight pre-harvest and five post-harvest quantitative traits. The pre-harvest traits measured were plant height, number of nodes per plant, node position of primary ear, length of the leaf subtending the primary ear, width of the same leaf, leaf area of the same leaf, number of tassel branches per plant, number of days from planting to first pollen shed and days to silk emergence. The post-harvest quantitative traits measured were ear length, ear circumference, and number of rows per ear, total kernels per ear, hundred grains weight and yield. Plant height was measured after flowering from the ground up to the ligule of the uppermost leaf. Nodes were counted from the first recognizable node above the ground surface. The primary ear is defined as the uppermost ear. Leaf width was measured at the widest point near the base of the leaf. Leaf length was measured from the ligule to the tip of the leaf. Number of tassel branches per plant was counted disregarding rudimentary branches. Ear length was measured from the base just above the shank to the tip of the ear. Ear circumference was measured from the broadest part of the ear near the ear base. Because several of the measurements involved subjective decisions, e.g., what constitutes a rudimentary tassel branch, all measurements were made by the first author.

In 2015, the traits measured were number of tassel branch, height, number of nodes, leaf length, position of the ear, days to pollen shed, days of silk emergence and the total number of kernel per ear.

Data analysis:

Data were organized and analyzed with R studio version 3.0.1 (Studio, 2012). The analysis of variance (ANOVA) was performed for each of the fifteen traits using two-way RCBD analysis as outlined below:

$$Y = \mu + Lineage\text{-}Generation + Block + e$$

Where, Y = Response variable, μ = Population mean, *Lineage-Generation*= Treatment effect, *Block*= Blocking effect and *e*= Random error effect. When ANOVA indicates that a lineage-generation effect is significant, a post hoc comparison was made using a Duncan Multiple Pairwise Comparison test.

Results and Discussions

Analysis of Variance showed that five traits among 15 traits were found to be significantly different (P-value < 0.001, 0.01 and 0.05) among at least one of the family (Table 4.1). These five traits were number of tassel branch, number of kernel per ear, number of nodes, leaf length and days to silk emergence. Four of the traits were found to be marginally significant (P-value equal or near to 0.05). They were plant height, the node position of the primary ear, days to pollen shed and yield. Six traits were found to be non-significant (P-value > 0.05). They were leaf width, leaf area, ear length, ear circumference, number of rows per ear and hundred-grain weight. Similar result was observed by Russell et al. in their studies on " Mutation affecting quantitative characters in long-time inbred lines of maize" (Russell et al., 1963) also shows that ear and grain traits were not significant.

In summer of 2015, partial replication of the project was conducted using those traits that were significant in the summer of 2014 as well as using those families that were significant with one another. Analysis of variance showed four among eight traits were significantly different with at least one family (Table 4.2). The traits were number of tassel branch, height, days to pollen shed and days for silk emergence. Total number of kernel per ear was marginally significant whereas number of nodes, leaf length, and position of the ear were non-significant.

For number of tassel branches family 07-06 was exceptional among family in 2014, even when compared to 07-09 and 07-10 (Figure 4.3A). The latter two families were direct descendants of 07-06 and were included because there were not sufficient kernels in 07-07 and 07-08. The earlier 07-06 family was included because increased tassel branch numbers were apparent prior to this test. We were curious to determine if this trait was transient so we regrew 07-06 and its self-progeny 07-07b in 2015. With the exception of one family, 07-06 was significantly different from all other lineages. Interestingly, it was also different from 07-07b, which exceeded all other families (figure 4.3B).

A simple hypothesis that could explain these observations is that the gene underlying this phenotype is semi-dominant nature. It might be that 07-6, the gene might be heterozygous and the mutation might take place making 07-7 homozygous dominant for the trait, resulting in the increase of number of tassel branch.

Total number of kernel per ear follows the criterion of a heritable polymorphism. Figure 4.4A shows that total number of kernels per ear for the two generations of family

02 are not significantly different from each other but are significantly different from other families including 07-09 and 07-10 (Figure 4.4A).

For number of days to pollen shed, phenotypic instability was seen. In 2014, family 08-6 took longest to shed the pollen (Avg. 80.7 DAS) whereas 08-5 shed its pollen around four days early (Figure 4.5A). A similar trend is found in 2015; 08-6 still being the slowest to shed the pollen whereas its selfed progeny 08-7 was four days early to shed its pollen (Figure 4.5B).

We found heritable polymorphic lines in 2015 that are in accordance with our hypothesis mentioned above. Families 07-6 and 07-10 are not different between each other but significantly different with family 01-7 and 01-6. However, instability seen among the generations of the lineage 07 i.e. 07-6 and 07-10 is significantly different to its selfed progeny 07-7 and 07-11 respectively indicating the genetic instability (Figure 4.5B). This trend of low (08-5), high (08-6) and the low (08-7) number of days taking to shed pollen indicates epigenetics switching occurring in different generations of the same lineage/family.

Number of days to pollen shed has a strong positive correlation with the number of silk emergence and the results obtained are also similar. In 2014, lineage 08-6 took longer for silk emergence (Avg. 80.1 DAS) whereas 08-5 had silk emerged around three days early (Figure 4.6A). A similar trend is found in 2015; 08-6 still being the slowest for days to silk emergence whereas its selfed progeny 08-7 was three days early for silk emergence (Figure 4.6B).

