Analysis of Two Maize Mutations that Arose After Exposure to Radiation from a Plutonium-Beryllium Source and Developing Molecular Markers for Antimorph AEI1-5180, An Alternative to AEI to Increase Amylose Content in Maize Endosperm

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ANALYSIS OF TWO MAIZE MUTATIONS THAT AROSE AFTER EXPOSURE TO RADIATION FROM A PLUTONIUM-BERYLLIUM SOURCE

AND

DEVELOPING MOLECULAR MARKERS FOR ANTIMORPH $AEI$-5180, AN ALTERNATIVE TO $AEI$ TO INCREASE AMYLOSE CONTENT IN MAIZE ENDOSPERM

BY

PRAMEELA AWALE

A thesis submitted in partial fulfillment of the requirements for the

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Major in Biological Sciences

Specialization in Biology

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2020
This thesis is approved as a creditable and independent investigation by a candidate for the master’s degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.
ACKNOWLEDGEMENT

This thesis is dedicated to my dad, who always believed in me more than I did in myself. I know wherever he is in this universe, he will be very proud, seeing me chasing my dreams.

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Finally, I would like to thank my husband for being there by my side in my good days and bad days as well.
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<td>Inbred line Iowa 73</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cM</td>
<td>Centimorgans</td>
</tr>
<tr>
<td>Mbp</td>
<td>Mega base pairs</td>
</tr>
<tr>
<td>SBE</td>
<td>Starch branching enzyme</td>
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<td>VCF</td>
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ABSTRACT

ANALYSIS OF TWO MAIZE MUTATIONS THAT AROSE AFTER EXPOSURE TO RADIATION FROM A PLUTONIUM-BERYLLIUM SOURCE

AND

DEVELOPING MOLECULAR MARKERS FOR ANTIMORPH AE1-5180, AN ALTERNATIVE TO AE1 TO INCREASE AMYLOSE CONTENT IN MAIZE ENDOSPERM

PRAMEELE AWALE

2020

This thesis consists of two independent projects dealing with two different aspects of maize. The first project is about the maize reproduction, where we try to uncover the candidate genes of two mutations that affect pollen viability and successful fertilization. The second project is about maize endosperm starch, where we developed molecular markers to characterize a mutant allele that knocks a major starch branching enzyme in maize.

Analysis of two maize mutations that arose after exposure to radiation from a plutonium-beryllium source

Maize is a monoecious and diclinous plant. Sexual reproduction takes place by the fusion of haploid male and female gametes produced in the anther and ovule, respectively. The male gametophyte is pollen, a trinucleate structure, that consists of two sperm cells and a vegetative cell. The female gametophyte is embryo sac, that consists of an egg cell, two synergids, two central cells and a variable number of antipodal cells. After pollination, the
vegetative cell germinates and forms a pollen tube. The pollen tube elongates through the style, penetrates the ovule, and finally burst after reaching embryo sac to release two sperm cells. One sperm cell fuse with the haploid egg cell in embryo sac and forms a diploid embryo, whereas the other fuses with two central cells to form a triploid endosperm. In the first project, we developed two mutant lines, PB1 and PB2 by exposing kernels heterozygous for r1 (colored 1) and c1 (colored-aleurone 1) color factors to a fast neutron source for three and six months, respectively. Two mutagenized plants when crossed with r1 and c1 testers, showed reduced transmission for the R1 marker through pollen, but normal transmission through female. Instead of 50%, the R1 transmission rate through pollen was 23.6% ±0.61% (mean ±se) for PB1, and 1.48% ±0.44% (mean ±se) for PB2. Comparison of whole genome sequences of PB1, PB2 and the unmutated parental line PB1, shows that 32 genes are affected by the deletions in PB1, whereas in PB2, 45 genes are affected. In PB2, genes that code for mechanosensitive ion channel protein, C2H2 type Zinc finger family protein and β-expansin have been previously reported to affect pollen tube germination and elongation, which makes them good candidates.

Keywords: r1 gene, Mutation, Deletions

**Developing molecular markers for antimorph AeI-5180, an alternative to aeI to increase amylose content in maize endosperm**

Maize endosperm consists of mostly starch, which is made of unbranched amylose and branched amylopectin residues. In most Midwestern dents, the endosperm starch is about 75% amylopectin and 25% amylose. Branch formation is catalyzed by starch branching enzymes. Starch branching enzyme IIB (SBEIIb) encoded by aeI (amylose extender 1), is
the primary starch branching enzyme that forms amylopectin. The amylose content increases up to 50% when \textit{ae1} is homozygous recessive. An antimorph allele (\textit{Ae1-5180}), was identified that acts in a dominant fashion to eliminate SBEIIb (Stinard \textit{et al.}, 1993). The essential portion of this mutant allele is a 3kb \textit{XhoI} fragment that includes \textit{Mutator1 (Mu1)} element flanked by an inverted duplication of the \textit{ae1} gene. Based on the restriction map (Stinard \textit{et al.}, 1993) we developed an approximate predicted sequence for \textit{Ae1-5180} using the known sequences of the wildtype \textit{ae1} gene and \textit{Mu1} transposon. Using this, we designed PCR primers targeted to amplify regions within a 3 kb \textit{XhoI} restriction fragment that appears crucial for the dominant action of \textit{Ae1-5180}. The primers amplify two DNA bands from samples expected to have \textit{Ae1-5180}, but none from B73. The sequence of the lower molecular weight band matches with 181 bp of \textit{Mu1} and 364 bp of \textit{Ae1} (545 bp). The higher molecular weight band also possesses 181 bp of \textit{Mu1} but includes 438 bp of \textit{Ae1} (619 bp). The additional 74 bp includes a restriction site for \textit{NotI}, which was predicted from the original restriction map. Sequencing results indicate that these primers reliably detect the presence or absence of the \textit{Ae1-5180} allele. Also, the insertion site of \textit{Mu1} in \textit{Ae1-5180} is now exactly defined.

Keywords: starch branching enzyme IIb (SBEIIb), \textit{ae1}, \textit{Mu1}, \textit{Ae1-5180}
Chapter 1

1 General Introduction and Literature Review

1.1 Maize gametophyte development

Maize is a monoecious and diclinous plant with the male inflorescence, i.e., the tassel, located at the top of the plant. Tassels are branched structures that bear florets that possess only stamens, which produce haploid pollen grains in its anthers. Germline development initiates with archesporial cells. In maize anther, they are the central rows of cells in each anther lobe. Archesporial cells undergoes several mitotic divisions, to form a column of sporogenous tissues in the center, and three-layered wall structure in between sporogenous tissues and epidermis. The innermost layer of the wall is the tapetum. The sporogenous tissue further divide to form microspore mother cells. The first cell of the male gametophyte is the microspore, which is produced by the meiotic divisions of microspore mother cell inside the anther. One microspore mother cell form four microspores arranged in the tetrad but are released from each other by degeneration of the tetrad wall as they develop into pollen grains. Meiosis does not occur simultaneously throughout the tassel, rather begin early from slightly above the base of central axis and proceed into both directions. The tip of the tassel is therefore the last part to shed the pollen. The tassel branches also follow similar pattern as central axis. Before shedding, the pollen goes through two mitotic division, the first division is asymmetrical, and forms a small generative and large vegetative nucleus within the pollen. The second mitotic division occurs only in generative nucleus and produces two sperm cells. Thus, the mature pollen grain is haploid and trinucleate when released from the anther. It is thick walled except at
single germ pore, which is the site of pollen tube germination (Kiesselbach, 1999; Zhou, Juranić, & Dresselhaus, 2017).

The female inflorescence is the ear, which develops from an axillary bud, which are located at each node of the shoot except the topmost one. Generally, there are multiple ears buds in a maize shoot, however in most cases, only the top one or two persists, and others degenerates (Kiesselbach, 1999). The florets of the ear possess only pistils, the female reproductive organs. where embryo sac, i.e., the female gametophyte, develops inside the ovule. Two anterior carpels facing towards ear tip develops into silk or style. The surface of silk is covered by numerous hairs, developed from epidermal cells. Silk elongates due to the continuous cell division at the base, until pollination and fertilization. Like microspore mother cells, megaspore mother cells develop from archaesporial cells. The embryo sac develops from the megaspore mother cell, which undergoes meiosis and form four megaspores in a linear tetrad form. Three megaspores closer to micropylar region degenerate and the remaining megaspore undergoes three consecutive mitotic division and form eight nucleated embryo sac. The first division forms two nuclei, which are separated by large vacuole. Each of the two nuclei divides twice more. Out of the eight nuclei, one from each end move to the center of the embryo sac to form the central cell. These “polar nuclei” remain unfused until fertilization. Three nuclei at chalazal end divide several additional times to form about 20 to 40 antipodal cells. The actual function of the antipodal cells have not been verified experimentally yet, but the high level of sucrose synthase activity found in antipodal cells at 0 days after pollination suggest that it may have nutritive function to the developing embryo (Wittich & Vreugdenhil, 1998). In the micropylar side, one nucleus enlarges to become the egg, while two others become synergids (Kiesselbach,

1.2 Pollination and fertilization

In maize, each pistil possesses an elongated style, commonly called silk. The stigma surface receives the pollen after they are shed by the anther. After landing, the pollen hydrate and germinate. Immediately after hydration, the vegetative nucleus elongates, and form a pollen tube that penetrates the silk and make it way to the embryo sac to fertilize the egg. Although several pollen grains germinate and form pollen tubes, only one pollen (with very few exception) can fertilize the female gametophyte. After reaching the embryo sac, the tip of pollen tube will rupture and release two sperm cells. One sperm fertilizes the egg forming the zygote that will develop into embryo.

