Screening and genotyping of *ga1* gene, and genotype x environment interaction of cross incompatibility in maize

by

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ABSTRACT

The steady increase of genetically-modified (GM) corn (*Zea mays* L.) in the U.S. makes production of transgene-free corn more difficult to achieve. A strategy to minimize cross-contamination of non-GM corn with GM corn is by using unilateral cross-incompatibility controlled by the Ga_1^S allele. This allele is transferred to desired germplasm through backcrossing before cross-incompatible Ga_1^S/Ga_1^S lines are obtained. The usefulness of this system in preventing cross-fertilization with foreign ga_1 pollen might be affected by a combination of environmental effects and heritability of the trait. Methods used for selection of cross-incompatibility in maize are based on differential incompatibility of plants to ga_1 pollen. Chapter 2 investigates the efficiency of SSR molecular markers in predicting zygosity at the ga_1 locus compared with conventional phenotyping. Chapter 3 evaluates the effect of genotype, environment, and genotype x environment effects on cross-incompatibility, and estimate its heritability.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation includes two individual experiments that addressed different aspects of cross-incompatibility in maize. The chapters are written in manuscript format for submission to scientific journals appropriate for the content of the paper. Chapter 2 explores the usefulness of molecular markers for selection of cross-incompatibility trait. Chapter 3 evaluates the environmental stability of Ga_1^S allele over different environments, namely years and locations. Figures, tables, and references are listed at the end of each chapter. The last chapter is a summary of general conclusions.

Literature Review

Discovery of *Ga1* Gene

Aberrant segregation of morphological and molecular markers with Mendelian inheritance in maize (*Zea mays* L.) has been attributed to action of gametophyte factors. A well-known mutation whose segregation is affected by gametophyte factor is sugary (su_1) mutation in the short arm of chromosome four. This recessive mutation is characterized by translucent and wrinkled endosperm when dry (Neuffer et al., 1997). Cross of inbred lines with sugary and starchy endosperm (i.e., su_1/su_1 x Su_1/Su_1) normally segregate 25% sugary in the F_2. Reciprocal backcrosses of F_1 with the sugary parent segregate 50% sugary whereas reciprocal backcrosses with the starchy parent do not segregate. However, Correns (1901) observed in F_2 of sugary x 'White Rice' popcorn, a 16% sugary instead of the expected 25% sugary. The result was confirmed by others who also observed F_2 sugary percentages of sugary x 'White Rice' popcorn crosses ranging from 15.1 to 16.2 (Jones 1924, Emerson 1934). The same phenomenon occurred

in the backcross *Su*$_1$/*Su*$_1$ x (*Su*$_1$/*su*$_1$) where less than 50% of the progeny segregated for sugary (Jones 1924; Emerson 1934). The reciprocal backcross (*Su*$_1$/*su*$_1$) x *Su*$_1$/*Su*$_1$ as well reciprocal backcrosses with recessive parent *su*$_1$/*su*$_1$ were normal with 50% of progeny being sugary (Jones 1924; Emerson 1934). These observations were interpreted as result of differential pollen-tube growth rate where gametes with the *Su*$_1$ allele are more competitive than gametes with the *su*$_1$ allele in silks of genotype *Su*$_1$/- (Jones 1924; Emerson 1934).

Emerson (1934) suggested that differential fertilization in crosses between sugary and 'White Rice' popcorn was not controlled by *Su*$_1$ allele itself but by a different allele linked to *Su*$_1$ in 'White Rice' popcorn. He observed in $F_{2:3}$ of sugary x 'White Rice' three different phenotypes producing less than 25% sugary (low sugary), 25% sugary (normal sugary), or more than 25% sugary (high sugary). Likewise, $F_{2:4}$ of low and high-sugary $F_{2:3}$ segregated into low, normal, and high-sugary phenotypes.

Mangelsdorf and Jones (1926) observed an aberrant segregation of defective kernel (*dek*) mutation in F_2 and subsequent self-pollinations of *Dek*/*Dek* x *dek*/*dek*. This mutation is recessive and it is characterized by reduced size in endosperm and embryo (Neuffer et al. 1997). In contrast with F_2 of sugary x 'White Rice' where a deficiency of sugary seeds was observed, the cross *Dek*/*Dek* x *dek*/*dek* resulted in excess of 25% defective in F_2 generation. The $F_{2:3}$ segregated with less than 25% defective (low defective), 25% defective (normal defective), and more than 25% defective (high defective). The authors concluded that abnormal segregation of defective kernel to sugary mutations were due to differential rate in pollen-tube growth caused by a common gametophyte allele linked to *dek* and *Su*$_1$ which they designated as *Ga*$_1$. The allele was

considered “dominant” because pollen tubes with the dominant allele grew faster than pollen tubes with the recessive allele on silks with heterozygous or homozygous dominant genotypes.

A common procedure for testing differential rate of pollen-tube growth consists of separating the silks of individual shoot ears in two portions (top and bottom) followed by pollination with a mixture of pollen. Mangelsdorf and Jones (1926) divided the silks of ear shoots of ‘White Rice’ popcorn and ‘Golden Bantam’ sweet corn in two portions which were pollinated with a mixture of pollen from both varieties. ‘White Rice’ has genotype *Su₁Ga₁*/*Su₁Ga₁* whereas ‘Golden Bantam’ has genotype *su₁ga₁*/*su₁ga₁*. In ear shoots of ‘Golden Bantam’, almost the same number of sugary seeds was observed in both portions of ear shoots. However, in ‘White Rice’ the number of sugary seeds observed was greater in top portion of ear shoots. The authors concluded that differential fertilization did not occur in sweet corn because the *Ga₁* allele was not present in the silks and thus, *su₁ga₁* pollen tubes were able to grow at the same rate as *Su₁Ga₁* pollen tubes. Pollen tubes with *ga₁* allele grew slower than pollen tubes with *Ga₁* allele in ‘White Rice’ resulting in less sugary seeds far from the ear tip.

Linkage of *Ga₁* and Other Markers in Chromosome Four

The abnormal segregation of sugary in F_2 of *Ga₁Su₁*/*ga₁su₁* and defective kernels in F_2 of *Ga₁dek*/*ga₁Dek* is due to linkage of *Ga₁* with *Su₁* and *dek*, respectively (Emerson 1934). Emerson (1934) reported a recombination frequency of 47% or 75cM between *Su₁* and *dek* loci. The author positioned the allele *Ga₁* between *Su₁* and *dek* because it affected both of these alleles almost equally.

The magnitude of deviation in sugary and defective seeds in F_2's of sugary x 'White Rice' and *Ga₁dek*/*ga₁Dek* is not only related to the frequency of recombination between *Ga₁* and marker loci but also to ratio in which *Ga₁* and *ga₁* gametes occur at fertilization (Mangelsdorf and Jones 1926). If there were no linkage between *Su₁Ga₁* and *Ga₁dek*, the disturbing effect of *Ga₁* would be zero and existence of gametophyte factors unsuspected (Emerson 1934; Nelson 1993). Also, if *Ga₁* and *ga₁* gametes were equally effective in fertilization, no disturbance in the ratio of other genes would be detected.

The recombination frequency between any two loci must be in the range of 0 to 50%, and the ratio of *Ga₁* to *ga₁* gametes occurring at fertilization may be 1:1 to 1:0. Different combinations of recombination frequencies and *Ga₁* to *ga₁* ratios may be responsible for observed deviations. Mangelsdorf and Jones (1926) estimated the recombination frequency between *Ga₁*, and *dek* and *Su₁* loci by creating a curve for each of these two intervals with different combinations of recombination and *Ga₁* to *ga₁* ratio that can produce, in theory, the observed deviation of sugary and defective kernels in two separate F_2 populations. The problem was to determine at what point on the *x*-axis an ordinate could be traced to intersect the two curves at points whose recombination values converted into map distance and combined would equal 75 cM, the genetic distance between *dek* and *Su₁*. A ratio of 40 *Ga₁*: 1 *ga₁* (i.e., 2.5% functioning of *ga₁*) resulted in 34.9% recombination frequency between *dek* and *Ga₁* and 28.5% recombination frequency between *Ga₁* and *Su₁* (Emerson 1934). The conversion of these recombination values into map units resulted in genetic distances of 42.4 cM between *dek* and *Ga₁*, 32.6 cM between *Ga₁* and *Su₁*, and a total of 75 cM between *dek* and *Su₁*.

The fact that *ga*$_1$ gametes are almost always excluded from fertilization on *Ga*$_1$/*Ga*$_1$ silks when in competition with *Ga*$_1$ was shown by pollination of 'White Rice' popcorn with a pollen mixture of *Ga*$_1$ and *ga*$_1$ (Emerson 1934). The average number of seeds resulting from fertilization of *ga*$_1$ gametes was calculated as 4%. The recombination frequency between *Su*$_1$ and *Ga*$_1$ assuming 4% effectiveness of *ga*$_1$ was estimated as 27.8%. Pollination of *ga*$_1$/*ga*$_1$ silks with pollen mixture of *Ga*$_1$ and *ga*$_1$ resulted in approximately 50% seeds of genotype *ga*$_1$/*ga*$_1$. No selective fertilization occurs in this case because the female plant is homozygous recessive.

Discovery of *Ga*$_1^S$ Allele and Cross Incompatibility in Maize

Demerec (1929) reported that pollination of 'White Rice' popcorn with a mixture of its own pollen and pollen from a plant of purple-shrunken endosperm resulted, on average, in 1% purple-shrunken seeds. However, pollination of purple-shrunken plants with a mixture of their own pollen and pollen from 'White Rice' produced, on average, 35% seeds of 'White Rice'. The author concluded that mechanism of differential fertility in 'White Rice' popcorn was due to cross incompatibility and not to differential pollen-tube growth between *Ga*$_1$ and *ga*$_1$ pollen on *Ga*$_1$ silks as postulated by Mangelsdorf and Jones (1926). Nelson (1952) questioned the identity of cross-incompatible popcorn used by Demerec (1929), and concluded the variety was a 'Pearl'-type popcorn and not 'White Rice'. The fact that cross incompatibility in 'Pearl' and 'White Rice' popcorns was controlled by similar gametophyte factor was supported by reduced sugary (12.4%) in F_2 of sugary x 'Pearl', and 50% sugary in the backcross of F_1 to sugary (Demerec 1929).

Another case of cross incompatibility similar to that observed in 'Pearl' popcorn was reported on inbred line D139 by Schwartz (1950). The author observed that

homozygous plants of D139 were completely sterile to *ga*$_1$ pollen even in the absence of competition by *Ga*$_1$ pollen. However, in 'White Rice' popcorn (*Ga*$_1$/*Ga*$_1$), pollination with *ga*$_1$ pollen resulted in full-seed set. The author designated the allele that controls cross incompatibility in D139 as *Ga*$_1^S$ because the allele had a stronger effect than *Ga*$_1$ allele. The F_2's of *su*$_1$/*su*$_1$ x D139 and *su*$_1$/*su*$_1$ x 'Pearl' produced 12.9 and 12.4% sugary, respectively (Schwartz, 1950). Nelson (1952) obtained 13.9 to 15.5% sugary in F_2 of *su*$_1$/*su*$_1$ x incompatible popcorns. Allelism of *Ga*$_1$ and *Ga*$_1^S$ was supported by crosses *Ga*$_1^S$/*ga*$_1$ x *Ga*$_1$/*ga*$_1$ and *Ga*$_1^S$/*Ga*$_1$ x *Ga*$_1^S$/*ga*$_1$ in which *ga*$_1$ gametes were poor competitors on heterozygous silks and the cross *Ga*$_1^S$/*Ga*$_1^S$ x *Ga*$_1$/*Ga*$_1$ was fully compatible.