Polymorphic lines are found in 2015. Family 07-6, 07-10 is not significantly different with each other but is significantly different with family (02-10, 02-11) and (01-6, 01-7) (figure 4.6B). However, as found in days to pollen shed, family (07-6, 07-10) is also significantly different with its selfed progeny 07-7 and 07-11 respectively (Figure 4.6B).

We hypothesized that the source of the polymorphisms could be due to mutations as simple as duplications and deficiencies. Continuous trait variation in natural and experimental populations is usually attributed to the actions and interactions of numerous DNA sequence polymorphisms and environmental factors (Lynch and Walsh, 1998). Mutation can be as simple as a deletion, duplication, inversion or translocation that occurs during different events of cell division including DNA replication. Mutations occurring during chromosome doubling occurs may arise from the homologous and non-homologous association between chromosomes (McClintock, 1933). If such non-homologous pairing gives rise to a genetic crossing over, the resulting gametes probably possess chromosomal structural dissimilarities. Further, zygotes derived from such gametes might be heterozygous for various types of chromosomal aberrations that could give rise to deletion or duplication in the resulting progeny. Reciprocal translocation would lead to semi-sterility and inversion would lead to a reduction in fertility (Sprague et al., 1960). These phenomena were seen in the early selfed lines generated from doubled-haploids seed. Further, the question might arise that if mutations of a normal inbred or doubled-haploid line are high, how would that affect the stability of performance and appearance? The degree of stability might be increased due to continued selection. Unusual or abnormal plants or kernels are generally avoided during harvesting

and seed preparation and might contribute to the stability of the lines despite high mutational changes.

Polymorphisms were seen among different generations of the same lineage, which may implicate epigenetics as a potential source of variation. Similar results were seen by Sprague et al. (1960) in their study. They observed significant differences between parents and progeny that were not retained in subsequent generations and considered it to be chance deviations, segregation or some form of reverse mutation. Epigenetic control of gene expression can be defined as a somatically or meiotically heritable alteration in gene expression that is potentially reversible and is not due to sequence modification (Holliday, 1994, 2006). Classically, the heritable basis of complex traits is thought to be solely due to the transmission from parent to offspring of multiple DNA sequence variants that are stable and causative (Lynch and Walsh, 1998). However, recent studies suggest that chromatin variation such as differential DNA methylation can also be inherited across generations independent of DNA sequence change (Bossdorf et al., 2008, Kalisz and Purugganan, 2004, Martienssen and Colot, 2001, Peaston and Whitelaw, 2006, Richards, 2006, 2008). The changes might be unstable or reversible somatically or through meiosis; however some are stable from generation to generation. A study conducted by Johannes et al., (2009) reported that epiRILS (Epigenetic Recombinant Inbred Lines (epiRILs) are lines derived from two parents with little DNA sequence differences, but contrasting DNA methylation profiles) used to conduct the study on epigenetic variation on complex traits showed variation and high heritability for flowering time and plant height, as well as stable inheritance of multiple parental DNA methylation variants (epialleles) over at least eight generations (Johannes et al., 2009).

Kaeppler and Phillips (1993) reported that DNA methylation patterns varied substantially among maize regenerated-derived families from the same cultured explant, with all families having unique methylation profiles across 20 single copy probes. Hence, one of the important questions that can be asked as a future perspective of the project is how this epigenetic mechanism contributes to the both stable and unstable variation in the quantitative traits.

Heritable polymorphisms were demonstrated by number of tassel branches, total number of kernel per year, days to pollen shed and days to silk emergence. Polymorphisms were also seen among different generations of same lineage (data not shown). This instability of phenotype may implicate epigenetics as a potential source of variation. As hybrids are developed from the inbreds, this study will be very useful for utilizing and selecting those useful inbreds that have a positive correlation with yield and yield attributing traits, disease and insect resistance traits. The next possible question to ask is to what extent these genetic changes will be expressed in any hybrid combinations in terms of yield and quality. The genetic changes in these families can be utilized for the improving the existing hybrids. This genetic variation might be a useful source of germplasm for breeders and geneticist. Further, the double-haploids are used as control and checks in various studies and it is important that they should be stable in the traits under study from generation to generation. Hence, the germplasm should be well evaluated before it can be actually used as a control or check.

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Table 4.1 Analysis of variance shows that five out of 15 quantitative traits are significantly different among at least one lineage in summer of 2014.

#	Traits	p-value	Symbol
1	Number of tassel branch	0.00	***
2	Plant height	0.05	.
3	Number of nodes	0.02	*
4	Position of ear	0.05	.
5	Leaf Length	0.04	*
6	Leaf Width	0.28	ns
7	Leaf Area	0.44	ns
8	Days to pollen shed	0.05	.
9	Days to silk emergence	0.02	*
10	Ear Length	0.23	ns
11	Ear Circumference	0.21	ns
12	Number of rows per ear	0.67	ns
13	Total no. of kernels per ear	0.01	**
14	Hundred grain weight	0.15	ns
15	Yield	0.08	.

*** ($p<0.001$), ** ($p< 0.01$) * ($p<0.05$) and ns = non-significant

Table 4.2 Analysis of variance shows that four traits among eight quantitative traits are significantly different among at least one lineage in summer of 2015.

