SORGHUM INTROGRESSION BREEDING UTILIZING *S. macrospermum*

A Dissertation

by

LESLIE CHARLES KUHLMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Plant Breeding

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Approved by:

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	David M. Stelly
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ABSTRACT

Sorghum Introgression Breeding Utilizing *S. macrospermum*.

(August 2007)

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Co-Chairs of Advisory Committee: Dr. William L. Rooney
Dr. David M. Stelly

Sorghum has been improved by plant breeders for yield, biotic and abiotic stress resistance, as well as quality traits by using germplasm from within the species. Interspecific hybridization can greatly increase the amount of genetic variation available to plant breeders for improvement. Interspecific hybrids between sorghum and the 19 species in the tertiary gene pool have, until recently, not been successful. The Australian species, *S. macrospermum*, was recently successfully hybridized with sorghum by using germplasm homozygous for the *iap* allele, which eliminated reproductive isolation barriers. The objectives of this research were to evaluate the potential for use of *S. macrospermum* in an introgression breeding program, determine the map position of the *Iap* locus, and backcross the *iap* allele into elite Texas A&M germplasm. Interspecific hybrids between *S. bicolor* and *S. macrospermum* revealed moderate levels (2.6 II per PMC) of allosyndetic recombination, indicating that introgression through genetic recombination is possible. Genomic relationships were sufficient to assign *S. macrospermum* the genomic formula AAB_1B_1YYZZ, Y and Z remain unknown. In backcrosses to *S. bicolor* using the female interspecific hybrid gamete and embryo

rescue, BC_1F_1 plants were recovered. They had high chromosome numbers ($2n$ = 35-70) and were male-sterile but three plants set backcross seed. Ninety-five percent of BC_2F_1 plants were $2n$ = 20 chromosomes and 75% of them contained *S. macrospermum* introgression. BC_2F_1 plants carried between 0-18.5% *S. macrospermum* introgression; in total 26% of the *S. macrospermum* genome was detected in the BC_2 generation. Three types of introgression germplasm were created: alien addition lines; alien substitution lines; and introgression lines. Recombinant chromosomes, containing *S. macrospermum* introgression sites, were identified in multiple introgression lines. The *Iap* locus was genetically mapped to sorghum chromosome 2 (SBI-02), flanking AFLP markers were 2.1 and 2.7cM away, one AFLP marker shared the same map position (0.0cM). A genetic stock, Tx3361, was created which has *iap iap* genotype and improved agronomic qualities such as short plant height, white seed color, non-pigmented testa, no awns, reduced lodging, early maturity, and backcross segregation of male-sterility (*ms3*). This research shows that *S. macrospermum* is now available to plant breeders for sorghum improvement.

DEDICATION

To my wife Kendra and my daughter Keira, I love you both.

ACKNOWLEDGMENTS

I would like to thank Dr. Bill Rooney for the opportunity to learn under his guidance. You have been a mentor and friend; it has been a true education, thank you. I would also like to thank Drs. David Stelly, Byron Burson, and Patricia Klein for being a part of this project and each contributing to my academic growth. To the late Dr. Jim Price, without your forethought in planning and directing the project, this research would never have happened. You are greatly missed.

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CHAPTER I

INTRODUCTION

Sorghum (*Sorghum bicolor* [L.] Moench) is an important food, feed, and forage crop with worldwide grain production in 2005 of 56,957,314 metric tons which ranks fifth among cereal grains (FAOSTAT data, 2006). Production in the U.S. accounted for approximately 10,000,000 metric tons of that crop which was valued at $715,000,000 (USDA 2006). Production limitations for sorghum in the U.S. include abiotic stresses such as drought and biotic stresses including insect and disease pressure. Plant breeders continually make progress in improving the crop for these and many other traits, including yield potential, but are ultimately limited by the amount of genetic variation available to them for the desired trait. Genetic variation is the lifeblood of plant breeding and without it genetic improvement is not possible. Identifying valuable new sources of germplasm is a key component to supply plant breeders with the genetic variation they need for improvement.

Wild species can be valuable sources of novel genetic variation for the improvement of yield, disease and insect resistance, and abiotic stresses (Goodman et al., 1987; Jiang et al., 1994; Jones et al., 1995; Jauhar and Chibbar, 1999). Interspecific hybridization can dramatically widen the available genetic pools so that novel genetic variation can be utilized by plant breeders. Sorghum breeders have traditionally used germplasm within the cultivated species for improvement (Duncan et al., 1991) but some

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work with *S. halepense* (L.) Persh. and *S. propinquum* (Kunth) Hitch. has occurred (Dweikat, 2005; Wooten, 2001). Interspecific hybrids with these two species are relatively easy to make and backcrosses can be produced that contain wild species introgression. Hybrids between sorghum and the 19 other species in the genus *Sorghum* had previously not been possible. Hodnett et al. (2005) determined that pollen-pistil incompatibilities were one cause of reproductive isolation within the genus. Wild species pollen on sorghum stigmas could germinate but their pollen tubes were inhibited stigmatically, thus fertilization did not occur. Price et al. (2006) showed that using germplasm homozygous for the *iap* allele reduced such incompatibilities and made recovery of previously unrecoverable hybrids relatively simple. One species in particular showed promise, interspecific hybrids with *S. macrospermum* Garber, a species native to Australia, grew vigorously and successfully reached maturity. No information is available regarding the feasibility of using *S. macrospermum* in an introgression breeding program. If introgression of *S. macrospermum* genetic variation is to be easily recovered in backcrosses with *S. bicolor*, genetic recombination between the parental genomes is required. The interspecific hybrid must also express enough fertility to recover backcrosses, which then must be evaluated to determine if and how much introgression is occurring. Thus, these and other questions need to be answered before *S. macrospermum* can be used in an introgression breeding program. The objectives of the research reported herein are:

(1) Produce interspecific hybrids between fertile *S. bicolor* (*iap iap*) and the wild species *S. macrospermum*. Analyze meiotic chromosome pairing and recombination in interspecific hybrids and determine if allosyndetic recombination occurs.

(2) Produce advance generation backcrosses of the interspecific hybrid using *S. bicolor* as the recurrent parent. Determine if introgression from *S. macrospermum* is present in advance backcrosses and localize detected introgression blocks to chromosomes.

(3) Map the genome location of the *Iap* gene in *S. bicolor* using existing genetic linkage maps and identify suitable molecular markers which can be used in marker-assisted selection to move the *iap* allele into other more complex genetic backgrounds.

(4) Backcross the *iap* allele into elite Texas Agricultural Experiment Station (TAES) germplasm for use as the parent in future interspecific crosses.

CHAPTER II

REVIEW OF LITERATURE

Structure of the Genus *Sorghum*

The genus *Sorghum* consists of 22 species taxonomically classified into five sections: *Eusorghum*, *Heterosorghum*, *Chaetosorghum*, *Parasorghum*, and *Stiposorghum* (Garber, 1950; de Wet 1978; Lazarides et al., 1991). Section *Eusorghum* is composed of *S. bicolor*, *S. propinquum*, and *S. halepense*, these three species are native to Africa and Southern Asia (de Wet, 1978). Sections *Chaetosorghum* and *Heterosorghum* are monotypic and consist of species *S. macrospermum* and *S. laxiflorum* Bailey, respectively. *Sorghum macrospermum* is found in a single region in the Northern Territory of Australia, north west of Katherine. *Sorghum laxiflorum* is more broadly dispersed and is found in Australia, Papua New Guinea, and the Philippine Islands (Garber, 1950; Lazarides et al., 1991). Ten species compose the section *Stiposorghum*: *S. amplum* Lazarides; *S. angustum* S.T. Blake; *S. brachypodum* Lazarides; *S. bulbosum* Lazarides; *S. escarinatum* Lazarides; *S. extans* Lazarides; *S. interjectum* Lazarides; *S. intrans* F. Muell. Ex Benth.; *S. plumosum* (R. Br.) P. Beauv.; and *S. stipoideum* (Ewart & Jean White) C.A. Gardner and C.E. Hubb. All of these are indigenous to Australia (Garber, 1950; Lazarides et al., 1991). The seven species of *Parasorghum* include: *S. grande* Lazarides; *S. leiocladum* (Hack) C.E. Hubb.; *S. matarankense* E.D. Garber & Snyder; *S. nitidum* (Vahl) Pers.; *S. purpureo-sericeum*

(Hochst. Ex. A. Rich.) Asch. & Schweinf.; *S. timorense* (Kunth) Buse; and *S. versicolor* Andersson. These species occur in Asia, Australia, Africa, and Central America (Garber, 1950; Lazarides et al., 1991).

The species *S. bicolor* contains three subspecies, subsp. *bicolor*, which contains 5 morphologically distinct cultivated sorghum races, subsp. *arundinaceum* (desv.) de Wet et Harlan, a widely distributed wild African complex, and subsp. *drummondii* (Steud.) de Wet, a complex of stabilized derivatives between cultivated sorghum and weedy relatives (de Wet, 1978). Cultivated sorghum was likely domesticated from subsp. *arundinaceum*, at least 2000-4000 BCE (Murdock, 1959; de Wet, 1978; Doggett 1988).

It is unknown what evolutionary sequence took place within the genus to firmly establish *S. bicolor* in Africa while 17 other species are endemic to Australia. There is some doubt as to whether the five sections within the genus correspond to evolutionarily relationships or are abstract constructions (Spangler et al., 1999; Dillon et al., 2001). Spangler (2003) used RFLP and multi-gene sequence data to suggest that *Sorghum* be divided into three separate genera containing only 13 species. Dillon et al. (2004) used combined ITS1/*ndhF* sequence data to construct a phylogenetic tree, and the results did not support the separation of the genus but did agree that three distinct clades existed within the genus. Price et al. (2005a) combined genome size and chromosome numbers with the ITS1/*ndhF* data and concluded that two distinct lineages exist within the genus. One lineage contains species that are $x = 5$ ($2n = 10$, 20, 30, and 40) and have large genomes containing large chromosomes; whereas, the second lineage has species with $x = 10$ ($2n = 20$ and 40) and have smaller genomes and chromosomes. According to this,

S. macrospermum and *S. laxiflorum* are the most closely related species to the *Eusorghum* section, all of which form the *x* = 10 lineage, and species from sections *Parasorghum* and *Stiposorghum* form the *x* = 5 lineage (Dillon et al., 2004; Price et al., 2005a). To date, this represents the most detailed description of the structure of the genus.

Genomic Relations within the Genus

The genus *Sorghum* contains species belonging to two separate lineages based on chromosome morphology and genome size: *x* = 5 and *x* = 10. The x = 5 lineage is typified by a large genome size and large chromosomes while the x = 10 lineage has smaller chromosomes and genome size. The evolutionary relationships between these two lineages and between species within the lineages have not been resolved. The *Eusorghum* section has received the most attention in elucidating the genomic relationships between species. To attempt introgression breeding, the genomic relationships between parental species must be understood, and without homology there will be little to no genetic recombination from which to recover introgression.

Sorghum bicolor (2*n* = 20) belongs to the *x* = 10 chromosome lineage within the genus, as do all species within *Eusorghum*, *Chaetosorghum*, and *Heterosorghum*; however, the name of the lineage does not accurately describe the genomic makeup of the species. *Sorghum bicolor* is generally regarded as a diploid and regularly displays normal meiosis with the chromosomes forming 10 bivalents at metaphase (Durra and Stebbins, 1952; de Wet, 1978; Sangduen and Hanna, 1984; Doggett, 1988). At low

frequencies, abnormalities do occur and they are usually observed as quadrivalents (Chin, 1946). During meiosis in haploid plants ($2n = 10$), chromosomes form one or more bivalents in approximately 10% of the pollen mother cells (PMCs) while the remaining chromosomes are univalents (Brown, 1943; Kidd, 1952; Endrezzi and Morgan, 1955). Multivalent formation in diploid plants and bivalent formation in haploid plants indicates that some homology exists between the chromosomes of the haploid genome. So while *S. bicolor* functions as a diploid, it is most likely tetraploid ($2n = 4x = 20$) in origin (Garber, 1950; Endrizzi and Morgan 1955; Celarier, 1958; Doggett, 1988). Recently, cytological evidence supported the conclusion that *S. bicolor* is a disomic tetraploid. Fluorescence in situ hybridization (FISH) of a large-insert genomic clone, BAC 22B2 and later a 1047-bp subclone, pCEN38, showed differential hybridization near the centromere. Ten homologous chromosomes had a strong FISH signal while the ten remaining homologous chromosomes had a weak but detectable signal, which led the authors to conclude that *S. bicolor* is composed of two subgenomes, $A_bA_bB_bB_b$ (Gomez et al., 1998; Zwick et al., 2000).