Allelism of *Ga*$_1$ and *Ga*$_1^S$ was supported by percent of sugary in backcrosses. Emerson (1934) obtained 30.3% and 67.8% sugary in *Ga*$_1$*su*$_1$/*Ga*$_1$*su*$_1$ x *Ga*$_1$*Su*$_1$/*ga*$_1$*su*$_1$ and *Ga*$_1$*su*$_1$/*Ga*$_1$*su*$_1$ x *Ga*$_1$*su*$_1$/*ga*$_1$*Su*$_1$, respectively. The weighted average of sugary on these backcrosses was 30.9%. Emerson (1934) assumed the percentage of sugary in these crosses was an indication of recombination frequency between *Su*$_1$ and *Ga*$_1$ assuming 0% functioning of *ga*$_1$ pollen when in competition with *Ga*$_1$. For example, sugary seeds in cross *Ga*$_1$*su*$_1$/*Ga*$_1$*su*$_1$ x *Ga*$_1$*Su*$_1$/*ga*$_1$*su*$_1$ are produced by recombinant *Ga*$_1$*su*$_1$ gametes in male parent. Schwartz (1950) obtained 25.8% sugary in similar crosses in which *Ga*$_1$ allele was substituted by *Ga*$_1^S$. This percentage was very similar to the recombination frequency of 27.8% calculated by Emerson (1934) assuming 4% functioning of *ga*$_1$ gametes. The difference between 30.9% and 25.8% sugary is due to the amount of *ga*$_1$ pollen which functions in competition with *Ga*$_1$. When *Ga*$_1$ and *Ga*$_1^S$ pollen grains compete, the latter has a definitive advantage in achieving fertilization on both *Ga*$_1$/*Ga*$_1$ and *Ga*$_1^S$/*Ga*$_1^S$ silks (Schwartz 1950).

Mechanism of Selective Fertilization and Gene Action by Ga_1^S

Kermicle and Evans (2005) showed that Ga_1^S allele controls nonreciprocal crossability with ga_1 allele by means of allele-specific congruence rather than active rejection. Failure of fertilization could reflect active rejection by the pistil of pollen containing a contrasting allele (incompatibility). Alternatively, the pistil could require the presence of a matching allele in the pollen (congruity) for a cross to be successful. The authors pollinated plants with genotypes Ga_1^S/Ga_1^S and ga_1/ga_1 with heteroallelic $Ga_1^S ga_1$ pollen grains to differentiate between these two possibilities. Heteroallelic pollen grains have two alleles in a locus. If there was active rejection, heteroallelic pollen would not be accepted; if presence of a matching allele is required, heteroallelic pollen would be accepted. The authors observed that all plants were successfully crossed with heteroallelic pollen indicating the requirement of matching allele in both pollen and pistil (Kermicle and Evans 2005). The incongruity model explain why plants with genotype Ga_1^S/Ga_1^S are incongruous to ga_1/ga_1 pollen as occurs normally in crosses between incompatible popcorns and dent corn.

Designation of Ga_1^S and ga_1 in pollen derives from behavior of pistils, since being haploid the question of dominance does not arise for pollen (Mangelsdorf and Jones, 1926; Kermicle and Evans, 2005). The allele Ga_1^S is considered dominant because heterozygous pistils select against ga_1 when Ga_1^S pollen grains are present in the silks. However, if Ga_1^S pollen is absent in silks, only Ga_1^S/Ga_1^S plants are incompatible to ga_1 pollen but not Ga_1^S/ga_1 or ga_1/ga_1 which would be compatible (Nelson, 1952). In this case, the trait shows recessive gene action. For the sake of simplicity, incongruous

genotypes (i.e., Ga_1^S/Ga_1^S) will be referred as cross-incompatible throughout this dissertation.

As mentioned before, Demerec (1929) claimed that selective fertilization in 'Pearl' popcorn was due to cross incompatibility rather than differential pollen-tube growth. The author hypothesized that cross incompatibility might be caused by inability of ga_1 pollen grains to germinate on silks with dominant allele Ga_1, inability of ga_1 pollen tubes to grow on silks with dominant allele Ga_1, or inability of ga_1 pollen tubes to reach the ovules on silks with dominant allele Ga_1. However, it was shown that ga_1 pollen can germinate on Ga_1^S/Ga_1^S silks, and pollen tubes of ga_1 and Ga_1^S can grow at the same rate on Ga_1^S/Ga_1^S silks (Schwartz 1950; Nelson 1952).

Gametophytic or sporophytic-cross incompatibility?

Kermicle and Evans (2005) indicated that female plants with genotype Ga_1^S/Ga_1^S are incompatible and semi-compatible to ga_1/ga_1 and Ga_1^S/ga_1 pollen, respectively. These observations indicate that behavior of pollen is controlled by genotype of individual pollen grains rather than parent sporophyte, hence the locus name *gametophyte factor-1*. Gametophytic control occurs only at the pollen level whereas control of pistil behavior is sporophytic, with Ga_1^S barrier being less strong in heterozygous than homozygous plants.

Pollen-tube growth is known to be critical in non-reciprocal cross incompatibility of ga_1 pollen grains on Ga_1^S/Ga_1^S silks (House and Nelson 1958). Lausser et al. (2010) studied in detail the Ga_1^S crossing barrier within maize and its close relative, *Tripsacum dactyloides*, at the cellular level. Growth of pollen tubes of *T. dactyloides* on Ga_1^S/Ga_1^S, Ga_1^S/ga_1, and ga_1/ga_1 silks was arrested within the first 4 to 8 cm from pollination site.

Most of these pollen tubes grew outside of the transmitting tract in a process called transmitting-tract mistargeting. However, pollen tubes in reciprocal crosses grew normally. Growth of *ga*$_1$ pollen tubes on *Ga*$_1^S$/*Ga*$_1^S$ silks was arrested after a shorter distance than *T. dactyloides* whereas no differences were observed in growth of pollen tubes of *Ga*$_1^S$/*Ga*$_1^S$ and *Ga*$_1^S$/*ga*$_1$ pollen on heterozygous *Ga*$_1^S$/*ga*$_1$ silks. Development of *ga*$_1$ pollen tubes on heterozygous silks was intermediate whereas pollen tubes of *Ga*$_1^S$/*Ga*$_1^S$ and *Ga*$_1^S$/*ga*$_1$ had normal growth. Pollen from all three genotypes of maize grew normally on *ga*$_1$/*ga*$_1$ silks. Lausser et al. (2010) suggested that lack of further growth support by sporophytic tissues might represent the major cause of tube arrest. The authors shown using RNA interference (RNAi) that female gametophyte (i.e., embryo sac) played an important role in guidance of pollen-tube growth in micropyle proximity.

Male-Only Function of *Ga*$_1^M$ Allele

Nelson (1952) conducted extensive testing of popcorn inbred lines and field corn inbred lines for cross incompatibility. He classified germplasm into three different classes based on number of kernels produced after cross pollination. The cross-neutral group is compatible with all inbred lines when used either as male or female. The cross-fertile group produces full-seed set when pollinated with any inbred line but its pollen can only fertilize inbred lines from the cross-neutral and cross-fertile groups. The third class, cross incompatible, successfully pollinate all inbred lines but is incompatible to pollen from the cross-fertile group. The genotype of incompatible popcorn lines was assumed to be *Ga*$_1^S$/*Ga*$_1^S$ because they behaved similarly to D139, the *Ga*$_1^S$/*Ga*$_1^S$ cross-incompatible inbred line used by Schwartz (1950), whereas the genotype of cross-fertile inbred lines

was assumed to be ga_1/ga_1 because they were similar to dent inbred line Hy in their behavior. The cross-neutral group, represented by 'White Rice' popcorn, was assumed to have the genotype Ga_1/Ga_1 because it could be fertilized by ga_1/ga_1 pollen and pollen of this group is capable of fertilizing Ga_1^S/Ga_1^S plants.

Jiménez and Nelson (1965) reported that male-only function on inbred line 4519 was controlled by a different gametophyte locus on chromosome 4 which they called Ga_9^M (the subscript denoted the number of gametophyte factors already identified at this time and the superscript denoted male-action). Using a full diallel design, the authors self-pollinated and intercrossed five F_1's of $Ga_1^SSu_1/ga_1su_1$, an F_1 of Ga_1Su_1/ga_1su_1, and an F_1 of $Ga_9^MSu_1/ga_1su_1$. The average percentage of sugary seeds in all self-pollinations was significantly lower than 25% except in the F_1 of $Ga_9^MSu_1/ga_1su_1$ where the percentage was 26.54%. Similarly, all reciprocal intercrosses between $Ga_1^SSu_1/ga_1su_1$ and Ga_1Su_1/ga_1su_1 resulted in less than 25% sugary. All intercrosses involving $Ga_9^MSu_1/ga_1su_1$ as the female parent resulted, on average, in 25% sugary. However, in intercrosses where $Ga_9^MSu_1/ga_1su_1$ was used as the male parent, less than 25% sugary was obtained. These results suggested that gametophyte factor carried by 4519 is restricted to the male (Jiménez and Nelson 1965).

Jiménez and Nelson (1965) tested the possibility of the factor being an allele of ga_1 locus with male-only function (i.e., Ga_1^M) or an allele in a different locus equidistant from su_1 locus and on the other side of su_1 (i.e., Ga_9^M). If the gametophyte factor on inbred line 4519 was allelic to Ga_1^M, then the three-way cross between sweet corn inbred P51 (ga_1su_1/ga_1su_1) and F_1 of inbreds 4519 ($Ga_1^MSu_1/Ga_1^MSu_1$) and D139 ($Ga_1^SSu_1/Ga_1^SSu_1$) [i.e., ga_1su_1/ga_1su_1 x ($Ga_1^MSu_1/Ga_1^SSu_1$)] would be expected to

produce progeny with only two genotypes (i.e., $ga_1su_1/Ga_1^{M}Su_1$ and $ga_1su_1/Ga_1^{S}Su_1$). However, if the gametophyte factor on 4519 was a different locus on the other side of sugary, the three-way cross $ga_9su_1ga_1/ga_9su_1ga_1$ x ($Ga_9^{M}Su_1ga_1/ga_9Su_1Ga_1^{S}$) would be expected to produce progeny with four genotypes (i.e., $ga_9su_1ga_1/Ga_9^{M}Su_1ga_1$, $ga_9su_1ga_1/ga_9Su_1Ga_1^{S}$, $ga_9su_1ga_1/Ga_9^{M}Su_1Ga_1^{S}$, and $ga_9su_1ga_1/ga_9Su_1ga_1$). Genotyping of progeny was made on the basis of expected percent of sugary seeds upon self-pollination and crossing of progeny with $ga_1su_1/Ga_1^{S}Su_1$ tester in reciprocal crosses (Table 1 and Table 2). All three-way crosses made by the authors resulted in three classes of progeny suggesting that Ga_9^{M} model was correct.

Ashman (1981) showed that Ga_9^{M} was actually an allele of *gametophyte factor-1* (Ga_1^{M}) and not a separate locus as claimed by Jiménez and Nelson (1965). The author made the cross $c_2ga_9su_1Ga_1^{S}/c_2ga_9su_1Ga_1^{S}$ x $c_2ga_9su_1ga_1/C_2Ga_9^{M}Su_1ga_1$ to verify the competitive advantage of Ga_9^{M} over ga_9 pollen grains on Ga_1^{S}/Ga_1^{S} silks. The percent of sugary seeds and c_2 kernels in this cross were 23% and 46.9%, respectively. A second cross, $c_2ga_9su_1ga_1/c_2ga_9su_1ga_1$ x $c_2ga_9su_1ga_1/C_2Ga_9^{M}Su_1ga_1$, serves to verify the lack of differential fertilization between Ga_9^{M} and ga_9 pollen grains on ga_9/ga_9 silks. Percentages of sugary and c_2 kernels were approximately 50 and 53%, respectively. The author concluded the gametophyte factor in this stock segregated as an allele of Ga_1 locus.