#	Trait	P-value	symbol
1	Number of tassel branch	0.00	***
2	Height	0.02	*
3	Number of nodes	0.25	ns
4	Leaf Length	0.47	ns
5	Position of ear	0.76	ns
6	Days to pollen shed	0.00	***
7	Days of silk emergence	0.00	***
8	Total number of kernel per ear	0.07	.

*** ($p<0.001$), ** ($p< 0.01$) * ($p<0.05$) and ns = non-significant

Figure 4.1 Partially fertile ear from S0 plant.

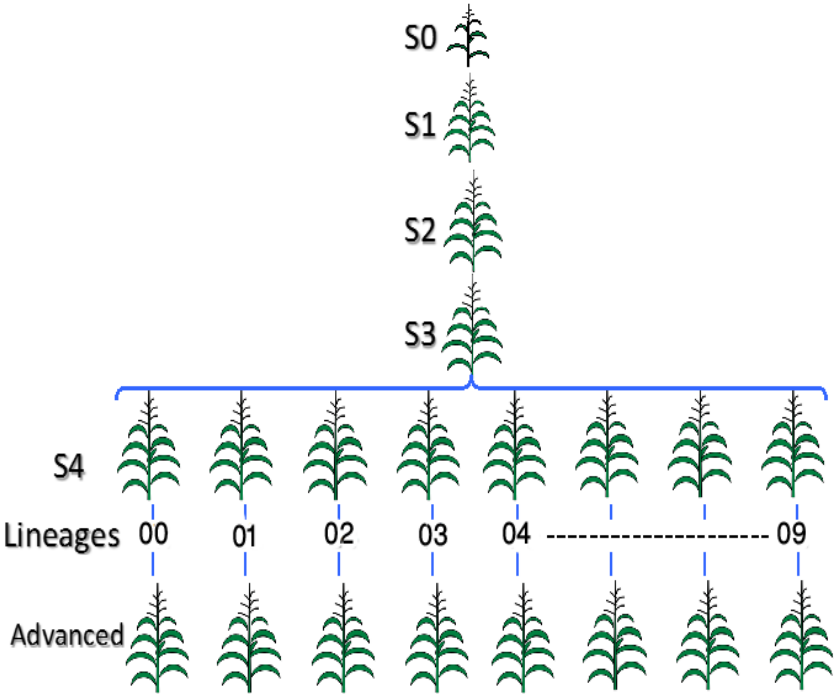


Figure 4.2 Scheme for generation of doubled-haploid lineages that are descended from a single doubled-haploid plant.