Another sperm fuses with the central cell to form the primary endosperm cell that develops into triploid endosperm. Fertilization takes place generally within 24 hours of pollination (Kiesselbach, 1999).

Pollen adhesion, hydration and pollen tube germination are usually not the specific mechanism in case of maize plant. Any incompatible pollen can land, hydrate, and germinate a pollen tube. However, there is a tight control in the pollen tube penetration and further growth. Alien non-grass pollen cannot penetrate through silk surface, whereas further growth of pollen tube inside the style is halted for related grass species and incompatible maize genotypes (Zhou et al., 2017).
1.3 Genes affecting Maize gametophyte development

1.3.1 Genes affecting germline initiation and differentiation

In initial phase of sex determination, growing points for both staminate and pistillate structure are present in tassel and ear primordia. Later, a programmed cell death of pistillate tissue in tassel and staminate tissue on ear take place leading to only one type of flower at each region. The tasselseed1,2,4,5,6 (ts1,2,4,5,6) genes cause abortion of pistillate tissues in the tassel, and mutation in these genes cause feminization of tassel. The ts2 gene is also expressed in ear and induce cell death of pistils, but another gene, silkless1 (skl), protects the functional pistils from cell death. In addition, mutant versions of ramosa3 (ra3) and tunicate1 (tu1) also caused pistil development in tassel. Two sets of genes anther ear1 (an1) and dwarf1,2,3,5,8,9 (d1,2,3,5,8,9) are expressed in ear and cause stamen abortion and development of female flowers. Mutations in these genes caused ears to have male flower structures instead of functional pistillate flowers. Dwarf genes are so named due to their effect in shortening plant height due to the hindered gibberellin synthesis pathway (Calderon-Urrea & Dellaporta, 1999; Chuck, Meeley, Irish, Sakai, & Hake, 2007; Dellaporta & Calderon-Urrea, 1993; Irish, 1996; Lebel-Hardenack & Grant, 1997; Zhou et al., 2017).

Genes have been identified that are important for the formation of archesporial cells, the precursor of microspore mother cells and megaspore mother cells in respective organ. For example, mutant msca-1 (male sterile converted anther 1) lack archesporial cells. In addition, the tapetum, middle layer and endothecium layers, that are found in functional archesporial cell, were replaced by structures consisting of parenchymal cells,
nonfunctional vascular strands and stomal structures in epidermis in later stage of development (Chaubal et al., 2003). This mutant was found only effecting male gamete formation. Another gene mutant, *multiple archesporial cells1* (*mac-1*), hinders the formation of archesporial cells from hypodermal cells in anthers and ovules. In *mac-1* mutant ovules, several hypodermal cells differentiated into archesporial cells in contrast to single hypodermal cell in wild type. This resulted in the formation of more than one embryo sac within an ovule, which led to partial sterility of ears. In anthers, this mutation hinders the periclinal division in cells of primary parietal layer, which is needed to form three layered wall structure surrounding archesporial cells. Instead, archesporial cells divided uncontrollably, leading to the failure to differentiate into functional microsporocytes (Sheridan, Avalkina, Shamrov, Batyea, & Golubovskaya, 1996; Sheridan, Golubeva, Abrhamova, & Golubovskaya, 1999).

1.3.2 Genes affecting Male Gametophyte development

The RNA sequencing from the whole anthers and pollen tissues of the maize showed the expression of 22,178 and 13,418 genes, respectively (Davidson et al., 2011). While some of the genes could overlap, this number stills shows numerous of genes involved in male gametophyte development. Another study reported the expression of 1848 transcription factor coding genes in the tassel, out of which 21 genes were specific to tassel (Jiang et al., 2012). The *outer cell layer 4* (*ocl4*) gene codes for a transcription factor, HD-ZIP IV, that plays a role in cellular differentiation of archesporial cells before meiosis (Vernoud et al., 2009). The mutation in this gene causes partial male sterility due to irregularly shaped non-viable pollen grains. Examination of the deformed pollen revealed that there was an extra subepidermal layer formed during differentiation of archesporial cell layers in mutants. A
number of male sterile mutants have been identified by (Timofejeva et al., 2013), where archesporial cell layer differentiation was affected, either due to malformed cell layers and premature layer degradation, or by addition of extra unnecessary cell layers. Mutants ems-63089 and mtm-0006 lacked middle and tapetal cell layers. Similarly, tapetal cell layer 1 (tcl-1) mutants did not form organized middle and tapetal layers but had extra cells between endothecial and archesporial cells. Two other mutants, ems-72063 and ems-72091, formed five layered anther cell walls by an additional periclinal division on tapetum and middle layer, respectively. Mutants ms*-6066 and ms*-6015 lacked binucleate tapetal cells, a typical feature of fertile anthers.

After differentiation of archesporial cells to pollen mother cells (PMC), PMC undergoes meiotic division to give rise to haploid microspores. PMC cells in am-1 (ameiotic1) mutants, fail to proceed through meiosis, rather enter mitosis. The am1 gene codes for a protein that is required by PMC to enter meiosis and proceed through early meiotic prophase stage (Pawlowski et al., 2009; Staiger & Cande, 1992). In another mutant, ms-8 (male sterile 8), reported by (Wang et al., 2010), the process of meiosis was normal, but the daughter cells soon collapsed after completion of meiosis. The dyads after first meiotic division were smaller and had gaps between cells. So, the mutant could release only sterile pollen with cellular debris and no functional nuclei.

Meiosis in PMC forms a pollen tetrad, which is eventually released by the degeneration of parental wall. After the release, each pollen divides asymmetrically to form large vegetative nuclei and small generative nuclei to form binucleate pollen. MAB1 (MATH-BTB domain protein) is required for this asymmetric cell division. The gene encoding MAB1 is expressed in the PMC during meiosis, and the meiosis to mitosis transition of
immature pollen. Lack of the MAB1 protein produced two nuclei of equal size, which hinders the pollen development afterwards (Juranić et al., 2012).

Following this first asymmetric, mitotic division, two layers of cell wall starts to form around immature pollen. The inner layer is called intine and outer layer is exine. The exine is formed by tapetal cells. Additional lipids and proteins are deposited on this layer as the tapetal cells start to degenerate and the pollen start to dehydrate and mature (Bedinger & Fowler, 2009). According to (Cigan, Unger, Xu, Kendall, & Fox, 2001), a gene expressed in tapetal cells, ms45, might have an important role in exine layer formation in pollen, because the ms-45 mutant plants did not have viable pollen. Further examination showed non-viable pollen could not form exine wall layer after first mitotic division.

1.3.3 Genes affecting Female Gametophyte development

A widely studied gene affecting female gametophyte development is indeterminate gametophyte 1 (ig1), which encodes for lateral organ boundaries (LOB) protein, responsible for restricting the proliferating phase of female gametophyte development in maize. A mutation in this gene causes nuclear division without control, leading to embryo sac abnormalities like extra egg cells, extra polar nuclei and extra synergids (Evans, 2007; Guo, Huang, Han, & Zee, 2004). Mutant ig1 plants can also produce haploid embryos after fertilization in a process called androgenesis (Kermicle, 1969). (Krohn, Lausser, Juranić, & Dresselhaus, 2012) in their study using RNAi knockdown, reported that ZmEAalL1 is required for strong expression of ig1 in antipodal cells. This gene is also necessary to protect the cell fate of antipodal cells and prevent them from converting into central cells in embryo sac. In addition, the diSUMO-like protein (2 domains of small ubiquitin related
modifiers) ZmDSUL, has been reported to express exclusively in the egg apparatus and zygote. In the female gametophyte, this protein was found to be essential for nuclei segregation and positioning in embryo sac, as shown by the mutant study using RNAi silencing of the gene encoding this protein.

1.3.4 Genes affecting Pollination and Fertilization

After the pollen lands on the stigma, the germinating pollen tube needs to penetrate the cellular walls of stigma and reach to the embryo sac to have a successful fertilization. Studies show two proteins identified to date, facilitate the penetration and growth of pollen tube inside the ovary. (Rubinstein, Marquez, Suarez-Cervera, & Bedinger, 1995) reported on \textit{pex1}, a gene with an extension-like domain and exclusively expressed in maize pollen. This protein was localized in a pollen tube with germinating pollen, making \textit{pex1} gene, a strong candidate involved in pollen tube growth. Another class of genes that code for expansin proteins have been reported to play role in pollen tube penetration inside the stigma surface.

Group 1 allergens in maize are pollen specific β-expansin group protein. There are fifteen different kinds, mapped to the genomic location in short arm of chromosome 2 and 3, and long arm of chromosome 5 and 9 in maize genome. Pollen from a \textit{Mu}-transposon insertion line in \textit{expb1}, a most abundant isoform in maize, has no effect on viability and pollen tube growth, but the efficiency to fertilize the embryo sac decreased significantly in a competing situation with wild type pollen. The reason for this inefficiency was elucidated as the pollen aggregation in higher rate, seen on stigma surface, which was not readily separable into individual pollen, as in wild type pollen. In addition, the mutant pollen tube did not
penetrate readily into stigma surface after germination, rather twisted and curl around silk hairs and penetrated through silk hairs rather than through silk surface. The wild type pollen tube outpaced the mutant pollen in the race to reach embryo sac. Furthermore, when all gene expansin coding genes were silenced using RNAi, more severe consequences were seen. 71% of transgenic plants were not fully fertile (<65 seeds set) and the male gametophyte transmission was zero for one transgenic line, even when the pollen was viable when checked. In addition, the anthers that normally developed in earlier stages shriveled and died just before the release of pollen (Valdivia, Sampedro, Lamb, Chopra, & Cosgrove, 2007; Valdivia, Stephenson, Durachko, & Cosgrove, 2009; Valdivia, Wu, Li, Cosgrove, & Stephenson, 2007).