Modification of Cross Incompatibility by Modifier Genes

Nelson (1952) obtained 13.9 to 15.5% sugary in F_2 of crosses between sweet corn (ga_1su_1/ga_1su_1) and four different cross-incompatible popcorn inbred lines ($Ga_1^{S}Su_1/Ga_1^{S}Su_1$). However, these values were significantly higher than 12.3%, the

sugary percent he obtained in cross of sweet corn x Ga_1^S/Ga_1^S inbred line D139. To explain this, Nelson (1952) suggested that cross-incompatible popcorns might have a different allele that behaved similar to Ga_1^S based on cross pollinations with pollen from dent inbred line Hy but different enough so that some ga_1 gametes function when in competition with dominant gametes carrying Ga_1^S. The other possibility the author suggested was that popcorn inbred lines might posses different sets of modifying genes rather than different alleles. The author crossed the sterile-popcorn inbred line 1001 KKB with a mixture of pollen from dent inbred line Hy, and pollen from cross-fertile popcorn line 1708. Inbred line 1001 KKB was incompatible with Hy pollen but compatible with 1708 pollen. However, a sister inbred line of 1001 KKB, 1001-52, was incompatible with 1708. To rule out the possibility of different alleles in Hy and 1708, three-way cross (Hy x 1708) x Hy was used as male in crosses with 1001 KKB. Assuming inbred lines in the single cross have different alleles (i.e., ga_{HY} and ga_{1708}), two different kinds of progenies would be expected from three-way cross. One type of progeny, ga_{HY}/ga_{HY}, would not be able to fertilize line 1001 KKB whereas the second type of progeny, ga_{HY}/ga_{1708}, would be able to fertilize inbred line 1001 KKB because one half of the gametes would be ga_{1708}. It turn out that inbred line 1001 KKB was incompatible with pollen from these two progenies and the author concluded difference between Hy and 1708 was likely due to different modifier genes rather than to different alleles.

To compare the effectiveness of compatible popcorn inbreds and field corn inbred lines in fertilizing cross-incompatible popcorns, Whiteley (1953) pollinated cross-incompatible inbred lines 15 (‘South American’ popcorn), Tc232 (‘Superb’ popcorn), and

W5 (‘Japanese Hulless’ popcorn) with pollen of forty different fertile popcorn inbred lines of ‘Supergold’, ‘Yellow ‘Pearl’, ‘Queen’s Golden’, and ‘Golden Amber’ varieties. Also, he pollinated fifteen incompatible lines derived from ‘South American’ and ‘Japanese Hulless’ varieties with pollen of Hy (‘Illinois High Yield’), WF9 (‘Reid Yellow Dent’), L317 (‘Lancaster’), 187-2 (‘Krug’), and 4Co63 (‘Four County White’). The cross-incompatible popcorns reacted similarly when pollinated by cross-fertile popcorns and dent field corns by producing less seed set than would be expected in compatible crosses. The variable number of kernels obtained from ear to ear within crosses was attributed to contamination by self pollination and/or to differences in silk lengths among ears at the time of pollination. The only exception was some lines from ‘Queen’s Golden’ and ‘Golden Amber’ had a tendency to produce more seeds on incompatible popcorns. Whiteley (1953) concluded that fertile popcorns and dent corn inbred lines carried the ga_1 allele whereas the incompatible popcorns carried the Ga_1^S allele. The author concluded that differences in seed set in hybrid combinations were due to modifier genes.

Inheritance Studies of Ga_1^S

Nelson (1952) and Whiteley (1953) conducted tests to determine the inheritance of cross incompatibility in popcorn on the basis of seed set with dent-corn pollen. They created segregating populations between fertile and cross incompatible popcorns. The F_1, F_2, and reciprocal backcrosses were detasseled and allowed to pollinate with dent corn pollen in isolation. The F_1 was completely fertile to pollen from dent corn. In backcrosses with cross incompatible line as male parent (i.e., Ga_1^S/ga_1 x Ga_1^S/Ga_1^S), two phenotypic classes would be expected in equal proportions among the progeny. The class

Ga_1^S/Ga_1^S would be cross incompatible with dent pollen whereas the class Ga_1^S/ga_1 would be fertile. When the number of cross incompatible and cross fertile progenies were pooled, Nelson (1952) and Whiteley (1953) obtained a ratio of 50% cross incompatible to 50% cross fertile progenies. However, in a few backcrosses the number of cross incompatible progenies obtained was greater than the number of cross-fertile progenies. The explanation given for the abnormal segregation was that Ga_1^S/ga_1 progenies show little to no seed set when pollinated with ga_1 pollen and thus, were indistinguishable from Ga_1^S/Ga_1^S progenies. The F_1 of these particular crosses also had low seed set or were partially receptive.

In backcrosses with cross incompatible line as female parent (i.e., Ga_1^S/Ga_1^S x Ga_1^S/ga_1), only cross incompatible progenies would be expected (Ga_1^S/Ga_1^S) assuming 0% effectiveness of ga_1 gametes when Ga_1^S gametes were also present in silks. In two of such backcrosses, the number of cross incompatible progenies ranged from 99.1 to 100%. However, Whiteley (1953) reported segregation for incompatibility in three similar backcrosses. He attributed the unexpected results to the effect of modifier genes in cross-fertile inbred line used.

Whiteley (1953) made reciprocal backcrosses with cross-fertile parents as recurrent parents. In such backcrosses (i.e., Ga_1^S/ga_1 x ga_1/ga_1 and ga_1/ga_1 x Ga_1^S/ga_1), all progeny were expected to be cross fertile because heterozygous individuals are known to be fully compatible with dent-corn pollen. However, the author obtained 0 to 13.6% cross-incompatible progeny when the cross-fertile parent (ga_1/ga_1) was used as male and 0 to 17.1% when used as female. Backcrosses that produced higher percentage of cross incompatible progenies only produced partial seed set in the F_1. The author concluded

that in some cases, heterozygous individuals might be indistinguishable from homozygote dominants in their reaction to ga_1 pollen by producing partial or no seed set at all.

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Table 1. Expected percent of sugary seeds on progeny of three-way cross *ga₁su₁/ga₁su₁* x (*Ga₁^M Su₁/Ga₁^S Su₁*) upon self-pollination, and reciprocal cross with tester *ga₁su₁/Ga₁^S Su₁*.

	Expected percent of sugary seeds		
Genotype of progeny	Self-pollination	Female[1]	Male[2]
ga₁su₁/Ga₁^M Su₁	25	25	14
ga₁su₁/Ga₁^S Su₁	14	14	14

[1] Tester used as male parent.
[2] Tester used as female parent.

Table 2. Expected percent of sugary seeds on progeny of three-way cross *ga*$_9$*su*$_1$*ga*$_1$/*ga*$_9$*su*$_1$*ga*$_1$ x (*Ga*$_9^M$*Su*$_1$*ga*$_1$/*ga*$_9$*Su*$_1$*Ga*$_1^S$) upon self-pollination, and reciprocal cross with tester *ga*$_1$*su*$_1$/*Ga*$_1^S$*Su*$_1$.

	Expected percent of sugary seeds		
Genotype of progeny	Self-pollination	Female[1]	Male[2]
ga$_9$*su*$_1$*ga*$_1$/*Ga*$_9^M$*Su*$_1$*ga*$_1$	25	25	14
ga$_9$*su*$_1$*ga*$_1$/*ga*$_9$*Su*$_1$*Ga*$_1^S$	14	14	14
ga$_9$*su*$_1$*ga*$_1$/*Ga*$_9^M$*Su*$_1$*Ga*$_1^S$[3]	-	-	-
ga$_9$*su*$_1$*ga*$_1$/*ga*$_9$*Su*$_1$*ga*$_1$	25	25	25

[1] Tester used as male parent.
[2] Tester used as female parent.
[3] No *a priori* basis for prediction.

CHAPTER 2: PHENOTYPING *VS* SSR MARKERS FOR PREDICTION OF ZYGOSITY AT GA1 LOCUS IN MAIZE

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Abstract

Discrimination of zygosity at ga_1 locus in maize (*Zea mays* L.) is a critical step for developing inbred lines that are incompatible to ga_1 pollen. Our objective was to evaluate the efficiency of simple-sequence repeat (SSR) markers in predicting zygosity at ga_1 locus. Twenty-eight SSR markers in close proximity to ga_1 were used to screen the recurrent parent B114 (ga_1/ga_1), the donor parent Mo508W/Mo506W (Ga_1^S/Ga_1^S), and the Ga_1^S/ga_1 hybrid. A backcross population was phenotyped for cross-incompatibility by a pollen-mix method and genotyped with molecular markers. Eighty-six percent of the population produced less than 10% seed set with ga_1 pollen whereas ten percent of the population produced 20 to 40% seed set with ga_1 pollen. Four percent of the population produced more than 60% seed set with ga_1 pollen. Segregation of SSR markers *phi021*, *umc2410*, and *umc1294* was significantly different from the expected ratio of 1:2:1. The SSR marker *phi021* correctly predicted 70% of genotypes at ga_1 locus whereas markers *umc2410* and *umc1294* predicted 65 and 48% of correct genotypes, respectively. The evidence suggests that use of molecular markers for the purpose of predicting zygosity at the ga_1 locus is inefficient.

Introduction

The genetic control of cross-incompatibility in maize (*Zea mays* L.) has been associated with numerous gametophytic genes. The ga_1 gene has received more attention because the allele Ga_1^S confers cross-incompatibility to ga_1 pollen (Nelson, 1993). The Ga_1^S allele has been found in some popcorn inbred lines and commercial hybrids grown in tropical and subtropical regions in Mexico (Nelson 1993; Whiteley 1953; de la Cruz et al. 2008). Dent and flint corn grown in the USA are of type ga_1/ga_1 (Nelson 1993). The Ga_1^S allele has been used for development of popcorn and sweet corn lines that are cross-incompatible to dent-corn pollen resulting in a reduction of cross contamination in grain-production fields (Perry, 1945; Ziegler and Ashman, 2001). The successful use of this genetic barrier to reduce cross contamination in specialty corns suggest that the system may be adaptable to the production of conventional corn grown under organic-farming conditions (Roseboro, 2008).

Conversion of compatible germplasm into incompatible has been accomplished through backcrossing. The first step is cross of incompatible ga_1/ga_1 with a Ga_1^S/Ga_1^S donor parent to produce F_1. The strategies used for the evaluation of zygosity at ga_1 in segregating populations are based on differential seed set resulting from crosses with ga_1 pollen. For example, Ziegler and Ashman (2001) screened and advanced popcorn populations segregating for Ga_1^S by crossing BC_1F_1 to the ga_1/ga_1 parent followed by self-pollination and crossing of BC_1F_1 to a ga_1/ga_1 stock that produce purple aleurone in seeds. BC_1F_1 progenies with lowest percent of purple seeds are classified as heterozygous Ga_1^S/ga_1. BC_2F_1 resulting from a heterozygous BC_1F_1 plant is used for subsequent backcrosses. A similar strategy consist in crossing BC_1F_1 to the recurrent

parent ga_1/ga_1 to produce BC_2F_1 and to the donor parent Ga_1^S/Ga_1^S to discriminate among the different genotypes present in BC_1F_1 (Thomas, 1955). Whiteley (1953) proposed to generate the F_2 of ga_1/ga_1 x Ga_1^S/Ga_1^S first followed by crossing to ga_1/ga_1 parent. The cross with ga_1/ga_1 parent serve to identify Ga_1^S/Ga_1^S genotypes because they are expected to produce few or no seeds with ga_1 pollen whereas Ga_1^S/ga_1 and ga_1/ga_1 genotypes are receptive to ga_1 pollen.

A common characteristic of the strategies discussed above is that progenies are classified either as compatible or incompatible to ga_1 pollen according to arbitrary seed-set thresholds. Furthermore, zygosity at ga_1 locus could be inferred by the amount of seed set produced by ga_1 pollen when Ga_1^S is present. Phenotypic separation of F_2 progeny with genotypes Ga_1^S/Ga_1^S, Ga_1^S/ga_1, and ga_1/ga_1 has been made by using pollen mixtures containing self-pollen and ga_1 pollen of blue corn (Thomas, 1955). This author reported that Ga_1^S/ga_1 genotypes were compatible to ga_1 blue pollen even in the presence of Ga_1^S pollen grains from self-pollination. Another study, however, suggest that ga_1 pollen grains are eliminated during self-pollination of Ga_1^S/ga_1 genotypes (Nelson, 1952). This author observed only Ga_1^S/Ga_1^S and Ga_1^S/ga_1 progenies resulting from the backcross Ga_1^S/Ga_1^S x (Ga_1^S/ga_1), and suggested that ga_1 gametes were completely eliminated in presence of Ga_1^S pollen. However, his results from self-pollination of Ga_1^S/ga_1 genotypes were inconclusive. These studies indicate that alternate methods for designation of zygosity at ga_1 locus are needed.