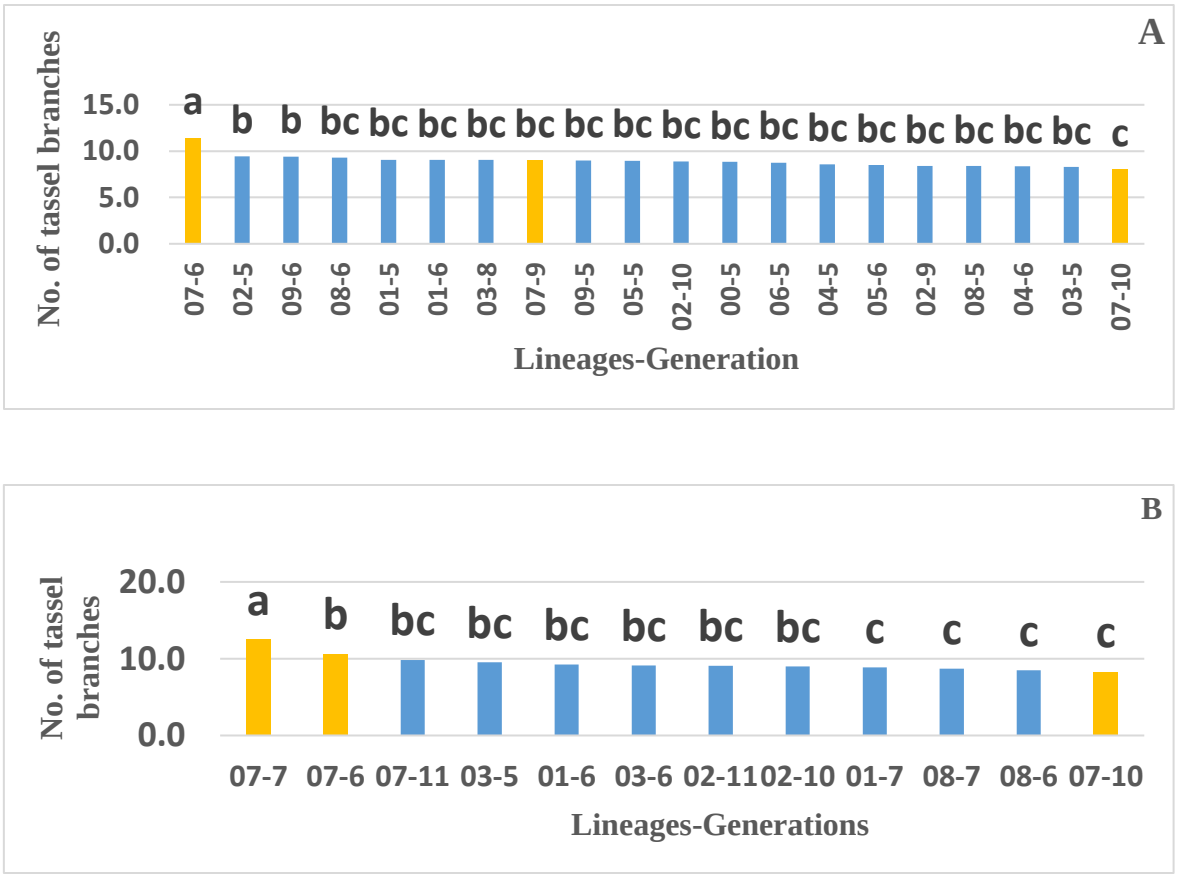


Figure 4.3 (A) ANOVA showing polymorphisms among doubled haploid families for number of tassel branch. Shared letters indicate no significant difference whereas different letters indicates significant differences. Yellow bars indicate that unstable phenotype among the different generation of same lineage. Data from 2014. (B) Data from 2015. Yellow bars indicate the unstable phenotypes of different generation within the same lineage.

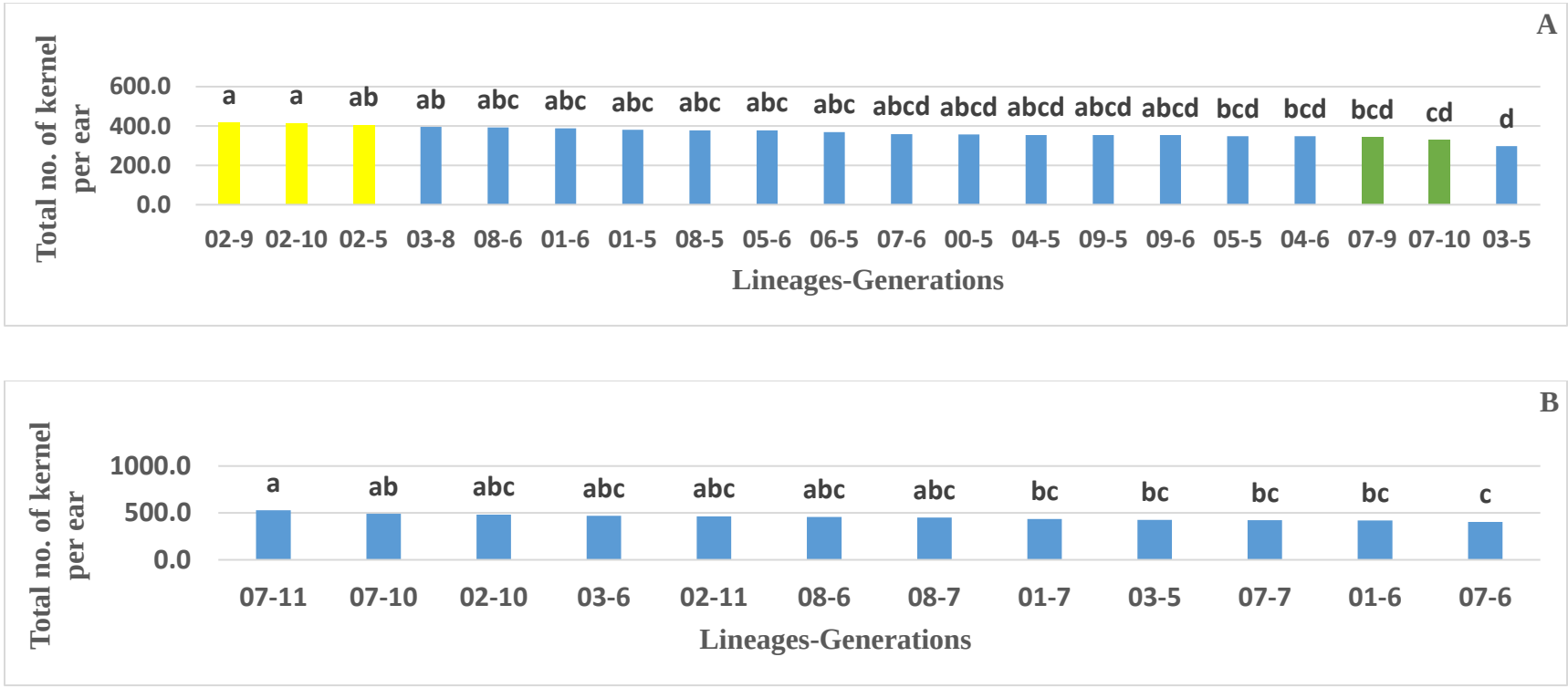
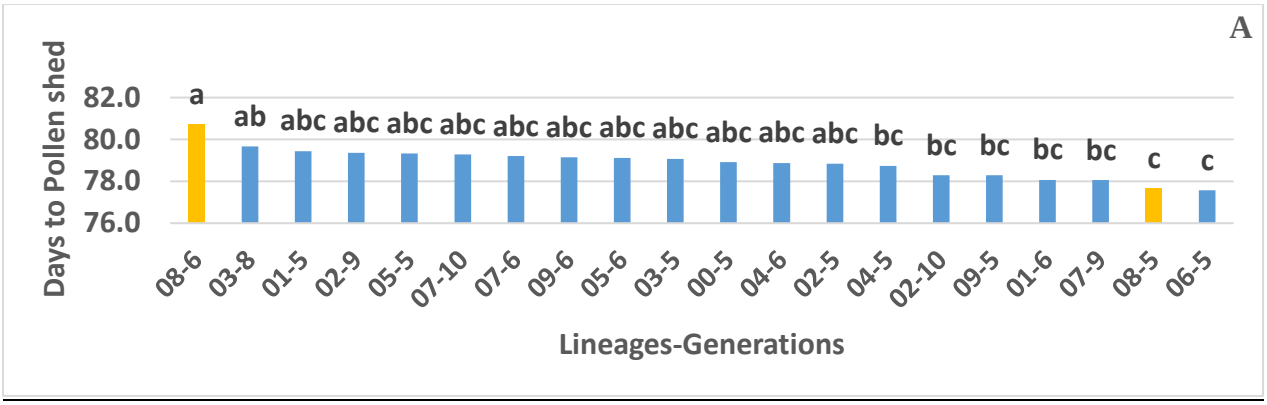