Pollen specific calcium dependent protein kinases (CDPKs) have been identified that regulate the pollen tube germination in both ways; positive as well as negative. A positively regulating calcium dependent but calmodulin independent protein kinase transcripts have been isolated exclusively from pollen. This kinase has a very high expression in late trinucleate stage and the level remains high in mature and germinating pollen. Invitro pollen germination was 40 to 75% with normal pollen tube growth, observed in the external calcium concentration between 0.5mM to 5mM. The concentrations of calcium ions beyond this range and CDPK inhibitors, inhibit pollen germination (Estruch, Kadwell, Merlin, & Crossland, 1994). A negatively regulating CDPK gene, zmcpk32 (calcium dependent protein kinase 32) is also reported, whose expression is highly upregulated in pollen at shedding. The accumulation of this protein inhibit the pollen germination and pollen tube growth, and the negative regulation is dependent on kinase activity of this protein (J. Li et al., 2018).
Many popcorn species have cross incompatibility genes, called *Gametophytic factors (ga)*. A strong allele, *Gal-S* of this factor is found on a Chinese popcorn species SDGa25, which blocks the pollen from majority of Chinese dent and flint maize lines that carry *gal* pollen. The female and male determinant of this *Gal-S* have been cloned (H. Zhang et al., 2012; Z. Zhang et al., 2018). Another factor, *Teosinte crossing barrier 1 (Tcb1)* identified in annual teosinte acts in similar fashion (Lu, Hokin, Kermicle, Hartwig, & Evans, 2019). All these factors confer unilateral cross incompatibility by encoding pectin methyl esterase, that modifies the cell wall of pollen tube inside the silk.

Once the pollen arrives near the micropylar region, a study has shown that the pollen tube was guided to the egg apparatus by a stimulus protein, encoded by *z*ea mays *EGG APPARATUS 1 (ZmEAI)*. This gene is expressed exclusively in the egg apparatus and codes for a 94 amino acid protein and has been reported to involve in guiding pollen tube through layers of micropyle into the egg apparatus (Márton, Cordts, Broadhvest, & Dresselhaus, 2005).

### 1.4 Generating Mutation with fast neutrons

Fast neutrons are produced when alpha or gamma radiation emitted from a radioactive, heavy element disintegrate the atomic nuclei of a light element, such as Beryllium. The most common alpha emitters are Radium, Polonium, Plutonium or Americium combined with light element such as Boron or Beryllium (Harvey, 2010). The combination of plutonium and beryllium produce fast neutrons in a reaction as follows:

\[
\frac{4}{2}\text{He} + \frac{9}{4}\text{Be} \rightarrow \frac{12}{6}\text{C} + \frac{1}{0}\text{n}
\]
Here, $^4_2\text{He}$ is the alpha particle emitted by Plutonium. Using the fast neutrons thus generated, mutations can be induced in plants. A common mutation caused by fast neutrons are large deletions (>1Mb) and chromosome rearrangement in genome (Gilchrist & Haughn, 2010). This mutagenesis technique is now used for both forward and reverse genetics. (X. Li & Zhang, 2002). Fast neutrons completely knock out the site of the mutation and avoids the need of transformation. However, the frequency of mutations is low and screening can be laborious and complicated by multiple gene deletion (Gilchrist & Haughn, 2010). Commonly used techniques for mapping the mutation produced by fast neutrons are Mapping by Mutation (MutMap), MutMap-Gap, mutant chromosome sequencing (MutChromSeq), exon capture, tilling, Mut-Ren-Seq, Whole Genome Sequencing and Genotyping by Sequencing (Kumawat et al., 2019).

### 1.5 Maize endosperm

A normal maize kernel is made up of 82-83% endosperm, 10-11% germ, 5-6% pericarp and 0.8-1% tip cap (Singh, Kaur, & Shevkani, 2014). Maize endosperm is mainly composed of approximately 70-85% of starch and a very less proportion of maize proteins. Based on starch granule size, structure and arrangement, maize endosperm can be horny and floury. Horny endosperm contains small starch granule, concentrated towards periphery and tightly packed. In contrast, floury endosperm consists of large sized starch granule, loosely packed towards the center of the endosperm (Singh et al., 2014). Biological function of starch is to serve as the source of energy to the developing seedling until it becomes able to make food by itself by photosynthesis. Starch in the maize endosperm is insoluble in water and found as granules in the stroma of amyloplast.
Amyloplast is a form of plastid where main reactions of starch biosynthesis take place. Starch is formed from two different types of glucan molecules: less or unbranched amylose and highly branched amylopectin. The main structural backbone in final semi-crystalline structure of starch is formed by amylopectin and the spaces between are filled by amylose, making the starch granules denser. The higher order arrangement of amylopectin is A-type starch polymorphs, which is common in all cereals (Imberty, Chanzy, Perez, Bulèon, & Tran, 1988).

1.5.1 Amylose

It is the linear or very less branched structure. Two glucose residues in amylose are linearly bonded by $\alpha$-1,4 glycosidic bonds. A molecule of amylose can have hundreds to thousand glucose residues, and comparatively very simple in structure and shorter in length than amylopectin. Amylose has the property of higher retrogradation in comparison to amylopectin, which makes it a resistant starch, which is not digested in small intestine and sent to colon (Singh, Inouchi, & Nishinari, 2006).

1.5.2 Amylopectin

Nearly 70-75% of starch granules in common maize lines is amylopectin. It is highly branched and complex structure made of glucose residues. It consists of linear chains of glucose residues connected to each other by $\alpha$-1-4 glycosidic bonds, and numerous branches within the linear chain connected by $\alpha$,1-6 glycosidic bonds. The linear chains of amylopectin can be variable in length, ranging from 7 to 50 glucose residues. Based on branching, glucan chains in amylopectin are classified as A, B and C chains. An A chain lies outward in the whole structure and unbranched. A B chain has more than one branch
within the chain. A C chain is branched like B chain but also has a free reducing end. Therefore, a C chain is only one in an amylopectin molecule (Martin & Smith, 1995).

![Diagrammatic representation of Amylose and Amylopectin.](image)

Figure 1.1 Diagrammatic representation of Amylose and Amylopectin.

Amylose has linear chain and very low branching; Amylopectin is heavily branched and consists of A, B and C chain. Modified from (Martin & Smith, 1995; Pfister, Zeeman, & Sciences, 2016).

### 1.6 Maize Starch Biosynthesis

Starch biosynthesis pathway is a complex pathway that starts on cytosol and completes in plastids. The plastids can be either chloroplasts or amyloplasts. The biosynthesis starts with sucrose being converted into fructose and UDP-glucose (L. C. Hannah & Boehlein, 2017). Sucrose, after series of reaction catalyst by different enzymes, is converted to ADP-glucose, which is the precursor for the starch synthesis process that occurs in plastids. The
formation of ADP-glucose is catalyzed by ADP glucose pyrophosphorylase. This step is the committed step in starch synthesis (Pfister et al., 2016). However, the rate limiting step of the starch synthesis is different across plants. In barley, the transport of ADP-glucose from the cytosol to plastid limits the starch synthesis (Tiessen et al., 2011). However, experiment in Rice overexpressing btl (brittle l) gene suggest that the expression of this gene is required to transport ADP-glucose to amyloplast (Cakir et al., 2016). ADP-glucose is then converted into starch by the action of three major group of enzymes: starch synthase, starch branching enzymes and debranching enzymes. Different enzymes are involved in synthesis of starch components; amylose and amylopectin which will be described later. A schematic representation of Starch biosynthesis is presented below in Figure 1.2.
Figure 1.2 Starch Synthesis Pathway in maize.

The major events that occur during starch biosynthesis in Cytosol and Plastid are shown above. Enzymes involved are abbreviated. Inv, invertase; Sus, sucrose synthase; UGPase, UDP-glucose pyrophosphorylase; AGPase, ADP-glucose phosphorylase; FK, fructokinase, PFK, phosphofructokinase; TPI, triose phosphate isomerase; PK, pyruvate kinase; PPtase, pyrophosphatase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; FBP, fructose 1,6 bisphosphatase; SS, starch synthase; SBE, starch branching enzyme; DBE, debranching enzyme. Adapted from (L. C. Hannah & Boehlein, 2017).
1.7 **Enzymes involved in Starch Biosynthesis**

Although many enzymes are involved in starch synthesis as shown in Figure 1.2, only the enzymes involved in the production of ADP glucose and process thereafter are explained in detail here.

1.7.1 **ADP glucose pyrophosphorylase (AGPase)**

In cereal endosperm, AGPase is found on cytosol. It is a heterotetramer protein with two large subunits and two small subunits. Larger subunits are regulatory, whereas two small subunits are functional domains. Enzyme activation takes place allosterically by 3-phosphoglycerate and inhibited by organic phosphate. This enzyme catalyzes the reaction in which glucose, 1-phosphate and ATP is converted to ADP glucose in cytosol as well in stroma of plastid.