Cross incompatibility in maize depends on genotypic constitution of silks and pollen grains, and the direction of hybridization (Table 1). Inbred lines of genotype Ga_1^S/Ga_1^S are incompatible to ga_1 pollen. However, the reciprocal cross is fully

compatible (Schwartz, 1950; Nelson, 1952). On the other hand, heterozygous individuals are receptive to both Ga_1^S and ga_1 pollen when applied separately. In this case, the Ga_1^S allele behave as a true recessive because Ga_1^S/ga_1 genotypes are indistinguishable from the ga_1/ga_1 genotypes. When a mixture of Ga_1^S and ga_1 pollen is used, however, the dominance relationship is inverted with heterozygous individuals being indistinguishable from Ga_1^S/Ga_1^S. Therefore, there would be a possibility of selecting erroneously heterozygous genotypes in a F_2 population with a pollen assay as if they were true incompatible lines of genotype Ga_1^S/Ga_1^S.

Use of co-dominant molecular markers might be an alternative for genotype discrimination in populations segregating for Ga_1^S allele. Marker-assisted selection for self-incompatibility has been used in stone-fruit breeding (Testolin, 2003). However, genotyping of maize populations with molecular markers for the purpose of discriminating between Ga_1^S/Ga_1^S and Ga_1^S/ga_1 genotypes for cross incompatibility has not yet been investigated. Therefore, the objective of our study was to evaluate the efficacy of simple-sequence repeat (SSR) markers in separating the genotypes Ga_1^S/Ga_1^S and Ga_1^S/ga_1.

Materials and Methods

Plant Material and Population Development

The inbred line B114 (ga_1/ga_1) was crossed to hybrid Mo508W/Mo506W (Ga_1^S/Ga_1^S), and the F_1 backcrossed to B114 (Figure 1). The inbred line B114 is a pedigree selection from CIMMYT pool 41, a genetically-diverse population developed for temperate climates (Hallauer et al., 2000). The pedigrees of inbred lines Mo508W

and Mo506W are H30-Ga_1^S and K6-Ga_1^S, respectively (Gerdes et al., 1993). The popcorn source of Ga_1^S allele in these pedigrees is unknown.

Fifteen B114 plants and fifteen BC_1F_1 plants were grown side-by-side. A pollen-mix technique was used for phenotypic selection of heterozygous Ga_1^S/ga_1 progenies in backcrosses (Thomas, 1955; Ziegler and Ashman, 2001). Pollen collected from individual BC_1F_1 plants was divided into approximately two equal amounts; one-half was used for self-pollination, and the remainder was used for backcrossing a single plant of B114 from which the BC_2F_1 was obtained. Approximately the same amount of pollen from 'Nokomis Blue' (*Z. mays* L.) was applied the same day to the silks of self-pollinated BC_1F_1 plants to produce a mixture of two different types of pollen on silks. 'Nokomis Blue' is a blue-kernelled derivative of the 'Nokomis Gold' population (Jaradat et al., 2010). The genes responsible for anthocyanin pigmentation in seed aleurone were expected to segregate independently from ga_1 gene as described by Neuffer et al. (1997). Plants with the fewest number of blue seeds were selected and classified as incompatible to ga_1 pollen. For example, a BC_1F_1 plant that produced 247 yellow and one blue seeds was classified as heterozygous Ga_1^S/ga_1 and its BC_2F_1 progeny retained for the next backcross. The same procedures were repeated for the next two cycles of backcrossing. One BC_2F_1 plant with 181 yellow and two blue seeds and one BC_3F_1 plant with 267 yellow and five blue seeds were classified as heterozygous.

A population of 96 BC_3F_2 was used in this study. This population was obtained by self-pollination of a heterozygous BC_3F_1 plant. All individuals of this population were self-pollinated and crossed to 'Nokomis Blue' as described above, and ears harvested and shelled individually. Separation of blue and yellow seeds that resulted from mix

pollinations was performed by using a Scan Master II color sorter (model SM-200IE, Satake USA, Stafford, TX). The machine consists of two closely spaced 10-channel chutes with a variable speed vibratory feeder. The 10-channel chutes were reduced to 2 channels to sort small amounts of seeds. The setup consisted of orange filters (-11), marigold (-1) backgrounds, and white lamps (Designer 3500K). Each sample was passed through the color sorter three times, beginning with the primary sort, resort of the reject fraction, and secondary sort of the blended accepts from the primary and resort passes. Percent of blue seed set per plant was calculated and used as a measure of cross-fertilization with *ga*$_1$ pollen grains.

SSR Genotyping

Twenty-four seeds of each B114 (*ga*$_1$/*ga*$_1$), Mo508W/Mo506W (*Ga*$_1^S$/*Ga*$_1^S$), and 96 BC_3F_2 were milled separately in bulk in a Stein M-2 seed grinder (Steinlite Corp., Atchison, KS). Genomic DNA was extracted from seed bulks by using lysis buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, and dd H_20), saturated phenol, chloroform: isoamyl alcohol (24:1), and isopropanol. DNA concentration was estimated with a NanoDrop (Thermo Scientific), and diluted to 10 ng/µl for PCR reactions. Twenty-eight SSR markers were selected from MaizeGDB database according to their proximity to *ga*$_1$ locus (Lawrence et al., 2005; Table 2). SSR markers were screened initially on parental DNA followed by BC_3F_2 population. Primer sequences were synthesized by Integrated DNA Technologies (Coralville, IA). DNA from each parent was mixed in equal proportions and used as a control. SSR markers that resulted in polymorphism between the two parents were used to screen the segregating population. The 25-µl PCR reactions consisted of 12.5 µl of 2X GoTaq Colorless Master

Mix (Promega Corp., Madison, WI), 1.25 μl of each primer (0.25 μM), 3 μl of diluted DNA (30 ng/μl), and 7 μl of molecular-grade distilled water. Four different annealing temperatures were tested on each marker to optimize the PCR reaction. These annealing temperatures were X=50°C, 55°C, 60°C, and 65°C. The amplification program consisted of 30 cycles of 1 m at 95°C, 1 m at X°C, and 2 m at 72°C in a PTC-100 thermal cycler (MJ Research, Watertown, MA). The PCR products were separated and scored on 4% MethaPhor gels (Lonza Rockland, Inc., Rockland, ME) stained with ethidium bromide.

Analysis

Segregation of SSR markers from the expected 1:2:1 ratio was tested with chi-square. Tests with *P*-value smaller than 0.05 were considered significant. For the purpose of evaluating the ability of SSR markers in predicting the zygosity at ga_1, we assumed that only two genotypes (i.e., Ga_1^S/Ga_1^S and Ga_1^S/ga_1) were segregating in our population and that Ga_1^S/Ga_1^S genotypes were less receptive than Ga_1^S/ga_1 genotypes to ga_1 pollen. The thresholds used to separate these genotypes on the basis of seed set with ga_1 pollen were 0 to 10%.

The ability of SSR markers to correctly identify genotypes Ga_1^S/Ga_1^S and Ga_1^S/ga_1 was calculated as percent of homozygous and heterozygous marker genotypes that produced seed sets within a threshold. The residual percent was the error rate of marker.

Results and discussion

A backcross population that was segregating for the Ga_1^S allele was phenotyped for cross-incompatibility using a pollen-mix assay. Eighty-six percent of the whole population resulted in 10% or less blue seeds per ear, 10% produced 20 to 40% blue

seeds per ear, and 4% of the population produced in excess of 60% blue seeds per ear with *ga*$_1$ pollen (Figure 2). Thirty-three percent of the population produced zero blue seeds whereas twenty-three percent of population produced 1% blue seeds (Figure 3).

Nelson (1952) observed over 99% of the progeny of backcrosses *Ga*$_1^S$/*Ga*$_1^S$ x (*ga*$_1$/*ga*$_1$ x *Ga*$_1^S$/*Ga*$_1^S$) and *Ga*$_1^S$/*Ga*$_1^S$ x (*Ga*$_1^S$/*Ga*$_1^S$ x *ga*$_1$/*ga*$_1$) being incompatible to *ga*$_1$ pollen. He concluded that *ga*$_1$ pollen grains produced by the F_1 were not able to fertilize the silks having the *Ga*$_1^S$ allele when *Ga*$_1^S$ pollen grains were also present resulting only in *Ga*$_1^S$/*Ga*$_1^S$ progeny. However, results from the same author after self-pollination of *Ga*$_1^S$/*ga*$_1$ genotypes were inconclusive. The F_2 was expected to segregate 50% incompatible (*Ga*$_1^S$/*Ga*$_1^S$) and 50% compatible (*Ga*$_1^S$/*ga*$_1$) to *ga*$_1$ pollen. However, the observed number of incompatible progeny fluctuated between 11.2 to 82.5% (Nelson, 1952). Our results with self-pollination of *Ga*$_1^S$/*ga*$_1$ support the model of differential fertilization proposed by Nelson (1952). In our study, all BC_3F_2 individuals were self-pollinated and crossed to a *ga*$_1$/*ga*$_1$ stock that confers colored aleurone in the seed. Ninety-six percent of our BC_3F_2 population would be considered incompatible to *ga*$_1$ pollen if plants with 40% or less blue seeds are classified as incompatible and plants with more than 70% blue seeds are classified as compatible (Figure 2). This result was close to the expected 100% incompatibility according to the model proposed by Nelson (1952) and suggest that heterozygous *Ga*$_1^S$/*ga*$_1$ individuals might have a preference for their own pollen over *ga*$_1$/*ga*$_1$ pollen due to the presence of *Ga*$_1^S$ allele in the silks. As mentioned before, incompatibility of heterozygous *Ga*$_1^S$/*ga*$_1$ silks depends on the genotypic constitution of pollen. If only *ga*$_1$ pollen is used, then heterozygous individuals would be compatible to *ga*$_1$. However, if *ga*$_1$ pollen is mixed with *Ga*$_1^S$ pollen, heterozygous

individuals would be incompatible to *ga1* and the dominance relationship reversed (Nelson, 1952). The thresholds used to classify plants as compatible or incompatible to *ga1* pollen are arbitrary. For example, Nelson (1952) and Whiteley (1953) classified progeny with 40% or less seed set as incompatible to *ga1* pollen and progeny that produced more than 70% seed set as compatible.

In our study, DNA from the recurrent parent B114 (*ga1*/*ga1*), the donor parent Mo508W/Mo506W ($Ga1^S$/$Ga1^S$), and a mixture of DNA from both parents was amplified with 28 SSR markers. Only SSR markers *phi021*, *umc2410*, and *umc1294* were polymorphic among the parents. The segregation of SSR markers in BC_3F_2 population was significantly different from the expected 1:2:1 ratio (*P*-value <0.0001; Table 3).

The higher number of individuals in our study with a marker genotype similar to $Ga1^S$/$Ga1^S$ and $Ga1^S$/*ga1* parents suggest a preference of plants for pollen containing the $Ga1^S$ allele over pollen with the *ga1* allele. The effectiveness of *ga1* pollen in fertilizing $Ga1^S$/*ga1* genotypes when competing against $Ga1^S$ pollen has been estimated as 0 to 4% (Emerson, 1934). Aberrant segregation of morphological and molecular markers in maize has been attributed to numerous gametophytic factors. For example, Sharopova et al. (2002) and Gardiner et al. (1993) attributed the abnormal segregation of multiple SSRs and restriction fragment length polymorphisms of chromosome 5 to the effect of *gametophyte factor 2* (*ga2*) gene. Lu et al. (2002) reported 18 segregation-distortion regions across the maize genome associated with at least three known gametophyte factors (*ga1*, *ga2*, and *ga8*).