Figure 4.4 (A) Heritable polymorphism demonstrated by total number of kernel per ear. Shared letters indicate no significant difference whereas different letters indicate significant differences. Yellow and green bars indicates lineage 02 and 07 respectively, are in accordance with the hypothesis of heritable polymorphism. Data from 2014. (B) Data from 2015.



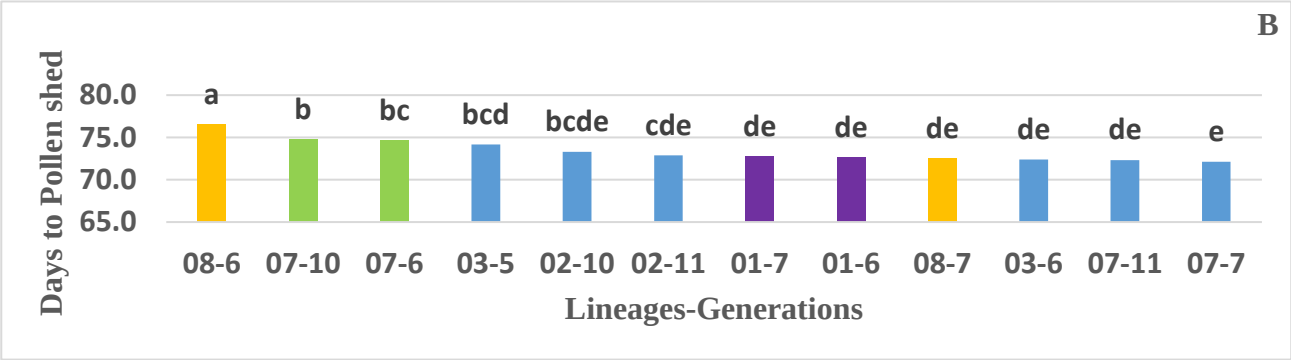


Figure 4.5 Fig (A) Showing polymorphisms among doubled haploid families for number of days to pollen shed. Data from summer 2014. Yellow bars indicate instable phenotype of lineage 08. (B) Data from 2015. Yellow bars indicate instable phenotype continues in lineages 08. Heritable polymorphic lines shown by lineage 07 (green bars) with respect to lineage 01 and 07 (purple and blue bars respectively)