1.7.2 **Starch synthases (SS)**

Five isoforms of starch synthase have been identified which are involved in starch biosynthesis in plants. Out of the five isoforms, SSI, SSII, SSIII, SSIV are soluble in stroma and synthesize amylopectin fraction of starch. The fifth isoform is granule-bound SS (GBSS) and will be explained later. In maize, SSI and SSIII (called DU1 earlier) are the major isoforms (Cao et al., 1999). However, recently SSV has been identified in maize but the involvement of enzyme on starch synthesis is yet to be confirmed (Liu et al., 2015). SS elongates the already existing chain with α,1-4 glycosidic linkage by using the glucose from ADP glucose. How the first unit of glucan chains is formed is still unclear. C-terminal catalytic domain of SS is highly conserved. It also has a N-terminal extension which is not
conserved among different species. SSI from maize extends shorter chains (A and B1 chains) to a certain length and stops. The further elongation of the chain is probably carried over by other SS enzymes (Commuri & Keeling, 2001). Deficiency of SSII in maize decrease the proportion of intermediate chain and increase in short glucan chains, suggesting than SSII is also needed to increase the chain length of short glucan chains (X. Zhang et al., 2004). The function of SSIII is not clearly defined yet.

1.7.3 Granule bound starch synthase (GBSS)

It is different from other SS in its location and function. It is exclusively bound in granule of plastids. It catalyzes the synthesis of amylose unlike other SS. A study using transgenic potato lines incapable of forming GBSS shows that amylose is synthesized in the matrix of starch granule formed by amylopectin and the synthesis increases with the level of GBSS in plant cells (Tiessen et al., 2011).

1.7.4 Starch branching enzymes (SBEs)

Three isoforms of SBEs are found in maize; SBEI, SBEIIa and SBEIIb (Tetlow & Emes, 2014). Studies in maize shows that SBEI prefers amylose as a substrate and can transfer long glucan chains as branches with degree of polymerization (DP) up to 30, more often DP ranging from 10-13. However, SBEII prefers amylopectin as a substrate and transfers short glucan chains (Guan & Preiss, 1993; Takeda, Guan, & Preiss, 1993). In cereals, SBEII is further divided into SBEIIa and SBEIIb, encoded by different genes. SBEIIb in monocots is found only found in endosperm whereas SBEIIa is found on other tissues as well. Study of branching enzymes (BEs) from rice endosperm shows that different isoforms have different chain length preferences. BEIIa transfers glucan chain length with a DP of
6 to 15. BEIIIB can only transfer chains of DP 6 and DP 7. In contrary, BEI can transfer chains with DP less than or equal to 40. In addition, BEIIa can attack inner chains of amylose and amylopectin only few times, but BEIIb cannot attack inner chains at all. BEI can often attack both inner and outer chains of amylose and amylopectin to form branching (Nakamura et al., 2010).

A study compared the enzymatic activity between normal maize line and low amylopectin starch (LAPS) maize line and found that the activity of SBEII is much lower, approximately 2% compared to normal high amylopectin maize. In addition, the starch morphology in LAPS was different than in normal maize endosperm starch. The starch granules were irregular and elongated in LAPS whereas starch granules had regular shape in normal maize lines (Sidebottom, Kirkland, Strongitharm, & Jeffcoat, 1998). SBEIIa is the main branching enzyme in leaves, which is essential for maintaining the diurnal cycle of transitory starch metabolism in leaves. Lack of this enzyme effects starch degradation at night, leading to starch hyperaccumulation and leaf senescence. The SBEIIa mutants also have different starch structure than normal plants. This finding suggests that SBEIIa is required enzyme to produce amylopectin structure in starch, that can be degraded during transitory starch metabolism (Susan L Blauth et al., 2001).

In maize endosperm, SBEIIb is the predominant isoform of SBE. A deficiency of SBEIIb in maize causes reduction in starch synthesis by 20%, change structure of starch granule forming long internal amylopectin chain lengths and less outer branched chains. The proportion of amylose content also increases in maize endosperm in absence of this enzyme (Boyer, Daniels, & Shannon, 1976). SBEI mutants in maize had altered branching pattern in amylopectin structures, which was not readily digested by amylase during seed
germination, thus resulting to the poor growth of seedlings (Xia, Yandeau-Nelson, Thompson, & Guiltinan, 2011). All SBEs contain the central catalytic A-domain, NH$_2$ and C terminal domain.

1.7.5 Debranching Enzymes

De-branching Enzymes are responsible for cleaving branching formed from $\alpha,1$-6 glycosidic linkage by hydrolysis, and releasing a linear chain. According to GlucanTrimming Model (Ball et al., 1996), excess branches are removed from pre-amylopectin, which then facilitates right chain elongation by starch synthases. Two types of debranching enzymes are known: iso amylases (ISA) and limit dextrinase (LDA). ISA1 and ISA2 removes branches from amylopectin. ISA3 and LDA facilitates starch debranching during its degradation (Pfister et al., 2016).

1.8 Genes involved in Starch Biosynthesis

The $bt2$ (brittle2) and $sh2$ (shrunken2) genes encodes for different subunits of AGPase enzyme. The mutants from both genes lack AGPase activity in the endosperm (L. Hannah & Nelson, 1976; Tsai & Nelson, 1966). The $wx1$ (waxy) locus in maize codes for granule-bound starch synthase I enzyme (GBSS I) (Huang, Tian, Zhang, & Huang, 2010; Nelson, 1962). Starch branching Enzyme IIb is the product of gene $ae1$ (amylose extender I) (Fisher, Boyer, & Hannah, 1993; Stinard et al., 1993). The $sbe1$ (starch branching enzyme I) codes for starch branching enzyme I (Baba et al., 1991). The waxy mutants have opaque endosperm consisting almost entirely amylopectin. The $ae1$ mutants form glassy tarnished endosperm with high amylose content than the wild type for this gene (Neuffer, Jones, & Zuber, 1968). There is no visible morphological differences in $sbe1$ mutant kernels
compared to wild type (S. L. Blauth et al., 2002). *bt1* (*brittle 1*) codes for BT1 protein, an adenylate translocator, that transports the ADP-glucose into the amyloplast stroma (Shannon, Pien, Cao, & Liu, 1998; Shannon, Pien, & Liu, 1996). The *Zmss1* (*starch synthase 1*) encodes for starch synthase I (SSI) enzyme in maize (Knight et al., 1998). Another gene *dull 1* (*du1*) codes for starch synthase II (SSII) in maize (Gao, Wanat, Stinard, James, & Myers, 1998).

### 1.9 *Ae1-5180*, mutant allele of *ae1* gene

The effect of SBEI enzyme is only visible when SBEIIb activity is absent in the endosperm (Yao, Thompson, & Guiltinan, 2004). The activity of SBEIIb can be eliminated when the *ae1* is in homozygous recessive form. A mutant allele of *ae1* has been identified that acts in dominant fashion over wild type *Ae1* allele and eliminates SBEIIb enzyme in endosperm. The cloning and structure of this allele has been described (Stinard, et al., 1993).

#### 1.9.1 *Ae1-5180* allele predicted structure

Based on the restriction map of *Ae1-5180* clones (Stinard, et al., 1993), this mutant allele consists of two copies of *Mul* insertion and three copies of part of *Ae1* sequences. One *Mul* sequence is flanked by two copies of *Ae1* sequences, one of which is the inverse duplication of another. Another *Mul* sequence is flanked by *Ae1* repeats on one side and wild type *Ae1* sequence in another side.
They were able to clone 2.3 kb and 3 kb *XhoI* restriction fragment which they mentioned to be a portion of 12.5 kb and 12 kb fragments, respectively. The wild type lacked both 2.3 kb and 3 kb *XhoI* fragments. However, the wild type revertant of *ae1* homozygous recessive mutants had 2.3 kb fragment but lacked 3 kb *XhoI* fragment. Therefore, the study concluded that for a functional *Ae1-5180* antimorph, presence of 3 kb fragment is probably crucial.

Figure 1.3 Restriction map of *Ae1-5180* clones.

A. Restriction map of the 12.5 kb *Ae1-5180* HindIII (H3) fragment. This is a simple insertion of 1.4 kb *Mu1* element (red hashed box) into *Ae1*. B. Restriction map of the 12 kb *Ae1-5180* HindIII (H3) fragment. This *Mu1* insertion is associated with an inverse duplication of *Ae1* fragments, represented by the dashed arrow. C. Restriction map of 3 kb *XhoI* (X) sub-fragment of the 12 kb fragment. Restriction sites: B = *BanII*; E = *EcoRI*; H = *HinfI*; H3 = *HindIII*; M = *MluI*; N = *NotI*; P = *PstI*; S = *SacII*; X = *XhoI*.

Adapted from (Stinard, Robertson, & Schnable, 1993)
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Chapter 2

2 Analysis of two maize mutations that arose after exposure to radiation from a Plutonium-Beryllium source

Abstract

Maize reproduces sexually and undergoes a short gametophytic cycle. It produces pollen grains in an anther, the male inflorescence, and an embryo sac in an ovule, the female inflorescence, by the process of microsporogenesis and megasporogenesis, respectively. Mature pollen is trinucleate and consists of two haploid sperm cells and a vegetative cell. One sperm cell fuse with a haploid egg cell in the embryo sac and forms a diploid embryo, whereas the other fuses with two central cells to form a triploid endosperm. The vegetative cell after pollination forms pollen tube and function as transmitting tract for sperm cells.