The highest percent of individuals with correct zygosity at *ga1* was identified with marker *phi021* (70) followed by markers *umc2410* and *umc1294* with 65 and 48,

respectively (Table 4). These percents were observed with a 1% threshold in the case of markers *phi021* and *umc1294*. For marker *umc2410*, the highest percent of individuals with correct zygosity was observed with thresholds of 1 to 3%.

Assuming differential fertilization between ga_1 and Ga_1^S pollen do not occur, the expected segregation of our population would be 1 Ga_1^S/Ga_1^S : 2 Ga_1^S/ga_1 : 1 ga_1/ga_1. In order to develop inbred lines of genotype Ga_1^S/Ga_1^S, which are non-receptive to ga_1 pollen, would be necessary to identify first the zygosity at ga_1 followed by self-pollination. Pollen mixtures containing self and ga_1 pollen have been used to discriminate among genotypes in segregating populations (Thomas, 1955; Ziegler and Ashman, 2001). As mentioned before, two studies reported contradictory results about the incompatibility of Ga_1^S/ga_1 individuals when a mixture of Ga_1^S and ga_1 pollen was applied to the silks. Thomas (1955) reported that Ga_1^S/ga_1 genotypes were receptive to ga_1 pollen resembling the behavior of ga_1/ga_1 genotypes. Plants of genotype Ga_1^S/ga_1 also have been reported as incompatible to ga_1 pollen similar to Ga_1^S/Ga_1^S genotypes (Nelson, 1952). These observations indicate that differentiation of genotypes using a seed-set criterion could lead to erroneous results. We tried to address this problem in our study by using molecular markers which have never been used for this purpose in maize. Our objective was to determine how well molecular markers can predict zygosity at the ga_1 locus. Our results suggest that SSR markers *phi021*, *umc2410*, and *umc1294* are not effective for the discrimination of Ga_1^S/Ga_1^S and Ga_1^S/ga_1 genotypes due to large error rates that fluctuated between 30 and 52% (Table 4). The wide range of seed sets observed among Ga_1^S/Ga_1^S and Ga_1^S/ga_1 genotypes in our population could be the result of different factors including variability in the pollen mixtures used to screen for

incompatibility and/or a dose effect of the Ga_1^S allele. Each plant of segregating population was phenotyped for cross-incompatibility using a mixture of its own pollen and ga_1 pollen of blue corn. These mixtures were not calibrated on ga_1/ga_1 controls to determine the viability of Ga_1^S and ga_1 in pollen mixtures as reported by Evans and Kermicle (2001).

A critical step to develop cross-incompatible lines is selection and increase of Ga_1^S/Ga_1^S genotypes and discard of Ga_1^S/ga_1 and ga_1/ga genotypes in advanced generations. For this purpose, a method that allows for screening and increase of Ga_1^S/Ga_1^S genotypes in a segregating population was devised using self-pollination and cross with a ga_1/ga stock that produce colored seed (Thomas, 1955; Ziegler and Ashman, 2001). This method is based on the assumption that Ga_1^S/Ga_1^S genotypes would produce fewer colored seeds with ga_1 pollen compared to Ga_1^S/ga_1 and ga_1/ga_1 genotypes due to differential fertilization among pollen grains. Our own results and those of Nelson (1952) suggest that Ga_1^S/ga_1 genotypes are incompatible to ga_1 pollen grains when mixed with Ga_1^S pollen grains. However, compatibility of Ga_1^S/ga_1 genotypes to ga_1 pollen also has been reported (Thomas, 1955).

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Figure 1. Scheme for backcrossing of Ga_1^S. The recurrent parent B114 (ga_1/ga_1) was pollinated with non-recurrent parent Mo508W/Mo506W (Ga_1^S/Ga_1^S). To identify heterozygous progeny in backcrosses each plant was self-pollinated and crossed with 'Nokomis Blue' (ga_1/ga_1). The self-pollinated progeny of BC_3F_1 (i.e., yellow seeds) was considered as the BC_3F_2 progeny.

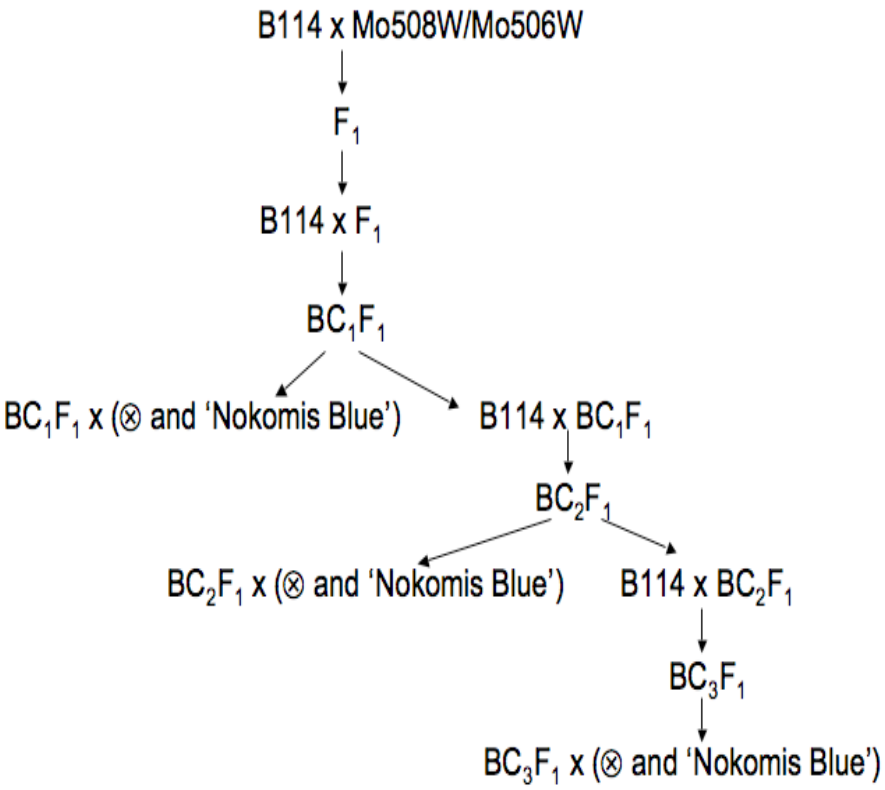

Figure 2. Distribution of 96 BC_3F_2 plants by percent blue seed set after self and cross-pollination with *ga*$_1$ pollen of 'Nokomis Blue'.

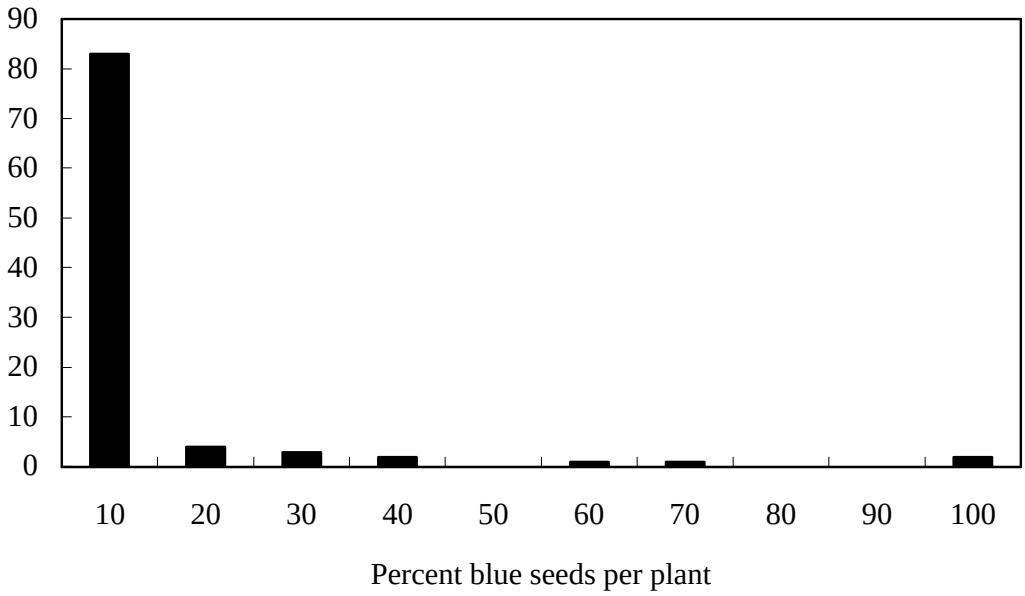

Figure 3. Distribution of 83 BC_3F_2 plants with 10% or less seed set after self-pollination and cross-pollination with *ga*$_1$ pollen from ‘Nokomis Blue’.

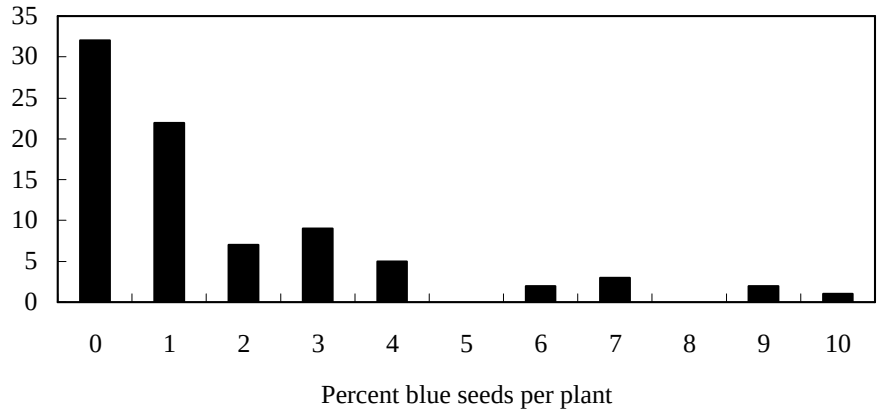

Table 1. Cross-incompatibility of maize silks to pollen of various genotypes according to Nelson (1952).

	Genotype of pollen grains		
Genotype of silks	Ga_1^S/Ga_1^S	ga_1/ga_1	$Ga_1^S/Ga_1^S + ga_1/ga_1$
Ga_1^S/Ga_1^S	Receptive	Non-receptive	Non-receptive to ga_1
ga_1/ga_1	Receptive	Receptive	Receptive to Ga_1^S and ga_1
Ga_1^S/ga_1	Receptive	Receptive	Non-receptive to ga_1[1]

[1] Reported as receptive to Ga_1^S and ga_1 by Thomas (1955).

Table 2. Primer name, bin position, and primer sequences of SSR markers used for the amplification of genomic DNA of cross-compatible and incompatible plants in maize.