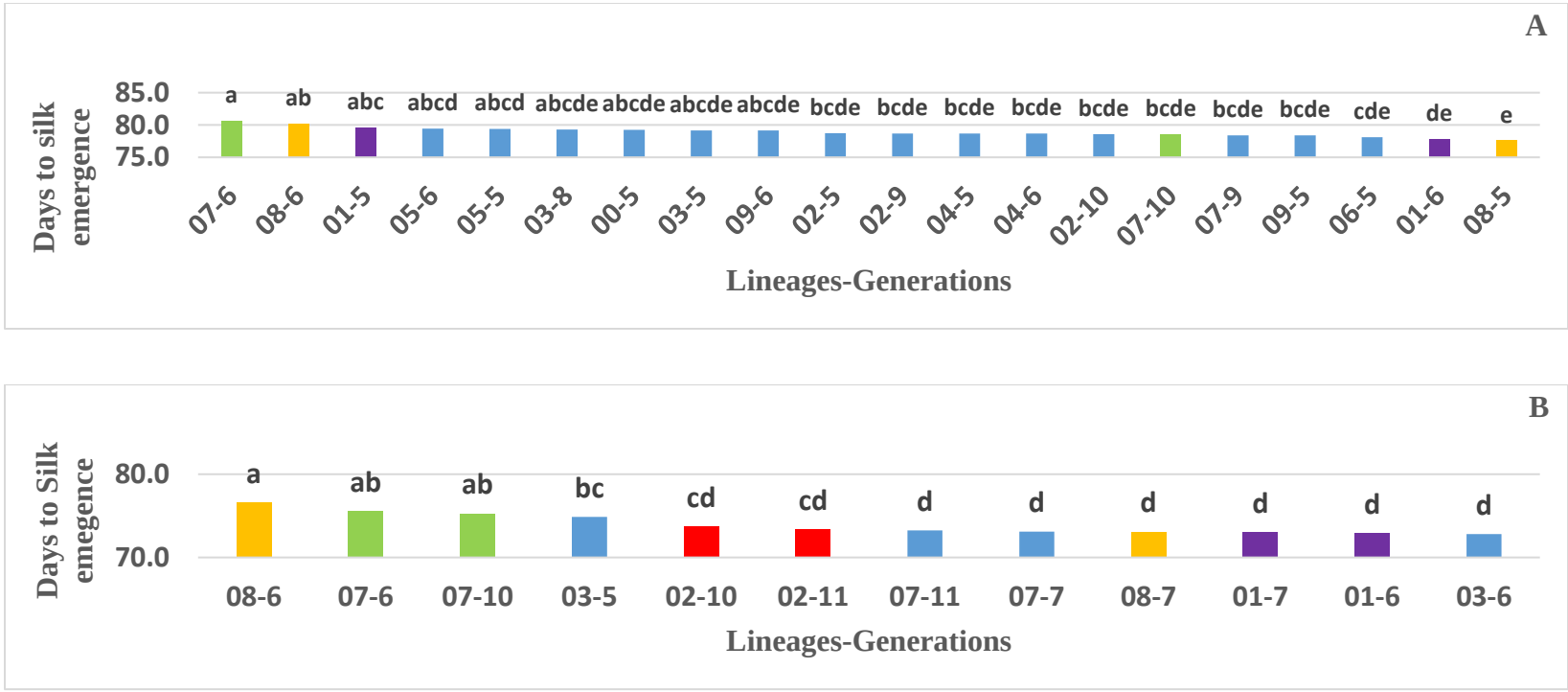


Figure 4.6 (A) Showing polymorphisms among doubled haploid families for number of days to silk emergence. Data from summer 2014. Unstable phenotypes showed by lineage 08 (yellow bars), by lineage 07 (green bars) and by 01 (purple bars). (B) Data from summer 2015. Lineage 07 (green bars) shows heritable polymorphic lines when compared to lineage 02 (red bars) and 01(purple bars)

Chapter 5 Conclusion and Future works

The maize gametophyte factor (*Ga1-s*) has the potential to prevent cross-contamination of organic corn, sweet corn and landraces with that of GM corn and hybrids. However, the strength to resist the *ga1* pollen by even the strong allele *Ga1-s* differs with genetic background. Quantitative trait loci (QTL) analysis was conducted using composite interval mapping and 1000 permutations. Analysis revealed one major QTL on chromosome 4S and one minor QTL on chromosome 1S in (B73 x Ky21) RILs population. Two major QTLs 5L and 10L were found on (B73x M162w) RILs. Understanding the effect of these modifiers genes will be helpful to understand cross-incompatibility in corn. The modifier genes hypothesis was supported by Kermicle et al. (2006) when they studied reproductive isolation among various *Zea mays* subspecies. They reported that when *Ga1-s/Ga1-s* plants were fertilized by *Ga1-s* and *Ga1-m* pollen, pollination was found more effective by *Ga1-s* pollen than *Ga1-m* and concluded that this preference may be strengthened by modifier gene differences between teosinte and maize, thereby providing partial reproductive isolation between the two.

Cross-incompatibility in maize occurs due to miscommunication of pollen-pistil interaction (Lausser et al., 2009). Studies have shown that pollen tube germination to *ga1* is normal on *Ga1-s* silks but pollen tube growth is unable to reach the ovule. One hypothesis may be tested: *ga1* pollen receives insufficient support from a *Ga1-s* sporophyte as the incompatible pollen tube grows along the silk. The silk recognizes the pollen as foreign and stops providing nutrients or enzymes that are essential for complete

pollen tube growth. An experiment can be initiated to answer this question. Near isogenic lines of *Ga1-s* can be either created or obtained from USDA-ARS National Germplasm Collection. Lines are crossed to create four separate crosses: *Ga1-s x Ga1-s, Ga1-s x ga1, ga1 x Ga1-s and ga1 x ga1*. Silk samples can be taken 0.5, 1, 3, 5, 7, 10 and 20 hours after pollination. Proteomic analysis through protein extraction from those silks will be helpful to understand the pollen-pistil interaction at cellular and molecular level. Differentially expressed protein in the crosses with respect to the cross to *Ga1-s x ga1* and their functional analysis will be helpful to understand the incompatibility.

Replication of the current protocol of QTL mapping is being used in another study of mapping the modifiers of the *Tcb1* where we are using inter-mated B73 and Mo17 (IBM) inbred lines. *Tcb1* was also mapped to short arm of chromosome 4, 44 cM from Ga1. *Tcb1* is only found in teosinte. The expression of the *Tcb1* has been found to be polymorphic in the parental lines of B73 and Mo17 in resisting the pollen that lacks the *tcb1*. The inter-mated recombinant lines of B73 and Mo17 has already been made. In summer 2016, about 100 IBM lines were used where in each of these lines, homozygous *Tcb1* in a W22 background was crossed. The efficacy of each of these F1s for resisting the pollen that lacks *Tcb1* will be tested in summer 2017.

Composite interval mapping (CIM) method was used for analyzing the QTL. One of the limitations with CIM is that it lacks the ability to detect the epistatic interaction among the identified QTLs. Multiple interval mapping can be use using the same data for determining the epistatic interaction among the identified QTLs. The QTL interval identified were around 20-25 cM. A simple and conventional fine mapping strategy that can be deployed is screening the mapping population with only two markers located on

either side of the target region. For instance, *PZA00683.4* and *PZA02358.1* are the two flanking markers located on either side of the target marker *PZA00975.1*, located at 25.9 cM on chromosome 4. Plants that do not show recombination between the two markers are rejected and those plants with recombination are retained. These plants and then can be analyzed with a large number of new markers and the markers located nearest to the target genes are identified. Once these markers are identified, marker-assisted introgression of the trait into the desired cultivars can be done. For those QTL with the phenotypic effects only known but no information on protein products, map-based cloning will be a promising technique for isolating and identifying such genes.