We exposed maize kernels heterozygous for $rI$ and $cI$ color factors to a fast neutron source, produced from a combination of Plutonium-Beryllium elements for three and six months, referred as mutant lines PB1 and PB2, respectively. Mutagenized plants were tested for reduced male gametophyte transmission, making reciprocal crosses with $rI$ and $cI$ testers. PB1 and PB2 lines showed reduced transmission of $R_I$ marker through pollen, but normal transmission through female. The $R_I$ transmission rate through pollen was $23.6\% \pm 0.61\%$ ($n = 3$) for PB1, and $1.48\% \pm 0.44\%$ (mean ± se, $n = 5$) for PB2, instead of $50\%$ transmission expected. The whole genome sequences of PB1, PB2 and unmutated P1 were assessed for potential mutations candidates unique in PB1 and PB2 and linked to the $R_I$ marker. In PB1, 32 genes are affected by the deletions within approximately 20 cM region proximal and distal of $rI$ whereas in PB2, 45 genes are affected within 1.5 cM region proximal and distal.
to *r1*. Two genes in PB1 and ten genes in PB2 are eliminated by deletions. In PB2, genes that code for mechanosensitive ion channel protein, C2H2 type Zinc finger family protein and β-expansin have been previously reported to effect in pollen tube germination and elongation. Further verification using molecular approaches is needed to determine the effect of mutation candidates in pollen viability and pollination.

Keywords: Pollen, *r1*, Mutation, deletion
2.1 Introduction

Maize is a monoecious and diclinous plant. The male inflorescence is tassel and located at the top of the shoot, whereas the female inflorescence is ear, which is located on the shoot node. Maize produce haploid pollen in its anther by the process of microsporogenesis, and haploid embryo sac in ovule by the process of megasporogenesis (Kiesselbach, 1999). The precursor cells for the initiation of germline is archesporial cells, which originate from hypodermal cells. The transition from hypodermal to archesporial cells failed in msca-1 (male sterile converted anther 1), that eventually resulted into the anther without mature pollen (Chaubal et al., 2003). After archesporial cell is formed, it divides and form sporogenous cells and primary parietal layer. The primary parietal layer divides to form secondary parietal layer and endothecium. Similarly, the secondary parietal layer divides to form middle layer and tapetum (Zhou, Juranić, & Dresselhaus, 2017). After three consecutive division, a three-layered wall structure forms between sporogenous tissue and epidermis in anther. The sporogenous tissue develops into microspore mother cells. In mac-1 (multiple archesporial cells1) mutants, the periclinal division during formation of wall layers is hindered. Instead, the archesporial cells divide uncontrollably and fail to differentiate to microspore mother cells (Sheridan et al., 1999). The microspore mother cell undergoes meiotic division and produces four pollen grains arranged in the form of tetrad. The pollen grains are released by the degeneration of microspore mother cell wall, enclosing the tetrad. Each immature pollen divides asymmetrically and forms large vegetative cell and small generative cell. Vegetative cell later forms pollen tube. The generative cell divides again and form two sperm cells. MAB1 (MATH-BTB domain protein) is essential for this asymmetrical division. Lack of MAB1 protein causes the
equational division of pollen and hinders pollen development in later stages (Juranić et al., 2012). As the pollen starts to mature, two layers of cell walls are formed, inner intine and outer exine. The tapetal degradation adds additional lipids and proteins to the exine layer (Bedinger & Fowler, 2009). Failure to form the exine wall layer can lead to the inviable pollen as seen in ms-45 mutants (Cigan et al., 2001).

Archesporial cells in ovule form megaspore mother cells, which undergoes meiosis and forms four megaspores in linear tetrad form. Unlike microspores, the three megaspores near to the micropylar region soon degenerate, and the functional megaspore divides thrice to produce eight nucleated embryo sac. Out of the eight nuclei, one from each end move to the center of the embryo sac to form the central cell. These “polar nuclei” remain unfused until fertilization. Three nuclei at chalazal end divide several additional times to form about 20 to 40 antipodal cells. The embryo sac then enlarges to become mature and ready for fertilization (Kiesselbach, 1999; Zhou et al., 2017).

As anther dehiscence, mature pollen is released and trapped by silk hairs. Soon, after the contact, pollen starts to hydrate, and the pollen tube starts to germinate. The pollen tube penetrates the stigma surface, elongates through the style, and grows through layers of maternal tissue before reaching embryo sac. *Zea mays* EGG APPARATUS 1 (ZmEA1) encodes for a stimulus protein, that guides the pollen tube in the micropylar region to the egg apparatus. Once in the egg apparatus, the pollen tube bursts and releases two sperm cells. One sperm nuclei fuse with egg cell to form diploid embryo, whereas other fuses with one central nuclei, which again fuses with the other nuclei to form triploid endosperm.
Therefore, for a successful pollination from male gametophyte perspective, a viable pollen is the first requisite. The viable pollen needs to be successfully delivered into the viable embryo sac for a successful fertilization. Therefore, failure to form viable pollen or failure to penetrate and grow inside maternal tissues, can cause sterility due to male gametophyte.

In this study, we are using whole genome sequencing approach to identify the potential candidate genes in two mutant lines named as PB1 and PB2. PB1 and PB2 were exposed to a fast neutron sources generated by a combination of Plutonium and Beryllium. The field experiment data shows reduced male gametophyte transmission linked to \( r1 \) marker in these mutant lines, but at a different frequencies This study will provide insight on candidate genes that affect male gametophyte transmission in maize and linked to the \( r1 \)-marker. Thus, further verification of the gene function using molecular techniques can provide valuable insights on how a mutation in a gene can affect the pollen viability in maize. This knowledge can be used in inducing male sterile lines in maize.

### 2.2 Methodology

#### 2.2.1 Plant materials

Maize kernels heterozygous for \( rl \) and \( cl \) color factors were exposed to a fast neutron source, produced from a combination of Plutonium-Beryllium elements in South Dakota State University Department of Physics in 2015. Two groups of kernels were made and subjected to fast neutrons bombardment for three or six months. Kernels subjected to three and six months are referred as mutant lines PB1 and PB2 respectively hereafter. Both lines were backcrossed to B73 for three and two generations, respectively. Mutagenized plants were grown in South Dakota and tested for reduced male gametophyte transmission. In a
subsequent generation, the mutant lines PB1 and PB2 were crossed onto \( r1 \) \( C1 \) and \( R1 \) \( c1 \) testers to determine whether the mutations were linked to \( r1 \) or \( c1 \). In both lines, the mutations were linked to \( r1 \) on the long arm of chromosome 10 (10L). Both lines were found to have reduced transmission of the \( R1 \) allele through the pollen; female transmission of this marker was normal. PB1, PB2 and the maize kernels from an unmutated \( R1 \) B73 ear (hereafter referred as P1) were grown up to two leaf stage in an incubator at 30°C before DNA extraction.

2.2.2 DNA extraction and quality control

DNA extraction for P1 was done using a protocol for high molecular weight DNA extraction using magnetic beads (Mayjonade et al., 2016). PB1 and PB2 DNA samples were previously extracted and sequenced (Burch, 2018). Fresh tissues immediately after harvest were frozen in liquid nitrogen and crushed into fine powder using mortar and pestle. To 100 mg of fresh tissue, 900 µl of freshly prepared lysis buffer and 5 µl of RNase A (100 mg/ml) was added. The mixture was immediately homogenized by inverting the tube 20 times and incubated at 65°C for an hour, inverting tubes 20 times, every 10 minutes of incubation period. After incubation, 300 µl of 5M potassium acetate was added, mixed and the mixture was then incubated on ice for 10 minutes. The mixture was centrifuged at 5000 x g for 10 minutes at room temperature and the supernatant was transferred to a new 2 ml tube without disturbing the pellet. Next, one volume of binding buffer and 200 µl of Sera-Mag beads at room temperature (5% solid, GE Healthcare, Pittsburg, PA, USA) were added to the supernatant and mixed immediately with gentle agitation for 10 minutes at room temperature. The beads were collected back by placing the tube on 3D printed magnetic tube rack with neodymium block magnets (B842, K &J Magnetics, Pipersville, PA, USA),
until the solution became clear. The sample was washed using 70% ethanol three times before air drying for a minute. 200 µl of preheated Buffer EB at 50°C was then added to the sample and the beads were resuspended and placed back into the magnetic rack overnight to ensure maximum DNA elution. DNA concentration and quality were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and further confirmed by standard Gel Electrophoresis (2% agarose gel).

2.2.3 Sequence and data analysis

P1 was sent to Novogene corporation (Sacramento, CA, USA) and sequenced using NEBNextR DNA library Prep Kit in Illumina Novoseq 6000 platform that generates pair reads of 150 bp. PB1 was sequenced at Macrogen (Rockville, MD, USA) with Illumina TrueSeq PCR Free Library Kit on the Illumina HiSeq X for 150 bp paired-end reads. PB2 was sequenced at Hudson Alpha Discovery using Chromium Genome Library Kit (10X Genomics, Pleasanton, CA, USA) in single lane Illumina HiSeq X generating 150 bp paired end reads.

Quality check and trimming for P1 and PB1 samples were done using FASTQC (Andrews, 2010). Reads were aligned to the reference genome B73_RefGen_V4 release-45 (ftp://ftp.ensemblgenomes.org/pub/plants/release-45/fasta/zea_mays/dna) using BWA-MEM. (Li, 2013). Duplicate alignments were marked and removed using SAMBLASTER (Faust & Hall, 2014). The discordant pair end alignments and split pair end alignments were extracted using samtools (Li, 2011) or bamtools (Barnett, Garrison, Quinlan, Strömberg, & Marth, 2011). Structural variants were called using LUMPY (Layer, Chiang, Quinlan, & Hall, 2014) using pre-extracted splitter and discordant reads.
PB2 sample was analyzed using the Long Ranger v2.2 (10X Genomics, Pleasanton, CA, USA). The whole genome mode paired with GATK 4.0.3 (https://gatk.broadinstitute.org/hc/en-us) was used to clean and sort fasta files, aligned sequenced reads to the B73_RefGen_V4 release-45 and call structural variants. Small deletions and large structural variants were called into different output file in this workflow.