Sorghum halepense ($2n = 40$) is a rhizomatous species and is considered to be one of the most noxious weeds found in U.S. and world agriculture (Holm et al., 1977; McWhorter, 1989). It has long been proposed to be a polyploid although the exact nature of its genomic constitution is still not resolved. Many authors have proposed genomic formulas for *S. halepense*: an autotetraploid (Casady and Anderson; 1952; Durra and Stebbins, 1952), an autooctoploid (Bennett and Merwine, 1966), and a segmental auto-allo-octaploid (Hadley, 1953; Tang and Liang, 1988). The difficulty in

assigning genomic constitution to *S. halepense* results from the lack of sharp differentiations between its polyploid genomes, i.e. significant homology exists between its subgenomes. Its meiotic behavior is not normal and multivalent frequencies vary widely between PMCs, its chromosomes associate primarily as bivalents and quadrivalents, which indicates *S. halepense* is at minimum an allo-polyploid (Hadley, 1953; Endrizzi, 1957; Bhatti et al., 1960; Tang and Liang, 1988). In crosses with *S. bicolor*, the hybrid ($2n = 30$) shows approximately equal numbers of univalents, bivalents, and trivalents (~ 4 I + 4 II + 6 III), which led Hadley (1953) to propose a genomic formula of AABB for *S. bicolor* and AAAABBCC for *S. halepense*. This formula predicts the interspecific hybrid (AAABBC) would form chromosome associations as: 5 III within the A subgenome, 5 II within the B subgenome, and 5 I with the remaining C subgenome. The hypothetical pairing is roughly close to the empirical results obtained by multiple researchers (Hadley, 1953; Endrizzi, 1957; Tang and Liang, 1988). This hypothesis breaks down when considering 40 chromosome hybrids AAAABBBC ($2n + n$) and 60 chromosome amphiploids AAAAAABBBBCC as fewer than expected trivalents (5 III) and quadrivalents (5 IV) form, respectively (Tang and Liang, 1998). Tang and Liang (1998) modified Hadley's model slightly by replacing genomes B and C with B_1 and B_2, respectively. Accordingly, the B_1 and B_2 genomes share high amounts of homology but are still distinct subgenomes; they also share some homology, though much less, with subgenome A. This modification allows the chromosomal associations to vary somewhat more in that it is allowable for B_1 and B_2 chromosomes to interact and pair more frequently as well as rarely to pair with A

chromosomes. An explanation for the 40 chromosome hybrid example that produces a deficiency of trivalents could be that the affinity to form B_1-B_2 bivalents is stronger than the affinity to form both B_1 trivalents and exclude B_2 chromosomes as univalents. This seems to be the most plausible explanation for the chromosome pairing observed in all ploidies of interspecific hybrids. Accordingly, *S. halepense* is considered ($2n = 8x = 40$) $AAAAB_1B_1B_2B_2$ and *S. bicolor* is considered ($2n = 4x = 20$) AAB_1B_1 (Tang and Liang, 1988).

Sorghum propinquum ($2n = 20$) is a rhizomatous species within *Eusorghum* that is native to Asia. It hybridizes readily with *S. bicolor* and produces fertile hybrids and F_2 progeny and shows normal chromosome pairing (Celarier, 1958; Doggett, 1988). There is a cytogenetic difference between the two species. The smallest chromosome in *S. propinquum* contains the nucleolar organizing region; whereas, it is present in the largest chromosome of *S. bicolor* (Magoon and Shambulingappa, 1961; Doggett 1988). It is considered a separate species from *S. bicolor* because of its markedly different rhizomatous habit and distinct geographic distribution (de Wet, 1978). It had been thought to be a progenitor of *S. halepense*, possibly hybridizing with *S. bicolor* followed by a doubling of chromosomes (Celarier, 1958; Magoon and Shambulingappa, 1961; Doggett, 1970), but this hypothesis is currently not resolved. Attempts to recreate *S. halepense* by doubling the chromosomes of *S. bicolor* x *S. propinquum* hybrids have not created plants that show similar chromosome associations. Molecular evidence tentatively suggests that *S. propinquum* shares little homology with *S. halepense* as very few RFLP fragments are shared between the two species (Chittenden et al., 1994). The

same authors produced a genetic map using RFLPs on F_2 progeny from a *S. bicolor* x *S. propinquum* cross and observed *S. bicolor* specific low-copy probes. They suggested that these species have diverged considerably from their most recent ancestor.

Genomic relationships between species in the rest of the genus have not been established, partly because hybridization between the species has previously not been possible. The plasmid pCEN38 offers the most data on relationships within the genus. Taxonomic distribution of pCEN38 is quite narrow. It is not detectable in maize, *Zea mays* L., and rice, *Oryza sativa* L., but is detected on most chromosomes of the sugarcane, *Saccharum officinarum* L., genome (Zwick et al., 2000). Research on its distribution within the genus *Sorghum* revealed that the sequence is present in *S. halepense* and *S. propinquum*, although at slightly lower copy number in the latter, lowly present in other species including *S. macrospermum* and *S. laxiflorum* and not detectably present in the remaining species (Anderson, 2005). This agrees with the close evolutionary relationships in the *Eusorghum* section and the considerable divergence of the other species.

Barriers to Interspecific Hybridization

Hybridization is a complex interaction between pollen and pistils that successfully culminates in fusion of male and female nuclei. Each step requires sensitive cellular coordination, including nutrient exchange, distinct guidance cues, and molecular communication to succeed (de Nettancourt 1997; Wheeler et al., 2001; Swanson et al., 2004). Simply described, pollination begins when pollen lands on the

stigma. It adheres to the stigmatic tissue and begins hydration. Then the pollen grain germinates and forms a pollen tube that penetrates the stigma tissue. Upon entering the stigma, the pollen tube grows intercellularly through the stigma transmitting tissue which leads from the outer stigma branches through the style and into the ovary. Passage through the micropyle allows access into the female gametophyte in the ovule for penetration and fertilization.

Not all pollen is treated equally, however, as the overall function of the pistil is to control the mating process. Some species suffer severe inbreeding depression, thus optimum mating would be exclusively cross pollination. In such species, self-incompatibility (SI) mechanisms serve to ensure cross fertilization by actively recognizing self pollen and rejecting it in favor of cross pollen carrying different genetic information. The multiallelic *S*-locus controls the specificity of pollen rejection in the widespread SI mechanisms present in the *Solanaceae*, *Rosaceae*, and *Scrophulariaceae* (de Nettancourt, 1997, 2001; Igic and Kohn, 2001). In the gametophytic SI system, pollen is rejected when the *S*-locus it carries matches the *S*-alleles in the diploid pistil (de Nettancourt, 1997). The SI system allows plants to identify pollen of the same genotype and reject it to ensure cross pollination.

Other species, growing among wild relatives, may need to avoid interspecific fertilization because the resulting progeny are less fit. Interspecific incompatibilities operate in these pistils to identify and reject pollen grains that are too distantly related. Interspecific cross incompatibility has been less studied than the SI interactions common in plants. Two main paradigms exist for understanding interspecific cross

incompatibility: incompatibility and incongruity. Incompatibility, as summarized by Hogenboom (1973), is a mechanism that through the inhibiting action of incompatibility genes, the reproductive relationship is nonfunctional. Incompatibility relies on active rejection of pollen identified as 'foreign', similar to the SI system previously described. Alternatively, incongruity does not rely on active rejection of pollen but is essentially a passive process in which non-functioning occurs due to a lack of genetic information about one of the partners. Incongruity is an isolating mechanism that results from evolutionary divergence. Hogenboom (1973) describes this as gene interactions between pistil and pollen; the female partner contains genes for barriers in the pistil and congruous male partners contain genes for penetrating those barriers by the pollen. An incongruous relationship exists when the male partner lacks a penetrative mechanism to overcome a certain barrier that exists in the female. Species that evolve new barrier mechanisms exert selection pressure on the male partners to contain new penetrative measures. Species that evolve in isolation from one another are more likely to be incongruent partners due to evolutionary divergence (Hogenboom, 1973). Barriers to interspecific crosses that are late-acting, those that do not inhibit pollen tube growth immediately, are more likely to be the result of incongruity as well (Heslop-Harrison, 1982). Barriers to interspecific hybridization are common in crop species and overcoming them is a prerequisite for utilizing wild species germplasm.

Multiple gametophyte loci, which cause cross-incompatibility within the species, have been described in maize. The *gametophyte-1* locus, whose *Ga1-s* allele confers nonreceptivity to *ga1* pollen, is used in popcorn varieties to prevent pollination by

nearby dent corn (Nelson 1993). In this system, *Ga1-s* silks inhibit *ga1* pollen tube growth in the style while allowing *Ga1* pollen to grow normally through the style and complete fertilization (House and Nelson, 1958). Similarly, *Teosinte Crossing Barrier1* (*Tcb1*) was identified in wild teosinte populations and prevents seed set from *tcb1* pollen while allowing fertilization by pollen carrying *Tcb1*. *Tcb1* inhibition acts pre-zygotically and is a gametophytic incompatibility system similar to *Ga1-s* (Evans and Kermicle, 2001). Kermicle and Evans (2005) used translocation stocks to produce heteroallelic pollen (containing both *Ga1* and *ga1* or *Tbc1* and *tcb1*), which functioned in the presence of *Ga1-s* and *Tcb1*, respectively, to determine that both crossing barriers are based on incongruity and not active rejection. That is both systems did not identify and actively reject the *ga1* and *tcb1* alleles, but instead both pistil barriers were overcome by the *Ga1* and *Tcb1* alleles. This supports the incongruity model for the manifestation of cross-incompatibility and not an *S*-locus type system of active rejection in maize.

Interspecific hybridization between pearl millet (*Pennisetum glaucum* [L.] R. Br.) and wild species within *Pennisetum* is very low (Dujardin and Hanna, 1989). Wild species pollen tube growth was inhibited by pearl millet stigmas and the lack of penetration into the micropyle was cause of the cross-incompatibility. The pollen inhibition manifested itself in two ways: stylar inhibition prevented pollen tubes from growing past the base of the style into the ovary for four species, while stigmatic inhibition reduced pollen penetration into stigmatic hairs and prevented tubes from reaching the base of the style for two other species (Mohindra and Minocha, 1991).

Pearl millet pollinated with sorghum inhibited pollen tube growth largely between the style and top of ovary although inhibition occurred in the stigmatic hairs and axis in progressively younger aged pistils (Reger and Sprague, 1983). The barriers preventing wide hybridization within *Pennisetum* may also inhibit sorghum pollen tube growth (Reger and Sprague, 1983).