Primer	Bin[1]	Forward Sequence	Reverse Sequence
umc1008	4.01	5'-TCTAGCTTGTGGTGGTGGTTGA-3'	5'-ACATGAGCACAAAGACTGACGC-3'
umc1022	4.01	5'-AACAAGTTTTGTTTGACAAGCCG-3'	5'-ATGATCACCCCGTCAGCG-3'
nc135	4.01	5'-CACAAAGAGCAGCCCACTTT-3'	5'-AAGTTGCTGACATCGATCCA-3'
umc2409	4.01	5'-GGACTGCTCTCATGGCTTCAG-3'	5'-CGAGAAGCCATCGTAAAAAGAAAA-3'
umc2150	4.01	5'-GTTGTTCACTTTCCAAAACCCTTG-3'	5'-GCCTTGTGCTTCTTGGAGTGTT-3'
umc1758	4.01	5'-CTTCCTCCTCACCTCACCTCCTAT-3'	5'-GGTAGCCAATCCTTCCTTCCTATG-3'
umc1757	4.01	5'-TTTTCTGCAGGGATAACATTTGTG-3'	5'-ATAGGAGGTGAGGTGAGGAGGAAG-
umc2410	4.02	5'-CAAGATCACCCAAGGT-3'	5'-AGCTACTGTGGACTGTGGACTGTG-3'
umc1509	4.02	5'-CTTTCTGCAGATTCACCGTTTCTT-3'	5'-TTGGTTCTTTTGACCATAGACAAGC-3'
umc1294	4.02	5'-GCCGTCAACGGGCTTAAACT-3'	5'-GCCTCCAGCTCTCTCGTCTCTT-3'
umc1288	4.02	5'-ATCCGGACAAATTGAACTTTCATC-3'	5'-ATAGATTCAGTGTTGGACCGAGGA-3'
umc1943	4.02	5'-GTGCTGCAGAATTCAACTCCTTC-3'	5'-ACCATTTCTGCGTTTCCACAGT-3'
Phi021	4.03	5'-TTCCAACTACGCAGGACAGTTCG-3'	5'-CTTGATCACCTTTCCTGCTGTCGCCA-
bnlg1126	4.03	5'-GAGATCGAAGGTCATGGCAC-3'	5'-ATGGTTCCTGGTTCAGATGG-3'
Phi096	4.04	5'-TCCACCATTTGACACTTAGGCA-3'	5'-GCGTAGGACGACCGTTGAA-3'
Phi074	4.04	5'-CCCAATTGCAACAACAATCCTTGGCA-3'	5'-GTGGCTCAGTGATGGCAGAAACT-3'
umc1117	4.04	5'-AATTCTAGTCCTGGGTCGGAACTC-3'	5'-CGTGGCCGTGGAGTCTACTACT-3'
mmc0471	4.04	5'-TTAGCACATTTGAAGAGTTTTG-3'	5'-TTTCCTTCACGTTTCTCTGT-3'
bnlg490	4.04	5'-GCCCTAGCTTGCTAATTAACTAACA-3'	5'-ACTGTAAGGGCAGTGGACCTATA-3'
umc1067	4.04	5'-ACTTGTACTACGCAGGACAGTTCG-3'	5'-AGCCTCTGTCTGGATGACTGAAC-3'
umc1088	4.05	5'-TCATCCTCCTAGCTCCTCTACTCG-3'	5'-AAAACAGTCAGCAGAACCCACTTT-3'
bnlg1937	4.05	5'-AATGCTCGGTCCACAGAATC-3'	5'-AACTGGAGCCAAAAGTGGTG-3'
bnlg1217	4.05	5'-AGCTGATCTGCACGTTGTTG-3'	5'-GCAGATCCACGCCATTTAAA-3'
umc1031	4.05	5'-TTGGGTTCATACCTCCTAGGAACA-3'	5'-ACGTGGACAACCAGTCTATCAACA-3'
umc1142	4.05	5'-CCGAAAACCCATTCTTCTAGCATC-3'	5'-GTGCGGTGTTCTCTCTTTCACTCT-3'
umc1317	4.05	5'-CCGACTCCGAGTAGCTTTCGT-3'	5'-CCGACTCCGAGTAGCTTTCGT-3'
umc1451	4.05	5'-GGTAGATCGAGAAAGGAGTGGACA-3'	5'-TTGCAAGAGCACACGACTAAGAAG-3'
Phi308090	4.05	5'-CAGTCTGCCACGAAGCAA-3'	5'-CTGTGGGTTTCGGTCTTCTT-3'

[1] Is an interval of ~20 centiMorgans (cM) between two fixed core markers that includes all loci from the top core marker to the next core marker.

Table 3. Phenotypic and SSR marker data of 96 BC_3F_2 plants.

	Number of seeds				SSR marker genotype[1]		
Plant	Yellow	Blue	Total	% blue seeds	phi021	umc2410	umc1294
1	243	0	243	0	b	b	h
2	149	3	152	2	h	h	b
3	162	2	164	1	a	h	h
4	169	6	175	3	b	b	b
5	295	0	295	0	b	b	h
6	64	19	83	23	h	h	h
7	253	7	260	3	.	.	.
8	265	0	265	0	b	b	h
9	152	15	167	9	h	h	h
10	0	11	11	100	.	.	.
11	149	3	152	2	b	b	.
12	58	1	59	2	h	h	h
13	116	24	140	17	h	h	h
14	339	6	345	2	b	b	b
15	291	8	299	3	b	h	h
16	278	0	278	0	h	b	h
17	256	17	273	6	b	b	h
18	259	0	259	0	b	b	h
19	225	1	226	0	b	b	.
20	303	9	312	3	h	h	b
21	198	0	198	0	b	h	b
22	248	2	250	1	b	b	b
23	13	0	13	0	a	h	h
24	227	2	229	1	b	b	b
25	220	1	221	0	h	h	b
26	281	4	285	1	a	h	h

[1] a=marker genotype of parent B114 (ga_1/ga_1), h= marker genotype of hybrid (Ga_1^S/ga_1), b= marker genotype of parent Mo508/Mo506 (Ga_1^S/Ga_1^S), .= marker data point not available.

Table 3. (continued)

	Number of seeds				SSR marker genotype		
Plant	Yellow	Blue	Total	% blue seeds	phi021	umc2410	umc1294
27	291	1	292	0	b	h	h
28	299	0	299	0	b	b	h
29	112	1	113	1	a	h	b
30	236	0	236	0	b	b	h
31	196	3	199	2	h	h	h
32	246	1	247	0	h	h	h
33	270	43	313	14	h	h	h
34	166	7	173	4	.	.	.
35	59	37	96	39	.	.	.
36	147	0	147	0	h	h	h
37	264	3	267	1	h	h	h
38	284	2	286	1	b	b	h
39	229	2	231	1	b	b	h
40	203	0	203	0	h	h	h
41	250	4	254	2	.	.	.
42	306	1	307	0	b	h	b
43	258	0	258	0	.	.	.
44	25	1	26	4	.	.	.
45	218	2	220	1	b	h	h
46	196	6	202	3	b	b	b
47	324	2	326	1	.	.	.
48	280	0	280	0	.	b	h
49	149	1	150	1	.	.	.
50	82	3	85	4	h	h	.
51	61	14	75	19	h	h	b
52	190	1	191	1	b	b	b
53	195	14	209	7	h	h	b
54	315	0	315	0	.	.	.
55	50	15	65	23	b	h	h
56	1	112	113	99	.	.	.
57	286	4	290	1	.	b	h
58	278	0	278	0	h	b	.
59	79	8	87	9	h	h	.
60	15	27	42	64	b	h	.
61	112	22	134	16	a	h	.
62	252	20	272	7	h	h	.
63	272	2	274	1	b	b	b

Table 3. (continued)

	Number of seeds				SSR marker genotype		
Plant	Yellow	Blue	Total	% blue seeds	phi021	umc2410	umc1294
64	128	36	164	22	h	.	.
65	233	15	248	6	h	h	b
66	254	1	255	0	h	h	h
67	276	3	279	1	.	h	b
68	129	71	200	36	.	a	b
69	267	6	273	2	.	b	h
70	249	9	258	3	.	h	h
71	355	0	355	0	b	b	b
72	169	0	169	0	h	h	b
73	259	2	261	1	b	h	b
74	225	1	226	0	h	h	b
75	101	0	101	0	.	b	b
76	223	2	225	1	b	b	b
77	228	0	228	0	a	b	h
78	191	14	205	7	h	h	h
79	256	8	264	3	h	h	b
80	247	2	249	1	b	b	b
81	47	60	107	56	h	h	b
82	235	1	236	0	h	b	b
83	164	5	169	3	.	b	h
84	294	0	294	0	b	b	b
85	29	1	30	3	h	b	h
86	115	1	116	1	b	b	h
87	165	7	172	4	b	b	h
88	221	0	221	0	b	b	h
89	350	0	350	0	b	b	h
90	197	23	220	10	h	h	.
91	339	0	339	0	b	b	h
92	230	10	240	4	h	h	b
93	85	1	86	1	b	b	b
94	174	2	176	1	h	h	b
95	308	0	308	0	.	h	b
96	293	4	297	1	b	b	h

Table 4. Prediction of zygosity at *ga1* locus with SSR markers. Values in bold indicate highest percent of correct hypothesis for each SSR marker.

Hypotheses[1]				phi021		umc2410		umc1294	
Correct		Incorrect		%C[2]	%I	%C	%I	%C	%I
b= 0%	h> 0%	b> 0%	h= 0%	54	46	60	40	45	55
b= 0-1%	h> 1%	b> 1%	h= 0-1%	**70**	30	**65**	35	**48**	52
b= 0-2%	h> 2%	b> 2%	h= 0-2%	69	31	**65**	35	47	53
b= 0-3%	h> 3%	b> 3%	h= 0-3%	69	31	**65**	35	47	53
b= 0-4%	h> 4%	b> 4%	h= 0-4%	67	33	64	36	47	53
b= 0-5%	h> 5%	b> 5%	h= 0-5%	67	33	64	36	47	53
b= 0-6%	h> 6%	b> 6%	h= 0-6%	67	33	64	36	47	53
b= 0-7%	h> 7%	b> 7%	h= 0-7%	63	37	60	40	47	53
b= 0-8%	h> 8%	b> 8%	h= 0-8%	63	37	60	40	47	53
b= 0-9%	h> 9%	b> 9%	h= 0-9%	60	40	58	42	45	55
b= 0-10%	h> 10%	b> 10%	h= 0-10%	59	41	57	43	45	55

[1] Based on % blue seeds produced by ga_1/ga_1 pollen; b= Ga_1^S/Ga_1^S, h= Ga_1^S/ga_1.
[2] Percent of individuals with correct (C) and incorrect (I) zygosity as predicted by SSR marker.

CHAPTER 3. GENOTYPE x ENVIRONMENT INTERACTIONS IN POPULATIONS POSSESING GA1S AND GA1 ALLELES FOR CROSS-INCOMPATIBILITY IN MAIZE

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Abstract

Use of cross-incompatibility in corn (*Zea mays* L.) by the Ga_1^S allele may reduce cross-fertilization in specialty and conventional organic corn with pollen from genetically-modified (GM) corn. For effective use, information about environment and genotype x environment effects on cross-fertilization by ga_1 as well as heritability of cross incompatibility in maize is necessary. Our objective was to obtain this information. Four population pairs (i.e., treatments) differing in their genotype at ga_1 were evaluated for cross-incompatibility with ga_1 pollen in different environments. Populations were derived by crossing the recurrent parents B116, PHG35, ARZM16035:S19 and CHZM05015:Mo17 to Ga_1^S-donor parent Mo508W/Mo506W. Two replicates of each treatment were grown in the center of 952 m^2 fields planted with purple corn as an adventitious source of ga_1/ga_1 pollen. Open pollination was allowed and amount of cross-fertilization estimated by averaging the percentage of purple seeds. Environment and genotype x environment effects were not significant. Contrasts to evaluate differences in cross-fertilization between Ga_1^S and ga_1 populations revealed that mean percentages of cross-fertilization in Ga_1^S populations of B116, ARZM16035:S19, and

CHZM05015:Mo17 were significantly lower than in *ga*$_1$ populations. The estimated broad-sense heritability on an entry-mean basis for cross incompatibility was 0.81. Results suggest differences in genotype at *ga*$_1$ played a major role in cross-fertilization of populations differing in their genotype at the *ga*$_1$ locus. Incompatibility may be selected effectively over a large array of environments and the *Ga*$_1^S$ system may be of value to reduce cross-fertilization with GM-corn pollen.

Introduction

In 2010, 35.5 million ha of corn were planted in the U.S., with 30.5 million ha planted with GM corn (http://www.nass.usda.gov, verified 3 August 2010). This represented 86% of the total acreage, and an increase of 34% of GM corn planted in the U.S. compared with 2005. As result of the steady increase in GM corn, it is becoming increasingly difficult to ensure the co-existence between GM and non-GM corn. This is of particular concern to organic corn growers and the organic industry, that need to maintain identity-preserved non-GM corn products.