All the structural variants from P1, PB1 and PB2 were subset to two different regions relative to the \( r1 \) (139779468bp-139791776bp) locus in chromosome 10 using VCFtools (Danecek et al., 2011). Previous data from our lab (unpublished) showed that the candidate mutations in PB1 line is about 24 cM away from \( r1 \), but for PB2, its only 1.5 cM approximately. However, for PB1, we also know that recombination of 24% is overestimation as we have evidence of incomplete penetrance. Taking the linkage map from Maize GDB as a reference (https://www.maizegdb.org/data_center/map?id=940889), we defined a region in the distal and proximal side of \( r1 \), where chance of finding potential candidate mutations was high. For PB1, approximately 20cM from \( r1 \) on proximal and distal side was considered as midpoint, and DNA sequences within 10cM left and right from the midpoint were assessed to find potential candidates in proximal and distal sides of \( r1 \). But, whenever there is a lack of information about gene within 10cM from midpoint, the nearest available gene coordinates were used. Similar approach was used for PB2, but the midpoint was determined at 1.5cM and length considered was 1cM left and right from midpoint. For PB1, DNA sequences of 31.5 Mbp (107000000 bp-138500000 bp) and 6.5 Mbp (143500000 bp-150000000 bp) on proximal and distal side of \( r1 \) respectively, were checked for mutation candidates. For PB2, DNA sequences of 1.2 Mbp (138500000 bp-
1397000000 bp) and 3.7Mbp (139800000 bp-143500000 bp) on proximal and distal side of \( rI \) respectively, were checked. The mutations in P1, PB1 and PB2 was set to include deletions only. Common mutations in P1, PB1 and PB2 within these regions were eliminated because it is unlikely that the same mutation causes different phenotypes. The functional consequences of the deletions was predicted using ENSEMBL Variant Effect Predictor (McLaren et al., 2016). The mRNA and protein expression of predicted candidate genes were observed using publicly available mRNA and proteomic data for maize vegetative and reproductive tissues.

2.3 Results

2.3.1 Reduced male gametophytic transmission of PB1 and PB2

In PB1, the \( R1 \) transmission rate through pollen was 23.6% ±0.61% (n = 3) and \( C1 \) was 50.7% ±1.49% (n = 3). In PB2 the pollen transmission for \( R1 \) marker was 1.48% ± 0.44% (mean ± se, n = 5), and \( C1 \) marker was 51.8% ±1.11% (n = 5) in PB2. (Burch, 2018). These phenotypic data show that the transmission of \( C1 \) is close to the expected ratio of 50%, whereas the transmission of \( R1 \) is far low than the expected ratio. These data provided evidence that the reduced male gametophyte transmission in mutants is linked to \( R1 \) allele.

2.3.2 Genome sequencing and identifying mutations

The sequencing depth of P1, PB1 and PB2 were 28X, 27.9X and 54.1X, respectively. Of the total reads, 99.78% in P1, 99.39% in PB1 and 98% in PB2 aligned to B73 reference genome V4. The total number of mutations called throughout the genome was 9541, 6193 and 7479 respectively for P1, PB1 and PB2 (Table 2.1). We checked different regions in
chromosome 10, around r1 to find out the potential mutation candidates as mentioned in
detail in Methodology. A total of 32 and 45 unique genes are affected by deletions in PB1
and PB2, respectively. The size of deletions ranges from few base pairs to 45,164 bp in
PB1 and 280,000 bp in PB2. The common consequences of deletions as predicted by
ENSEMBL Variant Predictor are upstream gene variants, downstream gene variants, intron
variations, feature truncation, intergenic region variants, 5’ and 3’ untranslated region
(UTR) variants, coding sequence variants and transcript ablations (Figure 2.1).

2.3.3 Identifying candidate genes in PB1 and PB2

In PB1, out of 32 genes affected, deletion of 21,231bp occurred in the region 135865341
bp to 135886572 bp contains two genes: Zm00001d026008 and Zm00001d026009, which
are completely lost. Zm00001d026008 codes for an uncharacterized protein. Zm00001d026009 encodes for transcription factor bHLH (basic helix loop helix). Loss of
function of the two genes mentioned may have caused mutant phenotypes in PB1.

In PB2, the largest deletion of 280,000 bp from 139330000bp to 139610000bp eliminates
nine different genes: Zm00001d026133, Zm00001d026134, Zm00001d026135, Zm00001d026137, Zm00001d026139, Zm00001d026140, Zm00001d026141, Zm00001d026142 and Zm00001d026143. All the large deletions are proximal to r1 and
within 1.5Mbp distance from r1 (139779468bp-139791776bp). Zm00001d026094 encodes a heat shock factor protein, Zm00001d026095 encodes sugar isomerase (SIS) family
protein and Zm00001d026108 encodes GRAS domain containing protein. Similarly, Zm00001d026133 and Zm00001d026134 encodes GDSL esterase/lipase, Zm00001d026135 encodes mechanosensitive ion channel protein, Zm00001d026137
encodes OSJNBa0084K11.10-like protein, Zm00001d026139 encodes calcium-dependent protein kinase and Zm00001d026140 encodes subtilisin-like protease SBT1.8. Zm00001d026141, Zm00001d026142 and Zm00001d026143 codes for the uncharacterized protein.

Apart from these deletions, there are other deletions that can affect the gene function either by mutating the promoter or enhancer regions or by affecting transcript splicing. A list of deletion sites for PB1 and PB2, along with genes affected and their predicted consequences are shown in Table 2.2 and Table 2.3.

2.3.4 Expression profile of potential mutation candidates

Publicly available expression data from Chettoor et al. (2014), Walley et al. (2016) and Warman et al. (2020) were used for to check the expression of mutation candidates across different maize tissues for PB1 and PB2 lines. Out of 32 total, nine candidates do not have any information on either mRNA or protein expression in PB1. Similarly, out of 45 totals, no information on expression of 16 protein coding candidates is available for PB2. The expression of the candidate genes that probably caused mutant phenotypes in PB1 and PB2 are shown in Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5 and Figure 2.6 using two publicly available datasets. In addition, expression of the candidate genes that are completely lost due to deletions in PB2 is shown in Figure 2.7. Genes that have higher mRNA expression in mature pollen than other maize tissues observed are Zm00001d026132, Zm00001d026133, Zm00001d026140 and Zm00001d026166.
2.4 Discussion

Zm00001d026140 (protein: subtilisin-like protease) is among the top 20% genes that are highly expressed in mature pollen. Its expression is about 799 times higher in mature pollen than in seedlings, higher in vegetative tissues than in sperm cells (Warman et al., 2020). Subtilisin is a serine protease, which has a unique arrangement of Asp, His and Ser triad in its active site (Schaller, 2004). This type of protease had been previously isolated from microspore of lily (LIM9). LIM9 was present in microsporocyte from late Zygotene stage of meiosis, reached the peak in tetrad stage and also present in young pollen grains (Taylor et al., 1997). An ortholog of LIM9 had been identified in tomato (TMP) with similar expression pattern (Riggs et al., 2001). The peak expression in tetrad stage and localization in tapetum hints that this protease may be involved in degradation of tapetal cells, an important process required for additional supply to thicken the exine layer of pollen. The thick exine layer is crucial for pollen dehydration and maturation. This protein may have role in interaction between pollen tube and stigma during pollen tube penetration and growth.

Mechanosensitive ion channel protein (encoded by Zm00001d026135) is a transmembrane protein that mediates the transport of ions in response to the mechanical stress such as osmotic pressure (Basu & Haswell, 2017). The presence of mechanosensitive channel protein in pollen is essential, most likely to maintain the balance of osmotic pressure inside pollen when the pollen hydrates, and the pollen tube starts to germinate after landing on stigma surface. A lack of a functional mechanosensitive channel at this stage can lead to inviable pollen, due to osmotic imbalance inside the cell and can cause the cell bursting, if the pressure inside the cell increases constantly. MSL8 (mechanosensitive channel of small
conducance-like 8) is a pollen specific mechanosensitive channel protein that is required to maintain pollen viability and prevent pollen from bursting due to osmotic pressure during pollen hydration, and also controls pollen tube germination in Arabidopsis. The pollen viability in msl8-4 mutants after two hours of hydration in distilled water was only 46% compared to 83-95% in wildtype. In addition, 26% of the germinated pollen tube burst compared to 3% in wild type (Hamilton et al., 2015).

Zm00001d026166 encodes a C2H2 type Zinc finger family protein. C2H2 type zinc finger motif consists of an α-helix and an antiparallel β sheet, and two cysteine and two histidine residues hold a Zinc ion in center. Most C2H2 type Zinc finger family proteins are transcriptional regulators, binding DNA (https://www.uniprot.org). A mutation in this protein is likely to cause inefficient transcription of DNA affecting gene expression. In Arabidopsis, several transcription factors related to C2H2 type zinc finger family protein have been studied for their function during pollen development. A T-insertion in DNA sequences of these transcription factors resulted in an abnormal phenotype in pollen. Two major pollen defects were observed; misarranged male germ unit in pollen ranging from 20 to 45%, and two-celled pollen, that contains regular vegetative nucleus and only one sperm cell like nucleus ranging from 5-15% in different transcription factor mutations within the C2H2 type zinc finger family protein (Renak, et al., 2012). This protein was exclusively detected in pollen. It could have been expressed earlier during mitotic cell division phase of immature pollen and retained in the mature pollen as well. Its role in pollen tube growth can be speculated.