Interspecific hybridization between wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) has long been known to be genetically controlled (Backhouse, 1916; Taylor and Quisenberry, 1935; Riley and Chapman, 1967). Lein (1943) concluded that two genes, Kr_1 and Kr_2, controlled interspecific hybridization between rye and wheat and that Kr_1 reduces hybridization more than Kr_2 (Riley and Chapman, 1967; Jalani and Moss, 1981). Riley and Chapman (1967) determined that hybridization is actively inhibited by the dominant alleles and not enhanced by the recessive alleles. Study into the site of action of the genes revealed that rye pollen tube growth is largely inhibited between the style base and the top of the ovary due to the *Kr*-genes and failure of seed set was a direct result of a lack of pollen tubes to enter the micropyle (Jalani and Moss, 1980, 1981). When pollinated with rye, Kr_2 reduces pollen tube growth but less severely than Kr_1. When pollinated with *Hordeum bulbosum* L., Kr_2 does not affect pollen tube growth while Kr_1 still strongly reduces pollen tube growth (Falk and Kasha, 1983). The *Kr*-genes are additive in effect and suppress pollen tube growth at different intensities, but interaction between species indicates the *Kr*-genes are likely independent hybridization barriers instead of duplications of the same. Hybridization barriers controlled by the *Kr*-genes are not always complete as some Kr_1Kr_1 Kr_2Kr_2 genotypes

can set limited hybrid seed when pollinated with rye (Riley and Chapman 1967; Jalani and Moss, 1981; Falk and Kasha, 1983). This effect indicates that multiple alleles or modifier genes also affect variation in crossability (Falk and Kasha, 1983).

Hybridization barriers are present in the genus *Sorghum* as evidenced by the multiple unsuccessful attempts at obtaining hybrids between sorghum and wild species beyond the section *Eu-sorghum* (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996). Sun et al. (1991) showed that in reciprocal crosses between sorghum and *S. versicolor* pollen tube growth was largely inhibited in the stigma and style of both crosses. Hodnett et al. (2005) studied pollen tube growth of 16 wild species beyond the *Eu-sorghum* section in crosses with sorghum. Pollen tubes were found to be inhibited at all points following germination prior to entry into the micropyle. The most frequent site of inhibition occurred between the stigma branches and the stigma axis while the remaining pollen tube growth was inhibited in the style prior to entry into the ovary. Laurie and Bennett (1989) found that when sorghum was pollinated with maize pollen, tube growth was inhibited in the stigmas. They identified a single accession (NR481) that lacked this barrier which allowed maize pollen tube growth to continue through the ovary to the micropyle. They designated the gene controlling this behavior *Iap* (Inhibition of Alien Pollen), and designated NR481 to be homozygous recessive for *iap*. Using this accession Price et al. (2006) reported that the *iap iap* genotype reduced pollen-pistil incompatibilities in the genus *Sorghum* and allowed hybrids to be recovered involving three wild species.

Interspecific Hybridization in Other Crops

Interspecific hybridization has proven to be a valuable tool in capturing genetic variation for improving traits in many important species. Plant breeders normally utilize elite and exotic germplasm within the cultivated species for trait improvement. These accessions form the primary gene pool, those that cross readily and produce fertile hybrids and progeny with the cultivated type (Harlan and de Wet, 1971). A secondary gene pool normally encompasses other species that hybridize with some difficulty; however, hybrids exhibit deleterious effects including partial sterility and low genetic recombination between genomes making gene transfer difficult. The tertiary gene pool encompasses other related species that exhibit significant barriers to recovering introgression due to difficulty in hybridization, hybrid lethality or sterility, and lack of genetic recombination. Thus, gene transfer is impossible without extreme techniques. Plant breeders routinely utilize germplasm in the closest gene pool that contains the necessary variation. Sometimes the primary gene pool does not contain variation for the desired traits; therefore, use of more distant gene pools becomes a necessity.

As previously discussed, significant interspecific hybridization barriers may exist between the cultivated species and members of the secondary and tertiary gene pools. If these sexual barriers can be overcome, there remain cytological barriers to recovering genetic variation from the wild species in the cultivated type. Introgression of genetic variation from wild species into cultigens through interspecific hybridization can occur in three ways: genetic recombination, alien translocations, and alien chromosome

additions. Alien chromosome addition and substitution lines are formed when the recurrent parent genome is recovered with either an addition of alien chromosomes or substitution of alien chromosomes for recurrent parent chromosomes (Singh, 2003). Alien translocation lines are formed when segments of alien chromosomes break and fuse to the recurrent parent's chromosomes. This process can occur naturally or be synthesized using radiation to induce chromosomal breaks (Sears, 1993; Singh, 2003). Genetic recombination occurs when the parental genomes share enough homology for homoeologous chromosomes to pair and recombine during meiosis. Chromosome pairing in the interspecific hybrid may occur in two forms: (1) autosyndesis, pairing occurs between members of the same parental genome, and (2) allosyndesis, pairing occurs between members of different parental genomes (Burnham, 1962). If chromosomes behave as univalents, do not pair during meiosis, or pair autosyndetically DNA is not exchanged between genomes and introgression through genetic recombination will not be possible. A requisite amount of homology between the different genomes will allow homoeologous chromosomes to recombine during meiosis, resulting in recombinant chromosomes carrying both donor and recurrent parent genetic material. All forms of introgression have been successful in transferring agronomically useful traits from wild relatives into cultivated species (Jiang et al., 1994; Pickering et al., 1995; Brar and Khush, 1997; Singh, 2003).

The interspecific hybrid must also display some level of gamete fertility if introgression is to be recovered. Interspecific hybrid fertility is not solely dependent on chromosome behavior at meiosis but may be influenced by cryptic structural differences,

complementary lethal genes, or differentiation in genes and chromosome structures (Stebbins, 1950). An example is a hybrid between *Primula verticillata* Forrsk. and *P. floribunda* Wall. which showed loosely paired bivalent chromosomes but was completely sterile (Newton and Pellew, 1929). Only after spontaneous chromosome doubling did the hybrid regain fertility. Complete chromosome pairing was seen in hybrids between *Triticum monococcum* L. and *T. urarta* Thum. ex Gandil. but again the hybrids were completely sterile (Dhaliwal and Johnson, 1982). Some level of interspecific hybrid fertility is required to recover introgression in backcross progeny.

Interspecific hybridization has been very successful in transferring useful traits from wild species in many crops including rice, wheat, oat (*Avena sativa* L.), cotton (*Gossypium hirsutum* L.), maize, tomato (*Solanum lycopersicum* L.), and soybean (*Glycine max* (L.) Merr.) (Brar and Khush, 1997; Shi, Leath, and Murphy, 1998; Sharma and Forsberg, 1977; Meredith, 1991; Stalker et al., 1977; Stamova and Chetelat, 2000; Riggs et al., 1998). In rice alone, at least 26 genes conferring disease and insect resistance, cytoplasmic male-sterility, and acid soil tolerance have been introgressed from distant *Oryza* species through interspecific hybridization. Homoeologous recombination has been implicated as the mechanism of introgression in this system (Brar and Khush 1997).

Few species have been more successful in exploiting interspecific hybridization as a means to novel genetic variation than wheat (*Triticum* ssp.); utilizing high crossability genotypes, wheat has been hybridized with over 50 species belonging to the *Agropyron* complex (Sharma, 1995). Bread wheat is an allohexaploid with three

genomes: A, B, and D (2*n* = 6*x* = 42, AABBDD) (Waines and Barnhart, 1992). Wild species with common genomes are found in *T. monococcum* (2*n* = 2*x* = 14, AA), *T. turgidum* L. (2*n* = 4*x* = 28, AABB), and *T. tauschii* (Coss.) Schmal. (2*n* = 2*x* = 14, DD). These wild genomes are fully homologous and pair normally during meiosis with bread wheat chromosomes of the same genome making them readily amenable to interspecific hybridization and recovery of introgression (Pathak, 1940; Gill and Kimber, 1974; Sharma, 1995; Jauhar and Chibbar, 1999). These species have contributed many genes for disease resistance that have contributed to wheat improvement (Feldman and Millet, 1995; Rong et al., 2000). Other species that contain genomes that are less homologous to those of wheat are still useful in transferring valuable genes. More distantly related genomes that exhibit lower rates of homoeologous recombination have still been useful as multiple disease resistance genes have been recovered (Sears, 1973; Riley et al., 1966; Ceoloni et al., 1988). A genetic system that controls homoeologous pairing in wheat can be used to induce such pairing. The *Ph1* locus in common wheat suppresses homoeologous recombination between the three genomes (A, B, and D), thereby making hexaploid wheat behave like a diploid during meiosis (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958). This locus also suppresses recombination between distant genomes that share some homology. The use of deletion lines which functionally renders the plants to *ph1* have been used to elevate the amount of genetic recombination between wild and cultivated genomes (Feldman, 1993; Jauhar and Chibbar, 1999).

Interspecific hybrids with parental genomes that do not recombine during meiosis can produce naturally occurring as well as irradiation induced translocation lines. These lines contain segments of alien chromatin interspersed in the cultivated genome. Fitness of alien-translocation stocks are normally reduced since alien segments are randomly incorporated with no respect to homology. Alien segments have reduced recombination in the cultivated parent; this makes the reduction of linkage drag difficult or impossible (Jiang et al., 1994; Jones et al., 1995; Singh 2003). Introgression progeny of this type have been a source for novel disease and insect resistance as well abiotic stress tolerance and male fertility (Friebe et al., 1993, 1996; Jiang et al., 1994; Jauhar and Chibbar, 1999; Singh 2003).

Many important crops have utilized wild species as a source of useful genetic variation. Introgression can occur through multiple mechanisms although homoeologous genetic recombination allows facile transfer of genetic variation.

Current Status of Interspecific Hybridization with *S. bicolor*

Interspecific hybridization with *S. bicolor* is not a new prospect. Within the *Eu-Sorghum* section, *S. bicolor* will readily hybridize with *S. propinquum*. Meiosis is normal in the hybrid ($2n = 20$) and fertile backcrosses are readily produced (Celarier, 1958; Chittenden et al., 1994). Wooten (2001) analyzed BC_3 lines and hybrids and determined that *S. propinquum* does contain useful alleles that could be used in the improvement of cultivated grain sorghum. Germplasm containing *S. propinquum*

introgression is still present in the TAES sorghum improvement program, but it has not yet been used in released and improved germplasms, lines, or hybrids.

Successful hybridization of *S. bicolor* with the other species in *Eusorghum*, *S. halepense*, has been reported numerous times; interspecific hybrids can be $2n = 30$ ($n + n$) or $2n = 40$ ($2n + n$) (Hadley, 1953; Endrezzi, 1957; Hoang-Liang and Tang, 1988). Chromosome pairing in the hybrids is irregular but gene transfer into $2n = 20$ backcross progeny is possible (Hadley, 1953; Hadley and Mahan, 1956). More recently, an interspecific hybrid with $2n = 20$ chromosomes was produced between a genetic male-sterile *S. bicolor* and *S. halepense*. The chromosome number was likely generated from fertilization by a rare monohaploid *S. halepense* pollen grain (Dweikat, 2005). This plant was fertile and showed normal segregation of polymorphic SSR markers in the F_2 generation. Interspecific hybrids between tetraploid sorghum ($2n = 40$) and *S. halepense* are more easily made and have been used in attempts to create perennial grain sorghums (Piper and Kulakow, 1994; Cox et al., 2002). None of the efforts to date have succeeded in producing agronomically useful germplasm.