Doubled-haploids offer an ideal source of experimental materials to study mutations affecting the quantitative traits in maize (Sprague, 1960). The stable changes observed among the families of the doubled-haploid lines are more probably due to genetic changes. Heritable polymorphisms have been found for number of tassel branches, total number of kernel per ear, number of days to pollen shed and number of days for silk emergence. These results encourage us to pursue molecular analysis that will help us to know the real cause of these polymorphisms. Gene expression analysis or sequence comparison of the polymorphic lines with that of reference genome through whole genome sequencing can be done. For instance, to identify genes governing number of tassel branches, RNA samples from the polymorphic lines can be extracted at the different time points of the tassel development and subjected to gene expression analysis.

The doubled-haploid project lacks estimates of genetic variance. To solve this, we are making F1s from lines that are divergent in a particular trait, for example, tassel branch number. We will self this F1s to make F2s that will help us to estimate

heritability. Also, phenotypic instability was common in most of the traits. So to better understand the instability, we will grow four or more generations of those unstable families for several traits under study. Study of the epigenetics of both stable and instable traits will be useful to dissect the underlying mechanism of complex quantitative traits under the study.

Appendix 1 ANOVA table of the traits measured in 2014

A. Traits significant at P < 0.05

1. ANOVA for number of tassel branch

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	26.23	1.38	3.85	0.00	***
Block	2	0.87	0.44	1.21	0.31	
Residuals	32	11.48	0.36			

2. ANOVA for total number of kernel per ear

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	43886	2309.80	2.68	0.01	**
Block	2	2028	1013.80	1.18	0.32	
Residuals	31	26695	861.10			

3. ANOVA for number of days to silk emergence

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	30.67	1.61	2.12	0.02	*
Block	2	17.04	8.52	11.20	0.00	***
Residuals	37	28.14	0.76			

4. ANOVA for number of leaf length

	Df	Sum sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	64.62	3.40	1.95	0.04	*
Block	2	28.06	14.03	8.05	0.00	**
Residuals	37	64.45	1.74			

5. ANOVA for total number of nodes

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	2.02	0.11	2.17	0.02	*
Block	2	0.23	0.12	2.34	0.11	
Residuals	37	1.82	0.05			

B. Traits that are marginally significant (P value near to 0.05)

1. ANOVA for plant height

	Df	Sum sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	783.50	41.24	1.88	0.05	.
Block	2	213.30	106.63	4.85	0.01	*
Residuals	36	791.70	21.99			

2. ANOVA for node position of the primary ear

	Df	Sum Sq	Mean Sq	F value	Pr (>)	
Lineage-Generation	19	2.43	0.13	1.88	0.05	.
Block	2	0.06	0.03	0.44	0.65	
Residuals	37	2.52	0.07			

3. ANOVA for number of days to pollen shed

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	31.10	1.64	1.85	0.05	.
Block	2	11.34	5.67	6.41	0.00	**
Residuals	37	32.71	0.88			

4. ANOVA for yield

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	1428.70	75.20	1.74	0.08	.
Block	2	349.10	174.55	4.04	0.03	*
Residuals	31	1340.60	43.24			

C. Traits found to be non-significant (P > 0.05)

1. ANOVA for leaf width

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	19	3.00	0.16	1.25	0.28
Block	2	0.60	0.30	2.35	0.11
Residuals	37	4.69	0.13		

2. ANOVA for leaf area

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	17487	920	1.04	0.44	
Block	2	7805	3903	4.41	0.02	*
Residuals	37	32729	885			

3. ANOVA for ear length

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Lineage-Generation	19	3.21	0.17	1.34	0.23
Block	2	0.21	0.10	0.83	0.45
Residuals	31	3.92	0.13		

4. ANOVA for ear circumference

	Df	Sum sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	19	1.88	0.10	1.38	0.21
Block	2	0.23	0.12	1.64	0.21
Residuals	31	2.22	0.07		

5. ANOVA for number of rows per ear

	Df	Sum sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	19	7.12	0.37	0.82	0.67
Block	2	0.22	0.11	0.24	0.79
Residuals	31	14.10	0.45		

6. ANOVA for hundred grain weight

	Df	Sum sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	19	20.57	1.08	1.50	0.15	
Block	2	10.06	5.03	6.99	0.00	**
Residuals	31	22.32	0.72			

Appendix 2 ANOVA table from the traits measured in 2015

A. Traits significant at P < 0.05

1. ANOVA for number of tassel branch

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	11	43.35	3.94	5.12	0.00	***
Block	2	1.45	0.73	0.95	0.40	
Residuals	22	16.94	0.77			

2. ANOVA for height

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	11	1101.1	100.1	2.92	0.02	*
Block	2	57	28.48	0.83	0.45	
Residuals	22	755.5	34.34			

3. ANOVA for days to pollen shed

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	11	58.77	5.342	5.49	0.00	***
Block	2	0.71	0.354	0.36	0.70	
Residuals	22	21.43	0.974			

4. ANOVA for days for Silk emergence

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	11	53.53	4.87	6.328	0.00	***
Block	2	0.22	0.11	0.146	0.86	
Residuals	22	16.92	0.77			