Zm00001d026169 is the beta-expansin 2 gene. Proteomic profiling of maize pollen coat showed presence of β-expansin (Wu et al., 2015). The expression data we used so that
this protein is widely expressed in maize tissues. Pollen specific β-expansin proteins have been previously reported and a mutation in a gene coding for this protein affected the efficiency of pollen tube to geminate and penetrate, specifically when there is competition from wild type pollen. Mutation in all similar proteins had severe impacts on fertility of mutant plants when propagated as male (Valdivia, et al., 2009). Since genes encoding pollen specific β-expansin have been mapped to four other chromosomes in maize, mutation in only one gene is less likely to have phenotypic consequences in pollen tube germination.

Genes do not need to be exclusively expressed in male gametophyte to be a candidate gene potentially causing reduced male gametophyte transmission. Any genes that are expressed in male gametophyte and other tissues can be a potential candidate. Since, pollen is haploid, any mutation that would otherwise be complemented in sporophyte is expressed. In addition, we are not sure that the exposure to the fast neutrons is the sole cause of mutation since mutation can be spontaneous or due to multiple environmental factors.
References


Figure 2.1. Functional consequences of unique deletions in PB1 and PB2 mutant lines.

The functional consequences were predicted using ENSEMBL variant predictor. The numbers over the graph is total number of respective consequences seen on the candidate genes at transcript level.
Figure 2.2. Heat map showing mRNA expression of mutation candidates for PB1.

The expression data were obtained from Supplementary table 2, Chettoor et al., 2014. Candidate genes that do not have information on expression were excluded from the plot.
Figure 2.3. Heat map showing mRNA expression of mutation candidates in PB1.

Few genes are expressed both in pollen and sporophytic tissues. mRNA expression is obtained from Walley et al., 2016.
Figure 2.4. Heat map showing protein expression of mutation candidates in PB1.

None of the candidate genes show expression in pollen. Expression data is obtained from Walley et al., 2016.
Figure 2.5. Heat map showing mRNA expression of mutation candidates for PB2.

The expression data were obtained from Supplementary table 2, Chettoor et al., 2014. Candidate genes that do not have information on expression were excluded from the plot.
Figure 2.6. Heat map showing mRNA expression of mutation candidates in PB2.

RNA expression data is obtained from Walley et al., 2016. Genes without any information on expression are excluded.
Figure 2.7. Heatmap showing mRNA expression for genes that are lost due to deletions in PB2.

Loss of the genes that have expression in pollen can have a detrimental effect on pollen viability. Expression data is obtained from Walley et al., 2016.
List of Tables-PuBe Mutation project

Table 2.1. Sequencing statistics for P1, PB1 and PB2 lines

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Table 2.2 Deletion sites, length, functional consequences, impact, genes affected, and gene distance from deletion site for PB1

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Chapter 3

3 Developing molecular markers for Antimorph *Ae1-5180*, An alternative to *Ae1* to increase amylose content in maize endosperm

Abstract

Starch structure mostly consists of highly branched (amylopectin) and largely unbranched (amylose). Branch formation is catalyzed by starch branching enzymes. The primary starch branching enzyme in maize endosperm is starch branching enzyme IIB (SBEIIb), which is encoded by *ae1* (*amylose extender 1*). In most Midwestern dentils, the endosperm starch is about 75% amylopectin and 25% amylose. The amylose content increases up to 50% when *ae1* is homozygous recessive. An antimorph, (*Ae1-5180*) was identified (Stinard *et al.*, 1993) that acts in a dominant fashion to eliminate SBEIIb. The essential portion of this mutant allele is a *Mutator1* (*Mu1*) element flanked by an inverted duplication of the 3’ portion of the *Ae1* gene. Based on the restriction map by Stinard *et al.*, we developed an approximate predicted sequence for *Ae1-5180* using the known sequences of the wildtype *ae1* gene and *Mu1* transposon. Using this, we designed PCR primers targeted to amplify regions within a 3kb *XhoI* restriction fragment that appears crucial for the dominant action of *Ae1-5180*. The primers amplify two DNA bands from samples expected to have *Ae1-5180*, but none from B73. The sequence of the lower molecular weight band matches with 181 bp of *Mu1* and 364 bp of *Ae1* (545 bp). The higher molecular weight band also possesses 181 bp of *Mu1* but includes 438 bp of *Ae1* (619 bp). The additional 74 bp includes a restriction site for *NotI*, which was predicted from the original restriction map.
Sequencing results indicate that these primers reliably detect the presence or absence of the 
\textit{Ae1-5180} allele. Also, the insertion site of \textit{Mul} in \textit{Ae1-5180} is now exactly defined.

Keywords: starch branching enzyme IIb (SBEIIb), \textit{ae1, Mul, Ae1-5180}
3.1 Introduction

Maize is a monocotyledonous plant, belongs to the Grass family and consists of a total of 10 chromosomes in its genome. Geographically, maize is believed to be originated from tropic regions of Central or South America (Kiesselbach, 1999). A teosinte species *Zea mays ssp. parviglumis* (hereafter called Parviglumis) shares the closest ancestor to maize as shown by genetic and archeological evidences (Sherry A. Flint-Garcia, 2017). Maize was domesticated around 9000 years ago in the Central Balsas River Valley in southwestern Mexico (Matsuoka et al., 2002). During domestication, an increase in the number of seeds and seed size were the major traits subjected to selection. Due to this reason, modern maize ears have large, soft, and larger number of kernels as compared to Parviglumis, which had very small and few kernels enclosed in hardened fruit case. In addition, maize kernels were also selected for kernel composition. Evidence shows that high starch and low protein were preferred. An increase of 34% is seen in starch content of modern maize in comparison to Teosinte (Sherry A Flint-Garcia, Bodnar, Scott, & Genetics, 2009). The genes involved in starch biosynthesis were also subject to selection. *bt2, sul* and *ae1* genes have low diversity compared to Parviglumis, indicating that these genes were selected during evolution (Whitt, Wilson, Tenaillon, Gaut, & Buckler, 2002). *sul* and *ae1* genes affect the structure of amylopectin. *ae1* gene codes for starch branching enzyme IIb (SBEIIb), which is a dominant enzyme in amylopectin synthesis in maize. Modern maize lines can be broadly divided into three categories: Dent corn, Sweet corn, and Popcorn. Most of the Dent corns have the amylopectin and amylose content of approximately 70-75% and 25-30% respectively in endosperm starch. Amylose is a resistant starch which is not digested in small intestine by the enzymes and carried out to
colon. In colon, it is fermented by microflora to release short chain fatty acids at the final product. The short chain fatty acids promote the function of colon viscera. Thus, it helps to prevent risk of type II diabetes and colon cancer (Singh, Kaur, & Shevkani, 2014). The amount of amylose can be increased to 50% when \( ae1 \) gene is in recessive form. H99ae is a Mid-western Dent with amylose content up to 50%. However, another variety, GEMS-0067, with recessive \( ae1 \) background publicly exists that has amylose content up to 70% in its endosperm starch. Another gene \( sbe1 \) which codes for starch branching enzyme I (SBEI) was found responsible for high amylose content in GEMS-0067 in recessive \( ae1 \) background and it has additive effect (A. Gyawali & Auger, 2018). DNA sequences comparison of GEMS-0067 with seventeen Mid-Western Dents and two teosinte species showed that \( sbe1 \) sequence from GEMS-0067 is identical to Parviglumis at amino acid level. It also showed six nucleotide polymorphisms with other Mid-Western Dents (Abiskar Gyawali, 2016). So, we hypothesize that it is the polymorphism in the \( sbe1 \), that is acting in a different manner during starch biosynthesis and thus increasing the proportion of amylose in endosperm starch. The effect of \( sbe1 \) is difficult to access when functional \( Ae1 \) is present. We use an antimorph \( Ae1-5180 \), which acts in dominant fashion to eliminate the SBEIIB enzyme, encoded by functional \( Ae1 \). We report the PCR method of cloning of \( Ae1-5180 \) allele. However, we were not able to predict the effect of \( sbe1 \) due to inconsistent phenotype in kernels with \( Ae1-5180 \).
3.2 Methodology

3.2.1 Sample generation

Kernels heterozygous for wildtype *Ae1* and *Ae1-5180* (hereafter denoted as */Ae1-5180*) was obtained from Maize Genetics Cooperation Stock Center, University of Illinois, Urbana/Champaign. Kernels from */Ae1-5180* were grown and were crossed with pollen from *Z. mays parviglumis* (hereafter called Parviglumis) on South Dakota State University Research Plot in 2015. In 2018, seeds from hybrid of Parviglumis and */Ae1-5180* were planted and crossed by pollen source expected to have *Ae1-5180* in winter nursery, Huawei. For the pollen source, glassy looking and shrunken kernels were selected from an ear which was heterozygous for *Ae1-5180* and selfed. The resulting ears from the cross ((*Parviglumis X */Ae1-5180*) X */Ae1-5180*) were used for accessing the presence of *Ae1-5180*. Glassy and shrunken kernels were selected from four different ears and grown on a Petri dish in dark conditions in an incubator at 30° C.

The DNA extraction was done at two leaf stage using the Genomic DNA Mini Kit (Plant) from IBI Scientific (IBI Scientific 7445, Chavenelle Road Dubuque, Iowa 52002). Extracted DNA samples from respective kernels were tested for the presence of *Ae1-5180* marker.