Until recently, hybridization between *S. bicolor* and species outside the section *Eu-Sorghum* have been unsuccessful (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996). This can easily be explained based on the recent results identifying pollen-pistil incompatibilities as the cause of reproductive isolation in the genus. A single hybrid was recovered between *S. bicolor* and *S. macrospermum* using standard cytoplasmic male-sterile (CMS) germplasm after much effort (Price et al.,

2005b). The interspecific hybrid was confirmed cytologically to be 2*n* = 30 and was intermediate in phenotype. Unfortunately this hybrid was male-sterile and meiotic recombination could not be studied. The recovery of this hybrid indicates that reproductive isolation barriers are strong but not absolute within the genus. Price et al. (2006) later used a CMS *S. bicolor* accession with genotype *iap iap*, which allows the growth of pollen tubes from foreign species into its pistils (Laurie and Bennett, 1989). This gene dramatically increased the ease and frequency of recovering interspecific hybrids. They reported, supported with cytological evidence, the production of three different interspecific hybrids between *S. bicolor* and *S. macrospermum*, *S. nitidum*, and *S. angustum*. Interspecific hybrids between *S. bicolor* and both *S. nitidum* and *S. angustum* did not survive to maturity, but the hybrids with *S. macrospermum* were vigorous. It was not possible to determine the meiotic chromosome pairing behavior of the interspecific hybrid and the genomic relationships between *S. bicolor* and *S. macrospermum* because of male-sterility in the hybrids. Intergeneric hybridization, specifically crosses between *S. bicolor* and sugarcane, has been successful. Most crosses used sugarcane as the female parent and sorghum as the pollen parent (Thomas and Venkatraman, 1930; Janakiammal and Singh, 1936); however, reciprocal hybrids have more recently been recovered (Nair, 1999). The compatibility of sorghum and sugarcane is less surprising since results involving the *Eusorghum* specific repeat CEN38 implicate related genomes in the polyploidy of sugarcane (Zwick et al., 2000).

Compared to other species such as wheat, rice, oat, barley, cotton, and soybeans where interspecific hybridization has produced tangible results; interspecific

hybridization in sorghum is in its infancy. Interspecific hybrids have just become a reality in species beyond *Eusorghum* and much research remains to prove its usefulness.

Description of *S. macrospermum*

Sorghum macrospermum ($2n = 40$) is the sole member of the *Chaetosorghum* section within *Sorghum* (Garber, 1950). It belongs to the $x = 10$ chromosome lineage within *Sorghum*, which are characterized by relatively small chromosomes, and it is one of two species most closely related to the *Eusorghum* section (Wu, 1980, Dillon et al., 2004; Price et al., 2005a). It is a robust annual plant that grows 1.8-3.8m tall, has a large loose panicle with long drooping branches, and large ovoid caryopses. Only a single population is known; it is distributed in a relatively small area of limestone outcrops north west of Katherine in the Northern Territory of Australia. The plants grow in soil pockets and crevices between boulders (Lazarides et al., 1991). The species has large seeds, relative to other wild sorghums, and is the only of the Australian wild species that has similar grain N and P concentrations to *S. bicolor* (Cook and Andrew, 1991). Coincidentally, or perhaps not, *S. macrospermum* played an important part in the diet of the Tagoman Aboriginal Tribe in the Katherine district (Arndt, 1961). The Tagoman gathered grains off the ground, which had shattered from the panicle, and they prepared the ground meal as grits after dehulling and cleaning. It is unknown whether the species underwent any selection by the aboriginal tribes, or if it only served as a wild source of cereal grain.

Research into agronomically useful traits held by Australian wild species has been limited as until recently because the genetic variation was inaccessible due to cross-incompatibilities. Some research has focused on the disease and insect resistance potential of the wild species. *Sorghum macrospermum* was shown to be either a non-host or has ovipositional non-preference to sorghum midge (*Stenodiplosis sorghicola* Coquillett) (Franzmann and Hardy, 1996; Sharma and Franzmann, 2001). It is not susceptible to sorghum downy mildew (*Peronosclerospora sorghi* Weston and Uppal (Shaw)) (Kamala et al., 2002) and has high tolerance to shoot fly (*Atherigona soccata* Rond.) (Sharma et al., 2005). Until recently research into useful traits from this species has had no direct application as hybridization was impossible. This species deserves more scrutiny if introgression into *S. bicolor* becomes possible.

Summary

A genetic system for producing interspecific hybrids with species beyond the *Eusorghum* section is now available. Using germplasm recessive for the *iap* allele allows pollen from foreign species to grow through the stigma, style, and successfully complete fertilization. Interspecific hybrids between *S. bicolor* and *S. macrospermum* display vigor and successfully reach maturity. No information is available about the genomic relations between the species and whether the interspecific hybrid retains enough fertility to recover backcross progeny. The present research will investigate the feasibility of using *S. macrospermum* in an introgression breeding program and determine methods for such research.

CHAPTER III

GENETIC RECOMBINATION IN THE INTERSPECIFIC HYBRID

Introduction

Sorghum is an important food, feed and forage crop in the U.S and around the world. The genus *Sorghum* contains five sections, of which *S. bicolor* is a member of the *Eu-Sorghum* section along with *S. propinquum* and *S. halepense*. The remaining four sections contain 19 species which are native to Africa, Australia, and Asia (Garber, 1950; de Wet, 1978; Lazarides et al., 1991). Breeding efforts have mainly used the primary gene pool of diverse germplasm within the *S. bicolor* species (Duncan et al., 1991). Limited efforts have been made to utilize the secondary gene pool consisting of the remaining species within *Eusorghum*. Interspecific hybrids are easily made with *S. propinquum* and with some effort *S. halepense*; however, few breeding programs have utilized the germplasm (Wooten, 2001; Dweikat, 2005). The tertiary gene pool contains all the remaining species within the genus, and despite many efforts to produce interspecific hybrids, hybridization was not successful until recently (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996).

Hodnett et al. (2005) determined that pollen-pistil incompatibilities were the reason for reproductive isolation between *S. bicolor* and species in the tertiary gene pool. Pollen tube growth of the wild species was arrested in the stigma and style preventing

successful fertilization. A single interspecific hybrid between cytoplasmic male-sterile *S. bicolor* (2*n* = 20) and the Australian species *S. macrospermum* (2*n* = 40) was recovered by Price et al. (2005b) which indicates that the barriers to hybridization were strong but not complete. Further research determined that interspecific hybridization efficiency could be improved by an order of magnitude simply by using *S. bicolor* homozygous for the *iap* allele. The *Iap* gene locus (*I*nhibition of *A*lien *P*ollen) causes pollen-pistil incompatibilities between *S. bicolor* and alien pollen species (Laurie and Bennett, 1989). Using this allele, Price et al. (2006) reported interspecific hybrids between *S. bicolor* and three tertiary gene pool species: *S. macrospermum*; *S. nitidum*; and *S. angustum*. Interspecific hybrids were verified morphologically and cytologically, but only hybrids with *S. macrospermum* survived to maturity.

The genus *Sorghum* is divided into two distinct lineages, *x* = 5 and *x* = 10 (Price et al., 2005a). The *x* = 5 lineage (2*n* = 10, 20, 30, and 40) consists of species with large genomes and chromosomes, while the *x* = 10 lineage (2n = 20 and 40) is characterized by small genomes and chromosomes. Both *S. macrospermum* and *S. bicolor* belong to the *x* = 10 lineage. The base chromosome number in *Sorghum* is generally regarded as *x* = 5, and research has shown that *S. bicolor* is likely an ancient tetraploid (2*n* = 4*x* = 20) with distinct but related subgenomes (Garber, 1950; Hadley 1953; Endrizzi and Morgan 1955; Celarier, 1958; Doggett, 1988; Tang and Liang 1988; Gomez et al., 1998; Zwick et al., 2000). Tang and Liang (1988) reviewed the data regarding genomic relationships between *S. halepense* and *S. bicolor*, and they designated the former as having the genomic formula $AAAAB_1B_1B_2B_2$ and the latter as AAB_1B_1. They also concluded that

subgenomes B_1 and B_2 share enough homology that homoeologous chromosome pairing can occur. Subgenome A shares much less homology with B_1 and B_2, but multivalents involving members of all three genomes are possible. Utilizing FISH, the most recent cytogenetic research assigned *S. bicolor* the genomic formula $A_bA_bB_bB_b$ with the subscript "b" representing *bicolor*, based on differential probe hybridization (Zwick et al., 2000). For this discussion, *S. bicolor* will be designated as AAB_1B_1. Little is known about the genomic relationships between *S. bicolor* and *S. macrospermum*. Wu (1980) studied the karyotype of *S. macrospermum* and suggested it was a polyploid with high amounts of similarity to the *S. bicolor* genome. That research found that the chromosome size of both species was similar and overlapped, and that *S. macrospermum* appeared to have two chromosomes for each individually identifiable *S. bicolor* chromosome. During meiosis *S. macrospermum* behaved as a diploid forming 20 II, although quadrivalents were observed. This suggests *S. macrospermum* is likely $2n = 8x = 40$, with an unknown genomic formula of WWXXYYZZ.

Sorghum macrospermum is the only member of the *Chaetosorghum* section and is native to the Katherine area in the Northern Territory of Australia (Lazarides et al., 1991). It is either a non-host or has ovipositional non-preference to sorghum midge (*Stenodiplosis sorghicola* Coquillett) (Franzmann and Hardy, 1996; Sharma and Franzmann, 2001). It is immune to sorghum downy mildew (*Peronosclerospora sorghi* Weston and Uppal (Shaw)) (Kamala et al., 2002) and is highly tolerant to shoot fly (*Atherigona soccata* Rond.) (Sharma et al., 2005). There is interest in using this newly compatible species in an interspecific breeding program for the improvement of *S.*

bicolor. The goal of an interspecific breeding program is to transfer useful *S. macrospermum* genetic variation to the *S. bicolor* genome. The most direct way for such introgression to occur is through allosyndetic recombination during meiosis, which is recombination between chromosomes of different parental genomes (Burnham, 1962; Jauhar and Chibbar, 1999). The level of allosyndetic recombination will largely be a function of the amount of genetic similarity between the different genomes (Singh, 2003). Homoeologous chromosomes, genetically related through ancestry, will pair if they retain sufficient homology with one another. Backcross progeny containing such recombinant chromosomes would possess *S. macrospermum* genetic variation. Autosyndetic recombination, which is recombination between chromosomes from the same parental genome, does not result in introgression within the *S. bicolor* genome because no exchange occurs between the chromosomes of the different genomes. Therefore, determining whether allosyndetic recombination occurs in a *S. bicolor* x *S. macrospermum* hybrid will provide insight as to the possibility of introgression occurring.

The objectives of this research were to determine the genomic relationship between *S. bicolor* and *S. macrospermum,* measure the frequency of allosyndetic recombination during meiosis, and recover backcrosses to the *S. bicolor* parent.

Materials and Methods

Plant Material

Interspecific hybrids were produced using *S. bicolor* accession 'NR481' (*iap iap*) as the hand-emasculated female parent and the wild species *S. macrospermum* (AusTRC Accession no. 302367) as the male parent. NR481 was the genotype in which the *Iap* gene was first identified (Laurie and Bennett, 1989). Pollinated florets set approximately 25% seed with a shrunken endosperm. Approximately 60% of the seeds germinated on agar media and the seedlings were subsequently transplanted into soil. The hybrids were identified at an early stage by their pubescent leaves, a trait absent in *S. bicolor* but present in both *S. macrospermum* and the interspecific hybrid. The putative hybrids were confirmed by determining their chromosome numbers ($2n = 30$). Morphology of the interspecific hybrids did not differ from previously published descriptions (Price et al., 2005b). Six hybrids, as well as control plants of the two parents, were grown in a greenhouse in the summer of 2004 in College Station, TX for cytological studies. Backcrosses were attempted by collecting anthers (non-dehiscent) from the interspecific hybrids, disrupting the anthers in a glass Petri dish, and dusting pollen onto CMS *S. bicolor* (*iap iap*) stigmas with a fine bristle paintbrush.

Traditional Cytogenetic Analysis

Immature panicles, with the flag leaf collar extended 3-4 inches above the last leaf collar, were harvested and fixed whole in Carnoy's solution (6 Ethanol : 3 Chloroform : 1 Glacial Acetic Acid) for a minimum of 24 hours and then transferred to

70% ethanol for storage. Florets were dissected and the anthers macerated on a glass slide in a drop of acetocarmine stain. A coverslip was placed over the stain and the slide heated over an alcohol flame before being squashed on a hard surface between filter paper. Pollen mother cells (PMCs) were examined under phase contrast microscopy using a Zeiss Axiophot microscope (Carl Zeiss Inc., Gottingen, Germany) at 1000-2000X magnification. Meiotic analysis of both parents and the interspecific hybrids consisted of counting the number of univalents, bivalents (both rod and ring conformation), trivalents, and quadrivalents in each PMC. Note of laggard chromosomes at anaphase was also taken. Images were taken using a Nikon Coolpix 4500 digital camera with a 57 to 38mm stepdown adapter through the microscope eyepiece. Data was analyzed using GLM in SPSS v11.5.