Cross fertilization among corn plants of neighboring fields has been amply documented. Goggi et al. (2006) measured level of cross fertilization in non-GM corn field at various distances from a GM-corn source with herbicide and insect resistance transgenes, and found the proportion of cross-fertilized seeds in the non-GM field to decrease exponentially with distance from a neighbor transgenic pollen source and linearly with wind speed and direction. At 1 m from the pollen source, mean percentage of cross fertilization in 2003 and 2004 were 29.9 and 17.0, respectively whereas average percentage of cross fertilization at 35 m from the pollen source was 0.4 in 2003 and 2004. At 100 m distance from the pollen source and beyond, percentage of cross fertilization

decreased to less than 0.1. However, the average of cross fertilization never reached 0% within 250 m from the pollen source. The study indicated that cross fertilization among corn fields can be an important source of adventitious mixtures between non-GM and GM corn fields.

To reduce cross-fertilization of non-GM with GM corn, numerous strategies have been devised including spatial and temporal isolation, physical barriers, and GM-crop-free zones (Devos et al., 2005). The larger the recipient field, the larger its own pollen mass will be. The pollen cloud, hanging over the recipient field, is a physical barrier and competitor for incoming pollen (Goggi et al., 2007). Use of border rows can reduce the amount of cross fertilization with incoming pollen. A difference in sowing dates may result in a difference in flowering time, hence also limiting cross fertilization. Another strategy is use of non-reciprocal cross-incompatibility by the Ga_1^S allele, which has been used in maize to reduce or eliminate the extent of cross-fertilization in certain specialty corns including sweet corn, popcorn, and white-endosperm corn (Perry, 1945; Poneleit, 2001; Ziegler and Ashman, 2001).

The Ga_1^S allele causes cross incompatibility in corn populations. The first case of gametophytic-cross incompatibility in corn was observed in 1929 by Demerec (1929), and subsequently by Schwartz (1950) and Nelson (1952). Cross incompatibility in maize is a trait controlled by a single gene Ga_1 with multiple alleles (Nelson, 1993). One of them, the Ga_1^M allele allows male-only function and has been found in 'White Rice' popcorn and some Mexican inbred lines (Nelson, 1952; de la Cruz et al., 2008). The Ga_1^S allele is characteristic of cross-incompatible inbred lines. The wild type recessive allele ga_1 is typical of cross-receptive germplasm and it is found in most North American

dent and flint corns (Nelson, 1993). Plants homozygous for Ga_1^S/Ga_1^S are incompatible to ga_1 pollen growing tubes, however the reciprocal cross is fully compatible (Schwartz, 1950). Incompatibility of heterozygous female plants Ga_1^S/ga_1 depends whether the ga_1 pollen is present alone or in competition with Ga_1^S in the silks. When only ga_1 pollen is present, there is no competition of Ga_1^S in the silks, and normal fertilization occurs. However, when Ga_1^S and ga_1 pollen grains compete on silks of the genotype Ga_1^S/ga_1, ga_1 pollen grains are at a disadvantage compared with Ga_1^S pollen grains (Nelson, 1952; Lausser et al., 2010).

These observations and the successful use of the system in other corn types to avoid pollen contamination, suggest that the system may be adaptable to the production of corn under organic-farming systems (Roseboro, 2008).

Observation of variable cross-fertilization with ga_1/ga_1 pollen on inbred lines with the genotype Ga_1^S/Ga_1^S in successive years led Nelson (1952) and Whiteley (1953) to suggest an important role of environment in the cross-incompatibility expression in maize. However, environmental effects on cross incompatibility have not been tested. Conventional corn in U.S. organic farming systems was grown on approximately 78,766 ha in 2008 (USDA, ERS, http://www.ers.usda.gov/Data/Organic/, verified 18 November 2010). The wide distribution of organic farms in the U.S. suggests that the $Ga1^S$ system might be incorporated into corn planted in a variety of environmental conditions.

Effective phenotypic selection in breeding programs is affected by heritability of the trait being selected (Fehr, 1991). Two types of heritability can be estimated, narrow-sense and broad-sense. Narrow-sense heritability estimates the importance of additive effects on total phenotypic variance whereas broad-sense heritability measures total

genetic effects (additive, dominance, and epistatic effects) as a function of total phenotypic variance. Narrow-sense heritability is more useful for plant breeders than broad-sense heritability because only additive effects are passed on from parents to offspring. However, estimation of narrow-sense heritability requires special mating designs that may not be commonly used in breeding programs. Estimates of heritability of cross-incompatibility in maize are desirable to improve efficiency of selection and to assess system usefulness. These estimates of heritability for cross incompatibility in maize, however, are not available.

As mentioned previously, published information suggests the importance of environmental variability on the expression of cross-incompatibility in maize (Nelson, 1952; Whiteley, 1953; Maletzky and Siritza, 1972). However, expression of cross-incompatibility by Ga_1^S over an array of different environments remains largely unknown. For the allele Ga_1^S to function as an efficient crossing barrier, stability of functioning across environments is a prerequisite for seed and grain production. Therefore, the objectives of our study were to compare the amount of cross-fertilization in maize populations segregating for the Ga_1^S allele and similar non-segregating ga_1 populations to determine environment and genotype x environment interaction effects in the expression of cross incompatibility and to estimate broad-sense heritability of cross incompatibility by using analysis of variance (ANOVA).

Materials and Methods

Plant Materials

Two inbred lines (B116 and PHG35) and two breeding populations (ARZM16035:S19 and CHZM05015:Mo17) were used in the study. Inbred lines B116

and PHG35 were pedigree selections from the F_2 of B97 x B99 (Hallauer et al., 2004) and G3BD2 x H7FS6 (USDA, ARS, NGRP, GRIN, http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1157409, verified 12 August 2010), respectively. The breeding populations were developed by crossing two high-yielding landraces from Argentina and Chile identified in the Latin American Maize Project with inbred lines developed as part of the Germplasm Enhancement of Maize project (Pollak, 2003).

The Ga_1^S allele for cross incompatibility was transferred into the inbred lines and breeding populations from the donor parent Mo508W/Mo506W (Figure 1). Recurrent parents were crossed with Ga_1^S donor as the male parent, and the F_1 backcrossed to the recurrent parents. Fifteen plants from recurrent parents and their respective BC_1F_1 progenies were grown side-by-side. A pollen-mix technique based on seed color was used for selection of heterozygous Ga_1^S/ga_1 progenies in backcrosses (Ziegler and Ashman, 2001). The technique consisted in dividing the pollen of individual BC_1F_1 plants approximately in two equal amounts; one-half was used for self-pollination, and the remainder was used for backcrossing a single plant of the recurrent parent to produce BC_2F_1 seed. Pollen from 'Nokomis Blue' (*Z. mays* L.) was applied at approximately the same amount to silks of self-pollinated BC_1F_1 plants to produce a mixture of two different types of pollen on the silks. 'Nokomis Blue' is a blue-kernelled derivative of the 'Nokomis Gold' population (Jaradat et al., 2010). The genes for color pigmentation in the seeds were expected to segregate independently from the ga_1 gene as described by Neuffer et al. (1997). The BC_1F_1 plants with the lowest percentages of purple seeds were assigned the genotype Ga_1^S/ga_1 and their BC_2F_1 progeny retained. For example, if two

BC_1F_1 sibs had 1% and 11% purple seeds, the sib with 1% purple seeds was considered as Ga_1^S/ga_1. A similar procedure was repeated in the following season with BC_2F_1.

Seeds from self-pollination of heterozygous BC_2F_1 and the recurrent parents were used in this study to evaluate cross-incompatibility with ga_1 pollen of lines with Ga_1^S and ga_1 alleles across different environments. Homozygous lines with the Ga_1^S/Ga_1^S genotype were still under development, therefore were not included in the research. All ears were harvested and shelled individually. Separation of purple and yellow seeds that resulted from mix pollinations was performed with a ScanMaster II color sorter (model SM-200IE, Satake USA, Stafford, TX). The machine consists of two closely spaced 10-channel chutes with a variable speed vibratory feeder. The 10-channel chutes were reduced to 2 channels to sort small amounts of seeds. The setup consisted of orange filters (-11), marigold (-1) backgrounds, and white lamps (Designer 3500K). Each sample was passed through the color sorter three times, beginning with the primary sort, resort of the reject fraction, and secondary sort of the blended accepts from the primary and resort passes.

Experimental Design and Data Analysis

Eight genotypes consisting of four populations segregating for the allele Ga_1^S and four corresponding populations with the genotype ga_1/ga_1 were used in the study. The experiment was conducted in two locations during 2008 and 2009. In 2008, the two locations were the Iowa State University Marsden farm and the Agricultural Engineering and Agronomy (AEA) farm, both near Ames, IA. These experiments were planted on 21 May 2008. In 2009, the two locations were the Iowa State University Hinds farm, near Gilbert, IA and AEA farm. Planting at the Hinds farm was 4 June 2009 and at the AEA

farm 30 May 2009. Predominant soils in the three locations were of the series Clarion, Nicollet, and Webster.

For analyses, each combination of year and location was considered a different environment in the statistical model. At each location, a field of approximately 952 m^2 was planted with 'Nokomis Blue' as the pollen source of type ga_1/ga_1 and two replicates of each population (treatment) were planted in 3.81 x 0.61 m plots in the center of the same field (Figure 2). The central plots were harvested individually and the shelled seed was collected in bulk for each. Yellow and purple seeds of each plot were then separated using a color sorter as before. Seeds of purple color and the corresponding percentage of purple seeds in each plot were calculated and an average was obtained for each population type (treatment).

Analysis of variance and linear combinations for differences in purple-seed averages among population types determined by PROC MIXED in SAS (SAS Institute, 2008). In this model, only genotype effects were considered fixed. The mixed-linear model was:

$$Y_{gek} = \mu + \alpha_g + \beta_e + \alpha_g*\beta_e + \varepsilon_{gek}$$

where, Y_{gek} = percent purple seeds on g^{th} genotype, e^{th} environment, k^{th} experimental plot

μ = overall mean

α_g = effect of g^{th} genotype

β_e = effect of e^{th} environment

$\alpha_g*\beta_e$ = genotype x environment interaction

ε_{gek} = residual

To calculate broad-sense heritability (H^2) of cross incompatibility on an entry-mean basis expected-mean squares were estimated by fitting a random model in PROC MIXED (SAS Institute, 2008). The eight genotypes were considered a random sample from a larger population of genotypes for the calculation of heritability. The formula we used to calculate H^2 on an entry-mean basis (Fehr, 1991) was:

$$\hat{H}^2 = \frac{\hat{\sigma}_g^2}{\frac{\hat{\sigma}_e^2}{rt} + \frac{\hat{\sigma}_{ge}^2}{t} + \hat{\sigma}_g^2}$$

where, $\hat{\sigma}_g^2 =$ total genetic variance

$\hat{\sigma}_e^2 =$ error variance

$\hat{\sigma}_{ge}^2 =$ genotype x environment variance

$r =$ number of replicates

$t =$ number of environments

Results and Discussion

Four pairs of populations differing in their genotype at the *ga1* locus were evaluated for cross incompatibility with *ga1* pollen in four different environments. We found no evidence of environment or genotype x environment (G x E) effects in cross-fertilization with *ga1* pollen of populations with the $Ga1^S$ and *ga1* alleles (Table 1). Environment and G x E effects were not significant at the 0.05 level ($P = 0.3168$ and $P = 0.1690$, respectively). Our results suggest that differences in cross-fertilization between

maize populations with the Ga_1^S and ga_1 alleles were affected by genotypic rather than environment or G x E effects.