3.2.2 Confirming the presence of *Ae1-5180* allele

The predicted sequence of *Ae1-5180* was developed based on the restriction map of *Ae1-5180* clones published on 1990’s (Stinard, Robertson, & Schnable, 1993) using the *ae1* gene and *Mu1* transposon sequence. The *ae1* gene sequence (GeneID: 542238) and the
*Mu*1 sequence (Genebank Accession X00913.1) were obtained from National center for Biotechnology Information (NCBI) database. Using the restriction map as reference, the restriction sites were assessed in the *ae1* and *Mu*1 sequence. Once the restriction sites shown on restriction map were found on physical sequence, the DNA sequences were used to construct predicted sequence of *Ae1-5180*. The entire length of 12 kb fragment and 12.5 kb fragment were not considered for developing PCR based markers. The predicted sequences of 3 kb and 2.3 kb *XhoI* fragment were used for designing primers.

Two primers were designed: Ae1mu4F and 5180R4 to amplify sequences from 3 kb *XhoI* fragment and 3’ side of 2.3 kb *XhoI* fragment. Another forward primer Ae1F was designed to pair with Ae1mu4F to amplify the sequences on the 5’ side of 2.3 kb *XhoI* fragments. Because, the 5’ side 3 kb fragment has inverse duplicated sequences of same *ae1* sequence in 3’ side, the same primer can be forward primer on one side and reverse on another. A total of 95 samples were tested for *Ae1-5180* marker.

For PCR analysis, the total reaction mixture of 25 µl reaction was made, which includes 1 µl of the DNA sample, 1.25 µl each of primers diluted to 10 µm concentration, 0.75 µl of 100% DMSO, 12.5 µl of High Fidelity Phusion PCR master mix (New England Biolabs, 240 County Road, Ipswich, MA) and 8.25 µl of ddH2O. PCR reaction conditions were 98°C, 30 seconds for initial denaturation, 98°C 10 seconds for denaturation, 63°C, 15 seconds for annealing, 72°C, 30 sec for elongation and 7 minutes for final elongation. PCR reactions were run for 35 cycles and final hold was at 4°C. Confirmation of the PCR bands were done by sanger sequencing from both sides of DNA.
3.3 Results

3.3.1 Precise DNA sequence of *Ae1-5180* clones

In the sample that contain *Ae1-5180*, the primer pair Ae1mu4F and 5180 R4 produced two different size bands. Sanger sequencing results showed the band were 545 bp and 619 bp. In addition, when Ae1F was also added, additional band of 258 bp was also produced. NCBI blast results confirmed that these sequences match to *Ae1* and *Mu1* sequences. In absence of *Ae1-5180* clones, primer pair Ae1F +5180 R4 produce 603 bp band amplified from functional *Ae1* sequences.

Figure 3.1: PCR bands from *Ae1-5180* DNA and functional *Ae1* DNA.

a. Lane 1 has two bands, 545 bp and 619 bp amplified using primer pair Ae1mu4F +5180R4. Lane 2 contains has an additional band of 258bp, when additional primer Ae1F was added with primer pairAe1mu4F+5180 R4 in PCR reaction. b. Lane 1 shows the 603 bp band amplified using Ae1F and 5180 R4 from functional *Ae1* gene.
Out of 95 samples tested for *Ae1-5180* marker, 2 were homozygous for *Ae1-5180* marker; 62 were heterozygous for *Ae1* and *Ae1-5180*, 17 were homozygous for functional *Ae1* and 14 samples showed band for *Ae1-5180* marker but were not tested for functional *Ae1* primer. All the samples for *Ae1-5180* marker were tested only using *Ae1mu4F* and 5180 R4 primer pair, that forms two bands at 545 bp and 619 bp.

### 3.4 Discussion

The 3’ side flanking the *Mu1* of the 3kb fragment, which is the part of 12 kb *XhoI* fragment, and 2.3 kb fragment, are identical (Figure 3). So, PCR amplification from 3’ side in both fragments should from identical bands. Therefore the 619 bp band should be from both fragments. The 545 bp band should be from the 5’ side of 3 kb fragment only because it has an inverse duplication of the 3’ side, thus can be amplified by using same primer pair used in 3’ side. The band on the 3’ side is expected to be bigger and has an additional *Nool* restriction site. Sanger sequences of the 545 bp and 619 bp bands aligned with the predicted sequences of 3 kb fragment. The presence of the *Nool* restriction site was also confirmed in extra 74 bp sequence found in 619 bp band, in line with the restriction map published in 1993. Careful visual observation of traces in 619 bp shows that trace contamination is seen, starting from extra 74 bp region of 619 bp and the alternative sequence match to 545 band at this region. This means the two bands processed for sequencing had some contamination from each other which is reflected during sequencing. The additional 258 bp band amplified is from 5’side of the 2.3 kb *XhoI* fragment by primer pair *Ae1F* and *Ae1mu4F*, which was also confirmed by sanger sequencing.
The cloning of *Ae1-5180* was successful but its efficiency in its function was questionable, when amylose to amylopectin ratio of the samples positive for this marker, were measured. We think *Ae1-5180* may not be eliminating the effect of SBEIIB as expected. To confirm this, kernels were taken from an ear heterozygote for *Ae1-5180* and selfed. The resulting kernels are expected to segregate 1:2:1 for homozygotes *Ae1-5180*, heterozygotes and homozygotes normal, respectively. Looking just at the phenotypes, the ratio looks plausible, since there were about three quarters of shrunken and glassy kernels. We tested three very normal looking kernels and three shrunken and glassy looking kernels for amylose to amylopectin ratio. The amylose to amylopectin ratio of these kernels were determined by using Iodine colorimetry (Knutson & Grove, 1994). The samples in 900 µl of 6.7 X 10^{-3} M Iodine-DMSO solution was incubated in a water bath at 70°C until the solution become clear. After 24 hours of total incubation time, the mean amylose to amylopectin ratio of normal looking kernels was 1.089633 with a standard deviation of ±0.000874, whereas the shrunken and glassy looking kernels had mean ratio of 0.9435 with a standard deviation of ±0.033147.

Apart from this ratio, the dark purple color seen, when the aliquot sample after the incubation was further diluted with Iodine-DMSO and ddH₂O. In presence of Iodine, amylose turns light blue and amylopectin turns purple when diluted in ddH₂O (needs citation). In fact, the mean amylose to amylopectin content of all the samples tested positive for *Ae1-5180* is less than mean of normal kernels. This implies that *Ae1* gene is still functional in these kernels and therefore the amylopectin content is higher than amylose. Also, these results also indicate that the glassy and shrunken kernel structure in samples tested positive for *Ae1-5180* is not necessarily due to the high amount of amylose, rather
something else is going on. We have no clear idea why the sample is glassy and shrunken other than because of high amylose. We also saw a trend that healthier and normal shaped kernel has higher ratio for amylose and amylopectin ratio irrespective of being negative for *Ae1-5180* whereas the shrunken and glassy samples have low amylose to amylopectin ratio irrespective of having *Ae1-5180*. 
References


Figure 3.2: Map of 2.3kb XhoI fragment showing simple insertion, with partial sequences including \textit{Mu}l insertion site to \textit{Ae}l sequences on both sides (green circles). Below the restriction map are the sequences showing \textit{Mu}l insertion site to \textit{Ae}l sequences on both sides (green circles), and the sequences underlined, are restriction sites. Sequences in black color are \textit{Ae}l sequences and red are \textit{Mu}l sequences with their respective length, within two underlined restriction indicated in brackets. The sequences on 5’ side is amplified with primer pair Ae1F+Ae1mu4F and on the 3’ side is amplified with primer pair Ae1mu4F +5180R4. Ae1mu4F is the reverse primer in the first case and forward in second case.
Figure 3.3: Map of 3kb *XhoI* fragment showing inverse duplication, with partial sequences including *Mul* insertion site to *Ael* sequences on both sides (green circles).

Below the restriction map are the sequences showing *Mul* insertion site to *Ael* sequences on both sides (green circles), and the sequences underlined, are restriction sites. Sequences in black color are *Ael* sequences and red are *Mul* sequences with their respective length, within two underlined restriction indicated in brackets. The sequences on both sides is amplified with primer pair *Ae1mu4F* +5180R4. PCR reactions produce two bands, the small band 545bp in 5’ side is inversely duplicated in 3’ side. The 3’ side has additional 74 bp, including a *NotI* restriction site within.
List of Tables - *Ae1-5180* project

Table 3.1 Primers used in PCR amplification of *Ae1-5180* fragments

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Chapter 4

4 Conclusions and Future Directions

4.1 PuBe induced Mutation Project

The potential candidate genes listed here are based on their expression in mature pollen. However, to affect the viability of pollen, it is not necessary that the mutation needs to be in mature pollen. It is highly possible that mutation affecting any stage of microsporogenesis can produce nonfunctional pollen. Alternatively, the pollen can be normal but the failure to effectively elongate the pollen tube inside the style can also affect the fertilization. Genes affected by a mutation in any of these stages mentioned above may have been probably missed due to the lack of expression data for these stages. The potential genes still need to be verified using reverse genetics techniques before concluding that mutation in these genes is casual for reduced male gametophyte transmission in maize.

Validation of potential candidate genes using expression data from various stages of microsporogenesis, comparison of gene expression between wild type plant and mutant in various pollen development stages and identifying function of the highly potential candidate genes using molecular techniques will be needed to done in future to further elucidate the casual genes.

4.2 Ae1-5180 Project

A different way to knock down a functional Ae1 gene needs to be explored. Use of transposable elements or CRISPR-Cas9 method can be potential option.