Fluorescent *in situ* Hybridization

Slides to be used with FISH (fluorescent *in situ* hybridization) were prepared in the same manner as above except the anthers were macerated in 20% acetic acid, and after squashing were immediately frozen at -80ºC. Plasmid CEN38, present on all *S. bicolor* chromosomes and visually absent from the *S. macrospermum* chromosomes, was used as a probe to visually differentiate the genomes (Zwick et al., 2000; Anderson 2005). Detection of the FISH probe followed a modified protocol of Jewell and Islam-Faridi (1994), as described by Hanson et al. (1995) and Kim et al. (2002). Purified CEN38 DNA was nick-translated with digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, IN). Meiotic chromosomes on glass slides were denatured in 70%

formamide in 2X SSC for 1.5 min at 70ºC, then dehydrated in 70 (-20ºC), 85 (RT), 95 (RT), and 100% (RT) ethanol, for 2 min each. The hybridization mixture (25ul per slide) contained 50ng labeled probe DNA, 50% formamide and 10% dextran sulfate in 2XSSC. The hybridization mixture was denatured for 10 min at 95ºC and chilled on ice. It was then added to the slide, sealed with rubber cement around a glass coverslip and incubated overnight at 37ºC. Following incubation, the slides were washed at 40ºC in 2XSSC and room temperature in 4XSSC plus 0.2% Tween-20, for 5 min each. Slides were blocked with 5% (w/v) BSA in 4XSSC plus 0.2% Tween-20 at room temperature. The digoxigenin-labeled probe was detected with CY3™-conjugated anti-digoxigenin anti-body. Slides were washed in 37ºC 4XSSC plus 0.2% Tween-20. Chromosomes were counterstained with 25ul DAPI with Vectashield®. Slides were viewed through an Olympus AX-70 epifluorescence microscope and images captured with a Macprobe® v4.2.3 imaging system (Applied Imaging Corp., Santa Clara, CA). Meiotic analysis of the interspecific hybrids consisted of counting the number of bivalents (rods or rings) and multivalents in each PMC as well as associations with CEN38.

Molecular Markers

DNA was extracted from fresh leaf tissue from 11 BC_1F_1 plants, their parents, and genetic map parents BTx623 and IS3620C using FastDNA Spin Kits (MP Biomedicals, Solon, OH). AFLP templates, using both *EcoRI*/*MseI* and *PstI*/*MseI* restriction enzyme combinations, were created using a modified procedure from Vos et al., (1995). The AFLP template, preamplification, and selective amplification reactions

of the *EcoRI*/*MseI* and *PstI*/*MseI* fragments were as described by Klein et al (2000) and Menz et al (2002), respectively. Thirty *PstI*/*MseI* and 15 *EcoRI*/*MseI* primer combinations were used to amplify fragments in the DNA samples. Amplification products were analyzed on a LI-COR model 4200 dual-dye automated DNA sequencing system. Electrophoresis conditions were as described by Klein et al (2000). Gels were analyzed by hand, bands were scored as unique to *S. macrospermum* if they were not present in the CMS female parents, unique bands shared between the putative backcrosses and *S. macrospermum* were identified as introgression bands. Percent introgression was calculated as the total number of introgression bands found for a particular plant divided by the total number of unique *S. macrospermum* bands found. This number is an estimate of the amount of the *S. macrospermum* genome that is present in the backcross plant.

Results and Discussion

Meiotic analysis of the parents revealed mostly normal meiosis. *Sorghum bicolor* (acc. NR481) had a mean chromosome pairing behavior of 10 bivalents per PMC and no multivalents were observed (Table 1; Fig. 1a). The *S. macrospermum* parent had an average of 19.96 bivalents per PMC with a single quadrivalent observed over 48 cells (Table 1; Fig. 1b). Some level of quadrivalent formation was expected in the *S. macrospermum* parent, although the level observed was lower than previously reported (Wu, 1980). Analyses of the interspecific hybrids reveal a mean of 3.54 bivalents per PMC, with 98% forming rods (Table 1; Fig. 1c). The number of bivalents ranged from

Table 1. Chromosome pairing behavior in parents and *S. bicolor* x *S. macrospermum* interspecific hybrids

			Bivalents			Multivalent Totals	
Genotype	2*n*	N	Mean†	Range	% Rod	Tri	Quad
S. bicolor	20	48	10.00^{B}	0	42^{B}	0	0
S. macrospermum	40	48	19.96^{A}	18-20	NA	0	1
S. bicolor x *S. macrospermum*	30	312	3.54^{C}	0-8	98^{A}	2	0
Hybrid 1	30	54	3.59^{C}	0-7	99^{A}	1	0
Hybrid 2	30	53	3.96^{C}	1-8	100^{A}	0	0
Hybrid 3	30	28	1.68^{D}	0-3	96^{A}	0	0
Hybrid 4	30	48	3.58^{C}	1-7	98^{A}	0	0
Hybrid 5	30	115	3.95^{C}	0-7	98^{A}	1	0
Hybrid 6	30	14	2.07^{D}	1-4	98^{A}	0	0
		$LSD_{.05}$	0.82		6		
		CV	26%		1%		

†Means followed by different superscript letters are significantly different ($p<.05$)

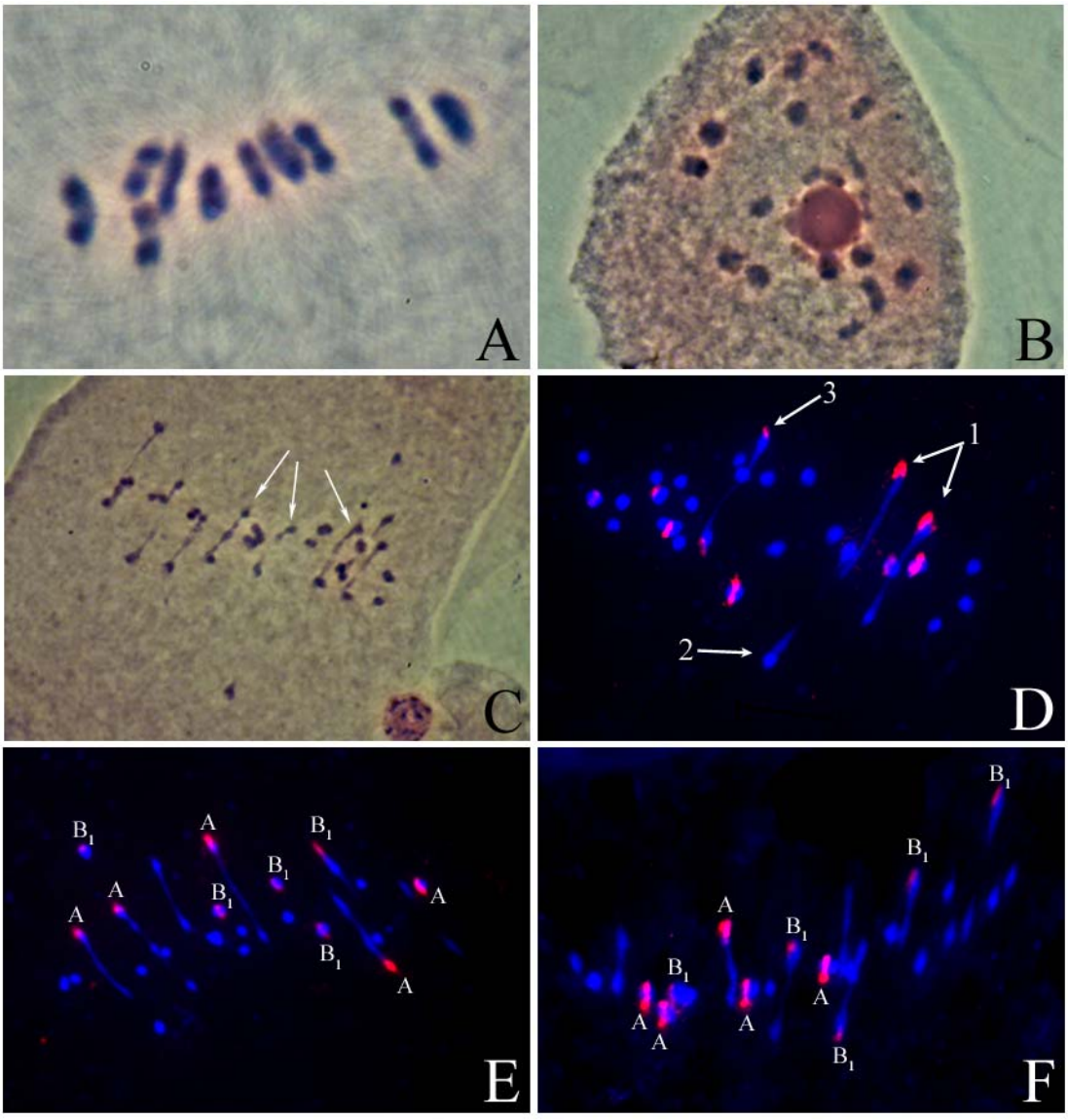

Figure 1. Pollen mother cells (PMCs) during meiosis of *S. bicolor*, *S. macrospermum*, and the interspecific hybrid (A-F). PMCs stained with acetocarmine used for the traditional analysis (A-C). FISH of hybrid PMCs using CEN38 which hybridizes strongly to the A subgenome and weakly to the B_1 subgenome of *S. bicolor* and does not hybridize to any chromosomes in *S. macrospermum* (D-F). (A) Metaphase I in *S. bicolor* showing 10 II, (B) Diakinesis in *S. macrospermum* showing 20 II, (C) Metaphase I in the interspecific hybrid with 8 II + 14 I, all bivalents are rod shaped, arrows show 3 rod bivalents. (D) FISH of hybrid PMC at metaphase I with (1) two allosyndetic bivalents, (2) one *S. macrospermum* autosyndetic bivalent, (3) one *S. bicolor* autosyndetic bivalent (B_1-B_1), the remaining chromosomes are univalents. (E) FISH of hybrid PMC at metaphase I showing five allosyndetic bivalents, four involving *S. bicolor* subgenome A and one involving B_1, two *S. macrospermum* autosyndetic bivalents, and 16 univalents. (F) FISH of hybrid PMC at metaphase I showing five allosyndetic bivalents, four involving *S. bicolor* subgenome B_1 and one involving A, two *S. macrospermum* autosyndetic bivalents, and 16 univalents.

0-8 with the most common configuration being 3 II + 24 I (26% of PMCs), and multivalents were rare. Two interspecific hybrids had significantly lower bivalent formation than the others, indicating that recombination may be influenced by factors such as environment or genetic background (Table 1). The observed range of bivalent formation in the interspecific hybrid rules out exclusive autosyndetic *S. bicolor* pairing as only five such bivalents are possible. Exclusive *S. macrospermum* autosyndetic pairing cannot be ruled out since some homology within the genome exists and 10 such bivalents are possible. Preferential formation of rod shaped bivalents is associated with less homology between genomes (Singh, 2003), which would likely be the case if such recombination were allosyndetic. With such a low amount of quadrivalent formation in the *S. macrospermum* parent, it is unlikely that all the bivalent formation in the hybrid can be attributed to autosyndesis within this parental genome. Traditional cytogenetic analysis cannot confirm allosyndetic recombination as discrimination of these parental genomes is not possible. This data does confirm that some recombination is occurring within the interspecific hybrid.