B. Traits found to be marginally significant (P value near to 0.05)

1. ANOVA for total number of kernel per ear

	Df	Sum Sq	Mean Sq	F value	Pr (>F)	
Lineage-Generation	11	42564	3869	2.04	0.07	.
Block	2	119	60	0.03	0.97	
Residuals	22	41669	1894			

C. Traits found to be non-significant (P > 0.05)

1. ANOVA for number of nodes

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	11	2.54	0.23	1.37	0.25
Block	2	0.18	0.09	0.54	0.59
Residuals	22	3.70	0.17		

2. ANOVA for leaf length

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	11	31.97	2.91	1.02	0.47
Block	2	5.44	2.72	0.95	0.40
Residuals	22	62.93	2.86		

3. ANOVA for nodes position of primary ear

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Lineage-Generation	11	1.55	0.14	0.66	0.76
Block	2	0.43	0.21	0.99	0.39
Residuals	22	4.74	0.22		

Appendix 3

Duncan multiple comparisons of significant traits measured in 2014

Lineage - generation	No. of tassel branch		Total kernel per ear		Leaf length		Height		No. of nodes		Primary ear Node position		Days to silk emergence		Days to pollen shed	
00-5	8.8	bc	357.5	abcd	81.1	ab	176.6	abc	14.2	abcd	8.3	bcd	79.2	abcde	78.9	abc
01-5	9.1	bc	381.2	abc	82.9	a	183.1	a	14.5	a	8.6	ab	79.6	abc	79.4	abc
01-6	9.1	bc	388.8	abc	79.5	bc	170.2	cd	13.9	d	7.9	d	77.8	de	78.1	bc
02-10	8.9	bc	415.6	a	80.7	abc	175.4	abcd	14.4	abc	8.4	abc	78.6	bcde	78.3	bc
02-5	9.5	b	405.4	ab	80.9	ab	174.6	abcd	14.2	abcd	8.4	abc	78.7	bcde	78.8	abc
02-9	8.4	bc	416.1	a	80.6	abc	179.8	abc	14.3	abc	8.4	abc	78.7	bcde	79.4	abc
03-5	8.3	bc	297.9	d	80.8	abc	179.6	abc	14.4	abc	8.4	abc	79.2	abcde	79.1	abc
03-8	9.1	bc	395.6	ab	81.1	ab	178.1	abc	14.5	ab	8.8	a	79.3	abcde	79.7	ab
04-5	8.6	bc	354.4	abcd	81.9	ab	177.6	abc	14.2	abcd	8.4	abcd	78.7	bcde	78.7	bc
04-6	8.4	bc	347.7	bcd	80.8	abc	178.1	abc	14.4	abc	8.6	ab	78.7	bcde	78.9	abc
05-5	9.0	bc	348.2	bcd	82.3	a	180.0	ab	14.4	abc	8.6	ab	79.4	abcd	79.3	abc
05-6	8.5	bc	377.4	abc	80.3	abc	171.1	bcd	14.2	abcd	8.3	bcd	79.4	abcd	79.1	abc
06-5	8.7	bc	369.1	abc	80.9	ab	177.5	abc	14.5	a	8.5	abc	78.1	cde	77.6	c
07-10	8.0	c	329.8	cd	81.3	ab	174.5	abcd	14.5	ab	8.6	ab	78.5	bcde	79.3	abc
07-6	11.4	a	359.2	abcd	82.6	a	167.4	d	14.4	abc	8.3	abcd	80.6	a	79.2	abc
07-9	9.0	bc	341.8	bcd	81.3	ab	175.3	abcd	14.1	bcd	8.0	cd	78.4	bcde	78.1	bc
08-5	8.4	bc	377.8	abc	80.7	abc	177.1	abc	14.3	abcd	8.3	bcd	77.6	e	77.7	c
08-6	9.3	bc	392.8	abc	78.3	c	180.5	ab	14.0	cd	8.4	abc	80.1	ab	80.7	a
09-5	9.0	bc	354.0	abcd	82.4	a	179.2	abc	14.5	a	8.4	abcd	78.4	bcde	78.3	bc
09-6	9.4	b	353.4	abcd	81.3	ab	176.8	abc	14.5	a	8.5	abc	79.1	abcde	79.1	abc

Duncan multiple comparisons of significant traits measured in 2015

Lineage-Generation	No. of tassel branch		Height		Days to pollen shed		Days to silk emergence	
01-6	9.2	bc	149.7	c	72.6	de	72.9	d
01-7	8.9	c	168.1	a	72.8	de	73.1	d
02-10	9.0	bc	165.3	ab	73.3	bcde	73.7	cd
02-11	9.1	bc	165.3	ab	72.9	cde	73.4	cd
03-5	9.5	bc	168.0	a	74.2	bcd	74.9	bc
03-6	9.1	bc	161.4	ab	72.4	de	72.8	d
07-6	10.6	b	163.1	ab	74.7	bc	75.6	ab
07-7	12.5	a	156.0	bc	72.1	e	73.1	d
07-10	8.3	c	157.3	abc	74.8	b	75.3	ab
07-11	9.8	bc	154.5	bc	72.3	de	73.3	d
08-6	8.5	c	163.4	ab	76.5	a	76.6	a
08-7	8.7	c	165.1	ab	72.5	de	73.1	d