Variability in cross-incompatibility of Ga_1^S/Ga_1^S genotypes to ga_1 pollen have been attributed to environmental effects; however, general conclusions about positive environmental effects have not been supported by statistical analyses. Nelson (1952), using a diallel design with 13 popcorn-inbred lines, observed inbred lines that were fully compatible with ga_1/ga_1 pollen one year but partially incompatible the following year and inbred lines that were partially incompatible one year but incompatible the next year. The author attributed these differences in cross incompatibility to environmental effects. The ga_1 gene in maize has been studied through the analysis of distorted segregation of markers linked to ga_1 with Mendelian segregation (Nelson, 1993). One such marker is the sugary (su_1) gene, with the dominant allele Su_1 being approximately 30 cM apart from the Ga_1^S allele (Emerson, 1934). When $Su_1Ga_1^S/su_1ga_1$ plants were self-pollinated, less than the expected 25% su_1 seeds were obtained because pollen tubes of $Su_1Ga_1^S$ pollen grains reached the ovules more often than pollen tubes of su_1ga_1 pollen grains (Mangelsdorf and Jones, 1926; Lausser et al., 2010). Nelson (1952) also observed differences in percent of su_1 seeds in lower and upper portions of self-pollinated ears of $Su_1Ga_1^S/su_1ga_1$ plants when pollinating either in the morning or afternoon. Maletzky and Siritza (1972) crossed $Su_1Ga_1^S/su_1ga_1$ plants reciprocally with a $Su_1Ga_1^S/su_1ga_1$ tester in multiple years and observed differences in the percentage of su_1 seeds in the progeny. The authors attributed such differences in sugary seeds to environmental effects at time of pollination. Nelson (1952) and Whiteley (1953) observed no differences in incompatibility of F_2 progenies of cross ga_1/ga_1 x Ga_1^S/Ga_1^S when plants were either

hand-pollinated with ga_1 pollen or detasseled before open pollination with ga_1 pollen. These observations were not substantiated with appropriate experiments and/or design analyses. Our work is the first report purposely designed to test environmental effects on the expression of cross-incompatibility.

Maize populations of B116, ARZM16035:S19, and CHZM05015:Mo17 with Ga_1^S allele were less receptive to ga_1/ga_1 pollen of 'Nokomis Blue' than their respective ga_1/ga_1 populations (Table 2). However, no evidence was found for difference in cross-fertilization between populations derived from inbred line PHG35. Average percent of purple seeds in populations of CHZM05015:Mo17 with Ga_1^S and ga_1 alleles were 16.25 and 47, respectively. In B116, 22.5% and 44.71% purple seeds were associated with Ga_1^S and ga_1 populations, respectively. The ARZM16035:S19 population with Ga_1^S had 8% purple seeds whereas the ga_1 population had 28.12% purple seeds. The difference in average percentage of purple seeds between the Ga_1^S population of PHG35 (25.62%) and that of ga_1 population (35.37%) was not significant.

Goggi et al. (2006) observed 29.9 and 17.0% cross fertilization in non-GM corn with GM pollen at a distance of 1 m from the pollen source. In our study, treatment plots were surrounded by a ga_1-pollen source (Figure 2). Percentages of cross fertilization we estimated in populations with the Ga_1^S allele were less than what Goggi et al. (2006) reported at 1 m distance from the pollen source. Genotypes with the Ga_1^S allele used in our study were BC_2F_2 populations with an expected segregation of 50% Ga_1^S/Ga_1^S and 50% Ga_1^S/ga_1 (Nelson, 1952). It has been reported that Ga_1^S/Ga_1^S plants are incompatible with ga_1 pollen, and that Ga_1^S/ga_1 plants are fertilized more often by Ga_1^S than ga_1 pollen tubes when both are present in the silks (Nelson, 1952).

The estimate of broad-sense heritability on an entry-mean basis for cross incompatibility was 0.81 (Table 3). The high heritability estimate for cross incompatibility suggest the trait might be selected effectively over different environments. A similar value for broad-sense heritability (0.84) was also reported by Luciano et al. (1965) for sunflower (*Helianthus annuus* L.). Broad-sense heritability estimates for cross incompatibility in other major crops have not been reported.

No system intended for containment of pollen from GM corn is 100% effective, including use of the cross-incompatibility system. The combination of different strategies, however, such as the use of Ga_1^S/Ga_1^S hybrids and the establishment of appropriate isolation distances may have synergistic effects in reducing cross-fertilization between GM and non-GM corn to levels close to zero, ensuring an effective protocol to maintain identity-preserved organic corn.

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Table 1. Analysis of variance for percentage of cross-fertilization with ga_1 pollen in maize populations with Ga_1^S and ga_1 alleles in four different environments.

Source of variation	DF	Mean square	F-test	*P*-value
Environment (E)	3	327.91	1.25	0.3168
Genotype (G)	7	1418.58	5.40	0.0012*
G x E	21	263.26	1.45	0.1690
Residual	31	181.31		

* Significant at *P*-value ≤ 0.05.

Table 2. Contrasts, *P*-values, estimated differences, and 95% confidence intervals for cross-incompatibility in maize populations differing in their genotype at Ga_1^S locus.

Contrasts	*P*-value	Estimated differences (%)	95% confidence interval
B116 (Ga_1^S/-) vs B116 (ga_1/ga_1)	0.0148*	11.10	(2.41 to 19.80)
PHG35 (Ga_1^S/-) vs PHG35 (ga_1/ga_1)	0.2442	4.88	(-3.59 to 13.34)
ARZM16035:S19 (Ga_1^S/-) vs ARZM16035:S19 (ga_1/ga_1)	0.0220*	10.06	(1.60 to 18.52)
CHZM05015:Mo17 (Ga_1^S/-) vs CHZM05015:Mo17 (ga_1/ga_1)	0.0011*	15.38	(6.91 to 23.84)

* Significant at $P \leq 0.05$.

Table 3. Expected-mean squares for percentage of cross-fertilization in maize populations with Ga_1^S and ga_1 alleles in four different environments.

Source of variation	DF	Mean square		Expected mean squares[1]
Environment (E)	3	327.91		
Genotype (G)	7	1418.58	M_1	$\sigma_e^2 + r\sigma^2_{ge} + rt\sigma^2_g$
G x E	21	263.26	M_2	$\sigma_e^2 + r\sigma^2_{ge}$
Residual	31	181.31	M_3	σ_e^2

[1] $\sigma_e^2 = M_3 = 181.31$
$\sigma^2_{ge} = (M_2 - M_3)/r = [(\sigma_e^2 + r\sigma^2_{ge}) - \sigma_e^2]/r = (263.26 - 181.31)/2 = 40.98$
$\sigma^2_g = (M_1 - M_2)/rt = [(\sigma_e^2 + r\sigma^2_{ge} + rt\sigma^2_g) - (\sigma_e^2 + r\sigma^2_{ge})]/rt = (1418.58 - 263.26)/8 = 144.42$

Figure 1. Diagram showing the backcross procedure utilized for development of BC_2F_2 populations. The recurrent parents (RP) were B116, PHG35, ARZM16035:S19, and CHZM05015:Mo17 and the source parent of Ga_1^S was the hybrid Mo508W/Mo506W. The BC_1F_1 and BC_2F_1 progenies with the lowest number of purple seeds resulting from pollination with their own pollen and the ga_1/ga_1 stock that confers purple aleurone ('Nokomis Blue') were classified as heterozygous Ga_1^S/ga_1. BC_2F_2 populations were created by self-pollination of heterozygous BC_2F_1 plants.

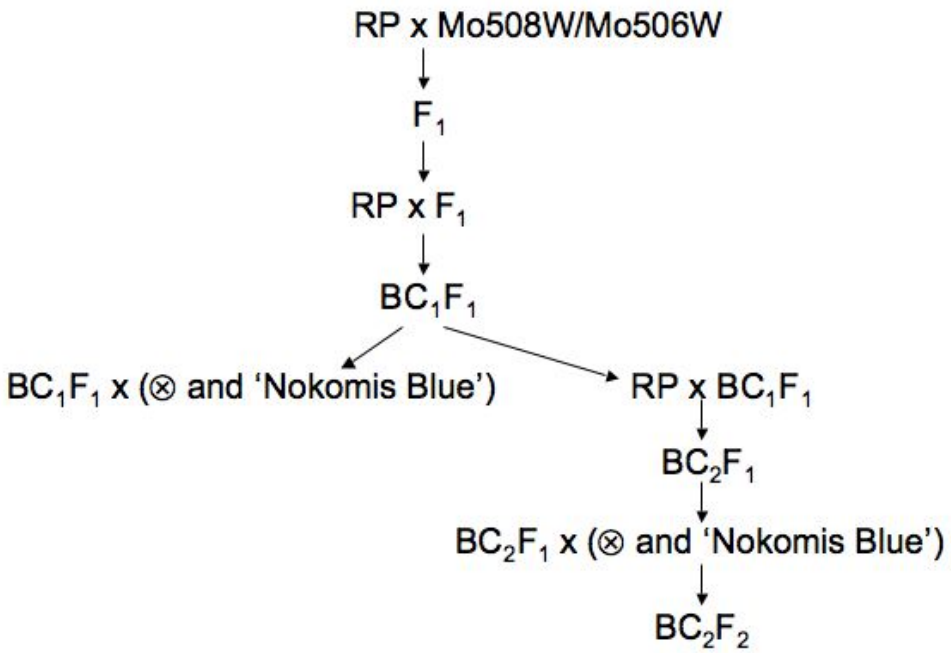
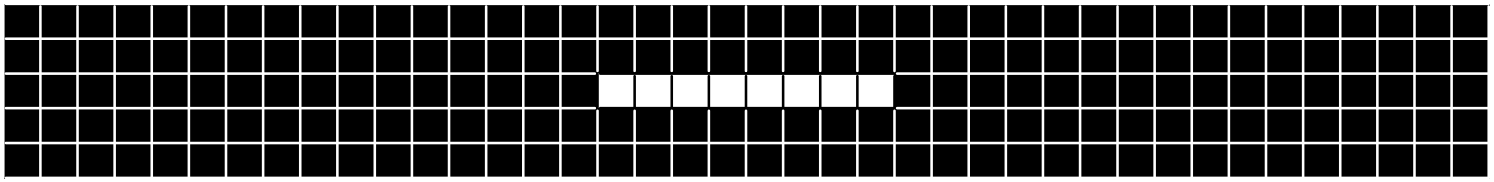


Figure 2. Field layout of experiment. Each cell represent a 3.81 x 0.61 m plot of either 'Nokomis Blue' (black) or treatment genotype (white). The eight genotypes in the center of the field consisted of four populations segregating for the allele Ga_1^S and four corresponding populations with the genotype ga_1/ga_1. 'Nokomis Blue' has the genotype ga_1/ga_1.



CHAPTER 4. GENERAL CONCLUSIONS

Our study supported previous observations of *ga1* pollen grains being less competitive than $Ga1^S$ pollen grains on silks of heterozygous $Ga1^S/ga1$ plants. This was shown by the segregation of 92 incompatible and only four compatible plants to *ga1* pollen in our BC_3F_2 population.

Eighty-six percent of the whole population resulted in 10% or less blue seeds per ear, 10% produced 20 to 40% blue seeds per ear, and 4% of the population produced in excess of 60% blue seeds per ear with *ga1* pollen. Thirty-three percent of the population produced zero blue seeds whereas twenty-three percent of population produced 1% blue seeds.

In our study, DNA from the recurrent parent B114 (*ga1*/*ga1*), the donor parent Mo508W/Mo506W ($Ga1^S/Ga1^S$), and a mixture of DNA from both parents was amplified with 28 SSR markers. Only SSR markers *phi021*, *umc2410*, and *umc1294* were polymorphic among the parents. The segregation of SSR markers in BC_3F_2 population was significantly different from the expected 1:2:1 ratio.

The highest percent of individuals with correct zygosity at *ga1* was identified with marker *phi021* (70) followed by markers *umc2410* and *umc1294* with 65 and 48, respectively. These percents were observed with a 1% threshold in the case of markers *phi021* and *umc1294*. For marker *umc2410*, the highest percent of individuals with correct zygosity was observed with thresholds of 1 to 3%.

We found no evidence of environment or genotype x environment (G x E) effects in cross-fertilization with *ga1* pollen of populations with the $Ga1^S$ and *ga1* alleles. Environment and G x E effects were not significant at the 0.05 level. Our results suggest

that differences in cross-fertilization between maize populations with the Ga_1^S and ga_1 alleles were affected by genotypic rather than environment or G x E effects.

Maize populations of B116, ARZM16035:S19, and CHZM05015:Mo17 with Ga_1^S allele were less receptive to ga_1/ga_1 pollen of 'Nokomis Blue' than their respective ga_1/ga_1 populations. However, no evidence was found for difference in cross-fertilization between populations derived from inbred line PHG35.

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