The FISH probe CEN38 does not visually hybridize to *S. macrospermum* chromosomes and differentially hybridizes to 10 of 20 somatic *S. bicolor* chromosomes (Zwick et al., 2000; Anderson 2005). Ten homologous *S. bicolor* chromosomes (SBI) show strong CEN38 signal, SBI-01, 02, 03, 05, and 06, and ten show weak signal, SBI-04, 07, 08, 09, and 10 (Kim et al., 2005b; J.S. Kim, Personal Communication). Zwick et al. (2000) suggested the differential hybridization corresponds to the subgenomes that make up *S. bicolor*; strong signal identifies subgenome A and weak signal identifies

subgenome B_1. The designation of CEN38 signal strength to subgenome was arbitrary since it was not based on genome homology to *S. halepense*, which was used to assign A and B_1 subgenomes (Hadley, 1953; Tang and Liang, 1988), but such designations will be used in this discussion. Meiotic FISH analysis of the interspecific hybrid revealed 4.3 bivalents per PMC, an estimate not significantly different from the traditional cytogenetic analysis. More importantly, it confirmed the presence of allosyndetic recombination between *S. bicolor* and *S. macrospermum* chromosomes, one chromosome with and one without CEN38 signal (Fig. 1, D-1). In fact, all three types of recombination were detected: allosyndetic; autosyndetic *S. macrospermum*; and autosyndetic *S. bicolor* (Table 2; Fig. 1, D-1-3). The frequency of allosyndetic recombination, 61% of bivalents or 2.6 II per PMC, indicates that significant homology exists between some chromosomes of *S. bicolor* and *S. macrospermum*. This agrees with Wu, (1980) who suggested that *S. bicolor* may be one of two ancestors of *S. macrospermum*.

Since the strong and weak CEN38 FISH signals differentiate *S. bicolor* subgenomes A and B_1, their behavior can be compared. In two different cells (Fig. 1, E and F), four A and four B_1 allosyndetic bivalents were separately detected. Together these represent 8 different *S. bicolor* chromosomes which are participating in recombination with the *S. macrospermum* genome. *Sorghum macrospermum* chromosomes cannot be differentiated in the present analysis; therefore, no definitive estimate can be given to the percentage of its genome that is subject to recombination

Table 2. Allosyndetic (*B-M*) and autosyndetic (*B-B* and *M-M*) recombination in a *S. bicolor* x *S. macrospermum* hybrid as revealed using FISH probe CEN38

	Bivalents				Trivalents
	B-M†	*B-B*	*M-M*	Total	Total
Sum (N = 46)	121	4	72	197	3
Mean‡	2.63^{A}	0.09^{C}	1.57^{B}	4.28	.07
% of Total	61.4	2.0	36.5		

† *B* and *M* represent *S. bicolor* and *S. macrospermum* chromosomes, respectively
‡ Means with different superscript letter are significantly different ($p<.05$)

with *S. bicolor*. It is likely that the 8 *S. bicolor* chromosomes are recombining with 8 different *S. macrospermum* homoeologous chromosomes. Therefore, at minimum approximately 80% of the *S. bicolor* genome and possibly 40% of the *S. macrospermum* genome is subject to allosyndetic recombination. Clearly, the moderate level of allosyndetic recombination makes recovering introgression in backcross progeny very likely.

A and B_1 *S. bicolor* chromosomes do not form allosyndetic bivalents with *S. macrospermum* at the same frequency. Chromosomes in subgenome A account for 70% of the allosyndetic bivalents in the interspecific hybrid (Table 3). So, while a majority of the genome has the capacity to recombine with *S. macrospermum*, subgenome A chromosomes are more likely to be involved in recombination. Thus, those chromosomes will be the likeliest to carry introgression into progeny.

Autosyndetic recombination occurred at a relatively high frequency within the *S. macrospermum* genome, 37% of the bivalents or 1.6 II per PMC, and at a very low frequency in the *S. bicolor* genome, 2% of the bivalents or 0.1 II per PMC (Table 2). This indicates there is significant homology within the *S. macrospermum* haploid genome, and relatively little within the *S. bicolor* haploid genome. The relative differences in autosyndesis are likely a result of multiple factors. *Sorghum bicolor* may have undergone significantly more diplodization, which reduced homology within its genome, than has *S. macrospermum* (Liu and Wendel, 2002). This may also indicate an older evolutionary age for the tetraploid nature of *S. bicolor* and a relatively more recent polyploidization event in *S. macrospermum*. It could indicate that the polyploidization

Table 3. Allosyndetic recombination involving subgenomes A and B_1 in a *S. bicolor* x *S. macrospermum* hybrid as revealed using FISH probe CEN38

	A-*M*†	B_1-*M*	Total
Sum (N = 19)	39	16	55
Mean‡	2.05^{A}	0.84^{B}	2.89
% of Total	70.9	29.1	

†*M* represents *S. macrospermum* chromosomes, A and B_1 represent chromosomes within subgenomes of *S. bicolor*

‡Means followed by different superscript letters are significantly different ($p<.05$)

of *S. bicolor* involved two separate genomes with very little homology, while the nascent genomes of *S. macrospermum* were much more related. Most likely all three describe the evolutionary history of these species. *Sorghum macrospermum* has been hypothesized to be an allooctaploid originating from chromosome doubling of two related *Eusorghum* species (Wu, 1980). *S. bicolor* is regarded as a highly diplodized allotetraploid originating from a chromosome doubling of two more divergent genomes (Tang and Liang, 1988; Gomez et al., 1998; Zwick et al., 2000).

Significant homology exists between the genomes of *S. bicolor* ($2n = 4x = 20$; AAB_1B_1) and *S. macrospermum* ($2n = 8x = 40$; WWXXYYZZ). Meiosis in the interspecific hybrid ($2n = 6x = 30$; AW B_1XYZ) shows recombination. FISH analysis reveals allosyndetic recombination between a minimum of 4 chromosomes from each *S. bicolor* subgenome. Recombination between the *S. bicolor* subgenomes and their *S. macrospermum* homoeologs is not regular; subgenomes A and B_1 average 2.1 and 0.8 allosyndetic bivalents per PMC, respectively (Table 3). Multivalents in the interspecific hybrid are very rare indicating that only the homoeologous chromosomes from the related subgenomes of the two species have the potential to pair during meiosis. From this data, it is suggested that the genomic formula for *S. macrospermum* is AAB_1B_1YYZZ. Subgenomes A_b and A_m, from now on additionally denoted by their species, share more homology than subgenomes B_{1b} and B_{1m}, indicated by the difference in pairing. Pairing between and within *S. bicolor* subgenomes in the interspecific hybrid was rare; one $A_b - B_{1b}$ bivalent, zero $A_b - A_b$ bivalents, and two $B_{1b} - B_{1b}$ bivalents were observed in 46 cells. A moderate level of *S. macrospermum* autosyndetic pairing does

occur in the hybrid, 1.6 II per PMC. If meiotic behavior of the A_b and B_b genomes is a guide, it is unlikely that the *S. macrospermum* autosyndetic pairing in the hybrid can be explained by $A_m - B_{1m}$, $A_m - A_m$, and $B_{1m} - B_{1m}$ chromosome associations. Therefore, they are likely occurring through the associations of the unknown subgenomes Y and Z. Autosyndetic pairing may be between these two subgenomes, Y_m-Z_m, or between one and either A_m or B_{1m}. Much like in *S. halepense,* in which Tang and Liang (1988) hypothesized moderate levels of $B_1 - B_2$ pairing, subgenomes Y or Z may be analogous to subgenome B_2. Thus, $B_{1m} - B_{2m}$ associations could explain the observed level of autosyndetic *S. macrospermum* pairing. Such conclusions about genomic relationships of subgenomes Y and Z are hypothetical. Research on the genomic relations between *S. macrospermum* and *S. halepense* could provide an answer.

Interspecific hybrid male fertility, measured by observing I_2-KI pollen stainability, indicated that normal pollen formation was rare; five fully stained pollen grains were observed in approximately 20,000 examined. Approximately, 3050 CMS *S. bicolor* florets were pollinated with interspecific hybrid pollen over 4 months; 13 putative BC_1F_1 seeds developed. All the putative backcross progeny developed on three heads that were pollinated in a 2 week period that coincided with low light intensity, an environmental condition known to affect the stability of the male-sterility. Thus, it is possible that "BC_1F_1" plants were the result of self-pollination or stray *S. bicolor* pollen, and not interspecific pollination. The putative BC_1F_1 progeny were grown to maturity and they did not differ morphologically from their maternal parent. Molecular markers were used in hopes of proving their interspecific paternity. DNA samples from 11

putative BC_1F_1 plants, their respective female CMS parents, *S. macrospermum*, and mapping parents BTx623and IS3620C were used to evaluate the presence or absence of *S. macrospermum* introgression. A total of 825 unique *S. macrospermum* bands were scored and some were found in the putative BC_1F_1 progeny. Introgression estimates were between 0-1%, and none of the introgression bands were shared by the mapping parents. Thus, the genomic location of the potential introgression is unknown (Data not presented).

Presence of introgression bands in these male produced backcrosses is not irrefutable evidence of their backcross status. All introgression bands were AFLP markers, and nothing is known regarding their sequence similarity, only that the fragments had similar mobility through the gel. Comigration of non-sequence related bands can be a problem for AFLP markers when comparing different biological species (Menchanda et al., 2004). It is possible that stray *S. bicolor* pollen from a genotype that produces non sequence-related AFLP bands of the same size as *S. macrospermum* was the male parent of the putative BC_1F_1s, thus making "introgression bands" not indicative of introgression. It is equally possible that these plants are actually backcrosses that contain between 0-1% *S. macrospermum* introgression. Regardless, "BC_1F_1" plants were male sterile like their CMS parent, and restoring fertility would require another cross and selfing generation. It was decided that recovering introgression in this manner was not ideal and another approach would be pursued. It may be necessary to use the interspecific hybrids as females in combination with embryo rescue, to avoid questions of pedigree in recovered backcrosses.

Summary

Allosyndetic recombination occurred between *S. bicolor* and *S. macrospermum* chromosomes at a moderate frequency (2.6 II per PMC) in the interspecific hybrid. Genome relationships were sufficient to suggest that *S. macrospermum* has two genomes which share homology with the genomes of *S. bicolor* and that the wild species has the genomic formula AAB_1B_1YYZZ, with Y and Z being unknown genomes. Interspecific backcrosses were sought among putative BC_1F_1 plants after pollinating sorghum with the interspecific hybrid, but irrefutable evidence of their hybridity remained elusive. Only extremely low levels (1%) of putatively alien AFLPs were observed, i.e., too low to confirm recovered progeny were different from selfs. Future research should focus on recovering introgression backcross progeny as the current research predicts recovering *S. macrospermum* introgression is probable.

CHAPTER IV

INTROGRESSION BREEDING AND ANALYSIS OF GERMPLASM

Introduction

Sorghum is an important food and feed crop around the world. The 2006 U.S. grain sorghum crop was valued at approximately $715,000,000 (USDA, 2006) and worldwide is the 5th most grown cereal grain. Plant breeders continuously improve the crop for yield potential, drought tolerance, disease and insect resistance, and other biotic and abiotic stresses. Genetic variation is the lifeblood of plant breeding so identification of useful new sources is a worthwhile endeavor.

Taxonomically, the genus *Sorghum* is separated in to 5 sections: *Eusorghum*, *Chaetosorghum*, *Heterosorghum*, *Parasorghum*, and *Stiposorghum* (Garber, 1950; de Wet, 1978). The cultivated species is grouped within section *Eusorghum* along with *S. propinquum* and the noxious weed *S. halepense*. Genetic improvements in sorghum have been made by utilizing genetic variation from within the primary gene pool, which contains all of the germplasm in the three subspecies of *S. bicolor*: ssp. *arundicum*, *bicolor*, and *drumondii* (de Wet, 1978; Cox et al., 1984; Duncan et al., 1991). The secondary gene pool is composed of the remaining two species in *Eusorghum*. Crosses between sorghum and *S. propinquum* are easily made, meiosis is normal in the interspecific hybrids, and progeny are fertile, but there has been little to no use of this germplasm in applied sorghum improvement (Wooten, 2001). Hybrids between

sorghum and *S. halepense* are more difficult to produce but are possible. Most efforts in utilizing *S. halepense* as a genetic resource have been devoted to developing perennial grain crops (Piper and Kulakow, 1994; Cox et al., 2002; Dweikat, 2005). The tertiary gene pool contains the 17 remaining species within the four other sections. Until recently, this gene pool was completely inaccessible and no hybrids had ever been recovered despite numerous efforts (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996).

The cause of reproductive isolation between sorghum and the tertiary gene pool was unknown until Hodnett et al. (2005) determined that it was due to pollen-pistil incompatibilities. Pollen tube growth of wild species was inhibited in the stigma and style which prevented successful fertilization. The reproductive barriers proved to be strong but not complete as Price et al. (2005b) finally recovered one interspecific hybrid between cytoplasmic male-sterile (CMS) sorghum and *S. macrospermum*. The efficiency of producing this hybrid improved dramatically by using a *S. bicolor* genotype homozygous for the *iap* allele. The *Iap* locus controls a pistil barrier that prevents foreign species pollen tube growth; whereas, the recessive genotype (*iap iap*) allows pollen tube growth of maize as well as wild sorghum species (Laurie and Bennett, 1989; Price et al., 2006). Price et al. (2006) recovered hybrids between sorghum and *S. macrospermum*, *S. nitidum*, and *S. angustum* but only the hybrids with *S. macrospermum* survived to maturity.

Sorghum macrospermum ($2n = 40$) is the only member of the *Chaetosorghum* section and it is native to the Katherine area in the Northern Territory of Australia (Lazarides et al., 1991). While this species does not possess any obvious phenotypic agronomically desirable traits, it does have significant pest resistance characteristics. It is either a non-host or has ovipositional non-preference to sorghum midge (*Stenodiplosis sorghicola* Coquillett) (Franzmann and Hardy, 1996; Sharma and Franzmann, 2001). It is not susceptible to sorghum downy mildew (*Peronosclerospora sorghi* Weston and Uppal (Shaw)) (Kamala et al., 2002) and has high tolerance to shoot fly (*Atherigona soccata* Rond.) (Sharma et al., 2005). These beneficial traits, as well as the possibility that it holds other valuable unique genetic variation, make it attractive to use in an introgression breeding program.

Until recently, the genomic relationship between *S. macrospermum* and *S. bicolor* was not known. Several authors have described *S. bicolor* ($2n = 4x = 20$; AAB_1B_1) as an ancient tetraploid; its genomic formula was derived by analyzing meiosis in hybrids with *S. halepense* ($2n = 8x = 40$; $AAAAB_1B_1B_2B_2$) (Hadley, 1953; Celerier, 1958; Tang and Liang, 1988). Meiotic chromosome pairing behavior in interspecific hybrids between *S. bicolor* and *S. macrospermum* revealed that moderate levels of allosyndetic recombination occurred and the genomic formula AAB_1B_1YYZZ was proposed for *S. macrospermum* ($2n = 8x = 40$) (Kuhlman et al., in review). Allosyndetic recombination was observed in subgenomes A and B_1, but the frequency was 2.5 times higher in subgenome A. The authors attempted to produce backcrosses using the interspecific hybrid as a male, but were not successful.

The tertiary gene pool species *S. macrospermum* is now available to plant breeders because hybrids can now be recovered by using specific *S. bicolor* germplasm (*iap iap*). The sorghum and wild species genomes undergo moderate levels of allosyndetic recombination; therefore, recovering introgression in backcross progeny is likely (Kuhlman et al., in review). The remaining obstacle to using this species in an introgression program is determining how to recover backcrosses. The objectives of this research were to produce $2n = 20$ introgression germplasm through backcrossing and to analyze introgression content in backcross progeny molecularly and cytologically.

Materials and Methods

Plant Material

Interspecific hybrids were produced by hand emasculating 'NR481', the *S. bicolor* parent, and pollinating it with the wild species *S. macrospermum* (AusTRC Accession no. 302367). Female plants set approximately 25% hybrid seed, which had shrunken endosperm. Approximately 60% of hybrid seed germinated on agar germination media and were transplanted into soil in small pots in a greenhouse during April, 2005 in College Station, TX. They were transplanted as growth demanded up to a final pot size of 15 gallons. Interspecific hybrids were tall (>4.5m) and photoperiod sensitive (initiating anthesis in September). Backcrosses were made using pollen from both the recurrent parent NR481 and BTx623.

Embryo rescue was necessary to recover backcrosses and was performed 15 to 20 days after pollination. Enlarged ovaries were removed from the florets and surface

sterilized in 30% bleach for 20 minutes. The soft pericarp tissue was removed and the immature embryos were placed in sealed Petri dishes on culture medium containing Murashige-Skoog basal salts and vitamins (Murashige and Skoog, 1962) supplemented with 10mg L-1 glycine, 10mg L-1 L-arginine, 10mg L-1 L-tyrosine, 100mg L-1 inositol, and 50 g L-1 sucrose, solidified with 0.7% plant tissue culture grade agar (Sharma, 1999). The dishes were placed in a growth chamber with 16 h light/8 h dark at 24ºC. Germinated embryos with good root growth and 2-3 leaves were removed from the media and transplanted into a fine textured soil mixture in pots. These were placed in a plastic tray with a clear dome lid inside the growth chamber with wet paper towels to ensure high humidity. As the plants grew, they were hardened off and transferred to the greenhouse.

Germplasm Evaluation

Male gamete viability was estimated by collecting anthers from flowering plants and macerating them in a drop of 1% I_2-KI stain on a glass slide. Slides were analyzed under a light microscope, and the numbers of pollen grains were counted and classified as fully stained, greater than 50% stained, less than 50% stained, and unstained. Plant height was measured in inches from the soil surface to the tip of the mature panicle. Some plants were also characterized for plant color, seed color, presence of awns, mid-rib type, number of days to 50% anthesis, and seed set. Field evaluation of selected BC_2F_1 progeny from family 101 was conducted in Weslaco, TX during the fall, 2006. Plants were self-pollinated and at harvest were evaluated for plant height and seed color.

Specific measure of seed set was not taken even though no plants were identified as sterile. Evaluation of BC_2F_1 progeny from all three families was done in a greenhouse in the winter of 2006 in College Station, TX.

Molecular Marker Evaluation

DNA was extracted from introgression backcross progeny and their parents using the FastDNA Spin Kits (MP Biomedicals, Solon, OH). AFLP templates, using both *EcoRI*/*MseI* and *PstI*/*MseI* restriction enzyme combinations, were created using a modified procedure from Vos et al. (1995). The AFLP template, preamplification, and selective amplification reactions of the *EcoRI*/*MseI* and *PstI*/*MseI* fragments were as described by Klein et al. (2000) and Menz et al. (2002), respectively. Twenty *Pst*/*Mse* and 12 *EcoRI*/*Mse* AFLP primer combinations were used to amplify fragments in the DNA samples. IRD-labeled SSR primers, obtained from LI-COR (LI-COR Inc, Lincoln, NE), were used in amplification reactions as previously described (Klein et al., 1998). Twenty-eight SSR primer combinations were run on the DNA samples, but only 11 (39%) showed transferability by producing a band in the wild species. Amplification products were analyzed on a LI-COR model 4200 dual-dye automated DNA sequencing system. Electrophoresis conditions were as described by Klein et al. (2000). Gels were scored manually; AFLP bands that were present in *S. macrospermum* and absent in the recurrent *S. bicolor* parents were scored as unique. Unique bands that were also shared by the backcross progeny were scored as introgression bands. The percent introgression was calculated by dividing the number of introgression bands a particular backcross

produced by the total number of unique *S. macrospermum* bands. This number is an estimate of the amount of the *S. macrospermum* genome that is present in the backcross progeny. Since backcrosses were produced using the female interspecific hybrid gamete, there is no question as to their authenticity as true backcrosses. Thus, introgression bands can be interpreted as actually representing transfer of *S. macrospermum* DNA into the progeny.

Cytogenetic Evaluation

Somatic chromosome spreads were prepared from root tips using a modified procedure from Andras et al. (1999). Root tips were harvested into a saturated aqueous solution of α-bromonapthalene for 1.75 h at room temperature in the dark. Pretreated root tips were fixed in 95% ethanol/glacial acetic acid (4:1 v/v) for 24 h and stored in 70% ethanol. Root tips were graded based on size standards of 0.0 – 1.0 mm. The terminal 1mm of several same sized root tips were dissected into a 0.5ml epitube, rinsed in water several times, hydrolyzed for 10 min in 0.2*M* HCl, and rinsed 10 min in distilled water. Cell walls were digested by adding 100ul of an aqueous solution of 3% cellulase (Onozika R-10, Yakult Honsha Co. Ltd., Tokyo) and 1% pectolyase Y-23 (Seishin Corp., Tokyo) at pH 4.5 for 1-2 h at 37ºC. Digestion times were based on empirically determined values for a particular size standard. Digestion was stopped by adding 400ul distilled water and centrifuging the cell suspension at 2500rpm (~400G) for 10 min. Using a drawn glass pipette, the supernatant was removed being careful not to disturb the pellet of cells. The cells were washed with water and centrifuged as at 2500rpm for

10 min., twice. After removal of the final wash water, 400ul of methanol/glacial acetic acid (4:1 v/v) was used to wash the cells followed by centrifugation at 2500rpm for 10 min., twice. After the final wash, all but ~50ul of the fixative was removed. The cells were resuspended in the remaining fixative, 2-8ul drops were placed on clean glass slides suspended over wet filter paper and allowed to dry. For chromosome counts, slides were stained with Azure Blue, made permanent with Permount, and analyzed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany). A minimum of four quality spreads of highly condensed chromosomes was used to determine the somatic chromosome number of individual plants.

Fluorescent and Genomic *in situ* hybridization (FISH and GISH) were used to visualize introgression in backcross progeny. Plasmid CEN38 was used as a FISH probe to visually differentiate *S. bicolor* subgenomes A and B_1 (Gomez et al., 1998; Zwick et al., 2000). Genomic *S. macrospermum* and *S. bicolor* DNA were used as GISH probes to detect introgression DNA in the backcrosses and to determine whether the chromosomes were recombinant. Detection of probes followed a modified protocol of Jewell and Islam-Faridi (1994), as described by Hanson et al. (1995) and Kim et al. (2002). Purified probe DNA was nick-translated with digoxigenin-11-dUTP or biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN). Slides with somatic chromosome spreads were prepared as described above. Chromosomes on glass slides were denatured in 70% formamide in 2X SSC for 1.5 min at 70ºC, then dehydrated in 70 (-20ºC), 85 (RT), 95 (RT), and 100% (RT) ethanol, for 2 min each. The hybridization mixture (25ul per slide) contained 50ng labeled probe DNA, 50% formamide, and 10% dextran sulfate

in 2XSSC. The hybridization mixture was denatured for 10 min at 95ºC and chilled on ice. It was then added to the slide, sealed with rubber cement around a glass coverslip, and incubated overnight at 37ºC. Following incubation, the slides were washed at 40ºC in 2XSSC and room temperature in 4XSSC plus 0.2% Tween-20, for 5 min each. Slides were blocked with 5% (w/v) BSA in 4XSSC plus 0.2% Tween-20 at room temperature. The digoxigenin and biotin-labeled probes were detected with CY3™-conjugated anti-digoxigenin anti-body and fluorescein isothiocyanate (FITC)-conjugated streptavidin, respectively. Slides were washed in 37ºC 4XSSC plus 0.2% Tween-20. Chromosomes were counterstained with 25ul DAPI with Vectashield® (Vector Laboratories, Burlingame, CA). Slides were viewed through an Olympus AX-70 epifluorescence microscope and images captured with a Macprobe® v4.2.3 imaging system (Applied Imaging Corp., Santa Clara, CA).

Results and Discussion

Breeding Methodology, Cytology, and Germplasm Phenotypic Evaluation

Twenty interspecific hybrids were produced and their identity was confirmed by morphology and chromosome number ($2n = 30$). At maturity, the hybrids flowered but the anthers were non-dehiscent. Normal I_2-KI staining pollen grains were rare and F_2 seed did not develop on 15 selfed panicles (approximately 3,000 florets). Previous attempts to recover backcross progeny using the male hybrid gamete were difficult and inconclusive (Kuhlman et al., in review). Interspecific hybrid panicles were pollinated with *S. bicolor* pollen, mostly from NR481 but a few also with BTx623. Backcross seed

development was rare; a single seed with well developed endosperm was observed but it was not viable. Thus, embryo rescue was used to recover backcross progeny. In total, 7009 florets were pollinated and dissected revealing 86 (1.2%) had embryo development of which 15 (0.2%) survived into adult BC_1F_1 plants (Figure 2).

All BC_1F_1s had awns and red plant color but varied in their height and vigor. BC_1F_1 108 was very short, extremely weak, and died shortly after producing a single panicle, while most of the BC_1F_1s showed moderately strong growth, produced multiple tillers, and were tall – some growing up to 5 meters at maturity (Table 4). BC_1F_1 plants had low male fertility with non-dehiscent anthers and non-viable pollen.

BC_1F_1 plants were backcrossed using NR481 pollen and embryo rescue was not needed as three BC_1F_1 plants (101, 102, and 107) set viable backcross seed (Table 4). Two other plants, 105 and 115, produced a single backcross seed that was not viable (Table 4).

BC_1F_1 101 was morphologically distinct from the others; it had wider leaves, larger florets, and features reminiscent of BTx623. It was rescued from a panicle that had been pollinated with a mixture of NR481 and BTx623 pollen. DNA was extracted from leaf tissue and SSR markers used to determine the parentage of the three seed producing BC_1F_1 plants. Multiple SSR and AFLP markers confirmed that BC_1F_1 101 arose from fertilization by BTx623 of the interspecific hybrid, instead of NR481. Evidence indicated that BC_1F_1 102 and 107 resulted from fertilization by the recurrent parent, NR481. Both BC_1F_1s produced solely with the recurrent parent had significantly less backcross seed set than did the BC_1F_1 with a mixed pedigree (Table 4). The

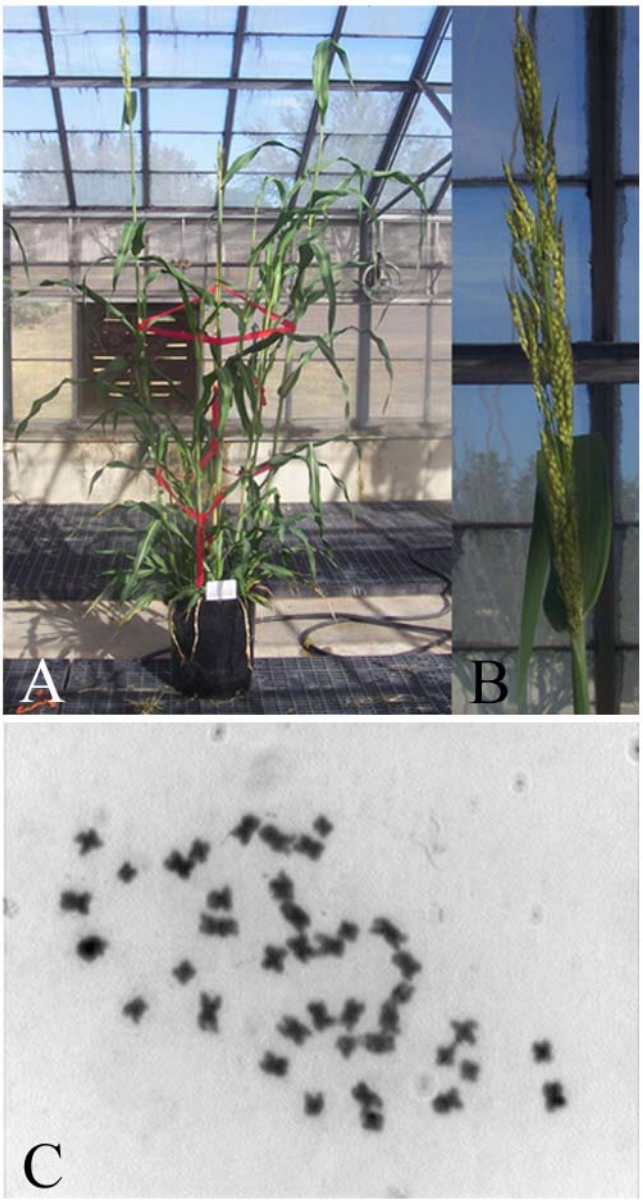

Figure 2. Interspecific BC_1F_1 generation with pedigree: (*S. bicolor* x *S. macrospermum*) x *S. bicolor*. (A) Vigorous growth of adult BC_1F_1 101 with (B) large panicle at maturity. (C) Somatic chromosome spread of BC_1F_1 106 with $2n = 38$ chromosomes.

Table 4. Chromosome number and phenotypic data of BC_1F_1 individuals ((*S. bicolor* x *S. macrospermum*) x *S. bicolor*) recovered using embryo rescue

BC_1F_1	(2*n*)	Ht†	Pl	Sd	Aw	Total Seed	Seed Set (%)
101	37	244	R	R	Y	126	2.99[A]
102	36	305	R	R	Y	28	1.65[B]
103	70	244	R	-	Y	0	0
104	60	198	R	-	Y	0	0
105	39	457	R	R	Y	1	0.06
106	38	305	R	-	Y	0	0
107	38	366	R	R	Y	36	1.94[B]
108		61	R	-	Y	0	0
109	38	366	R	-	Y	0	0
110	39	366	R	-	Y	0	0
111		183	R	-	Y	0	0
112	36	305	R	-	Y	0	0
113	38	274	R	-	Y	0	0
114	35	198	R	-	Y	0	0
115		183	R	R	Y	1	0.36

†Ht, Pl, Sd, Aw are height (cm), plant color, seed color, and awns respectively. Seed set is after pollination by *S. bicolor*.

Seed set percentages followed by different letters are significantly different ($p<.05$)

increased seed set in BC_1F_1 101 may be due to increased heterozygosity resulting from its mixed pedigree.

Chromosome numbers of the BC_1F_1 plants ranged from 35 to 70 (Table 4; Figure 2). Such high chromosome numbers resulted from irregular meiosis in the interspecific hybrid (Kuhlman et al., in review). BC_1F_1 plants with chromosome numbers between 35 and 39 likely resulted from the transmission of 25-29 chromosomes through the female gamete and 10 chromosomes through the *S. bicolor* gamete. Transmission of 25-29 chromosomes from plants with $2n = 30$ is best explained by the formation of a restitution nucleus composed of the univalents during meiosis. Under this hypothesis, some chromosomes would pair at meiosis, and those undergoing recombination would form bivalents at metaphase I and subsequently separate and move to the spindle poles. The remaining chromosomes would form univalents, some of which might distribute themselves to the poles via spindle attachment, while others would remain at the metaphase I plate and other intermediate positions. In cells with a pole-to-pole distribution of univalents, a restitution nucleus would sometimes form between the two poles, and the product would contain all or most chromosomes. Meiosis II typically conserves chromosome numbers of meiosis I products, so variable chromosome numbers among restitution and partial-restitution products from meiosis I would translate to megagametophytes with various chromosome numbers. Restitution nuclei have been implicated in transmission of univalents in multiple species (Singh, 2003). The two plants with $2n = 60$ and 70 chromosomes may have been produced due to meiotic irregularities (Singh, 2003) resulting in tetraploid ($2n = 60$) female gametes.

Parthenogenesis of such a “4n” egg would result in 2*n* = 60 progeny or fertilization of such an egg would result in 2*n* = 70 progeny. BC_1F_1 104 (2*n* = 12*x* = 60), is hypothesized to be a naturally produced allododecaploid. It displayed slow growth and very stiff leaves, but unfortunately it did not show any fertility and backcrosses were not recovered.

Three BC_2F_1 families consisting of 45 seed from the three partially fertile BC_1F_1s (101, 102, 107) were planted and evaluated. Pollen samples were taken from plants of each family and scored for pollen stainability. All three BC_2 families had significantly lower mean pollen stainability than NR481. Family 101 had higher pollen stainability than 102 and 107, which were not different (Table 5). BC_2F_1 families 102 and 107 displayed significantly lower seed set (1.3% and 1.4%) than family 101 and NR481 (87% and 94%), which were not different (Table 5). The vastly lower seed set from families 102 and 107 made obtaining selfed seed difficult and limited the evaluation of the BC_2F_2 generation. Lower pollen stainability and high sterility for two BC_2 families may be a manifestation of *S. macrospermum* introgression. These two families have only NR481 in their pedigree and are different from the recurrent parent; thus, introgression is a likely candidate to cause the altered phenotype. Another explanation could be that genetic or epigenetic DNA changes in the recurrent parent genome which resulted from interspecific hybridization, are responsible for the sterility and not introgressed DNA from *S. macrospermum*. Separating these hypotheses is impossible with the present data, regardless the phenotypic change resulted from the interspecific hybridization.

Table 5. Phenotypic data and *S. macrospermum* introgression estimates of BC_2F_1 individuals ((*S. bicolor* x *S. macrospermum*) x *S. bicolor*) and the recurrent parent. Phenotypic data for BC_2F_2 progeny are given for some individuals

		Individual BC_2F_1 Plant Data										BC_2F_2 Progeny Data			
BC_1 Family	BC_2F_1	2*n*	Dy†	Pl	Sd	Aw	Ht	Mr	% INT‡	% PS	% SS	Ht§	Aw	Sd	Mean % SS¶
101	201	20	62	R	R	Y	102	D	0.38	62.6	95.0	S	-	R	-
	202	20	57	R	R	N	193	J	0.00	-	95.0	SEG	-	R	-
	203	20	55	R	R	N	183	D	0.57	63.0	73.0	SEG	-	SEG	-
	204	-	55	R	R	Y	180	D	0.00	70.4	95.0	SEG	-	SEG	-
	205	-	-	R	R	N	196	D	0.19	72.7	80.0	T	-	R	-
	206	20	-	R	R	N	168	D	1.72	40.4	56.0	T	-	R	-
	207	20	56	R	R	N	175	D	0.00	55.8	95.0	T	-	SEG	-
	208	-	55	R	R	N	157	D	0.00	-	95.0	SEG	-	SEG	-
	209	20	-	R	R	Y	168	D	18.56	-	72.0	T	-	SEG	-
	210	-	56	R	R	N	124	D	0.19	-	95.0	SEG	-	SEG	-
	211	20	58	R	R	Y	180	D	0.19	-	95.0	T	-	SEG	-
	212	20	43	R	R	N	160	J	0.19	56.8	95.0				
	213	20	41	R	R	N	224	D	0.00	-	88.0				
	214	20	41	R	R	Y	206	D	0.00	-	95.0				
	215	20	39	R	R	Y	201	D	0.00	-	75.0				
	216	-	48	R	R	N	211	D	0.39	-	95.0				
	217	-	40	R	W	N	165	D	0.00	-	95.0	SEG	SEG	W	57
	218	-	43	R	R	N	163	D	0.00	57.1	84.0				
	219	-	41	R	W	Y	224	D	0.00	-	95.0	SEG	Y	W	52
	220	20	39	R	W	Y	198	D	0.00	-	82.0	T	Y	W	63
	221	20	39	R	R	Y	193	D	0.19	-	95.0				
	222	21	40	R	R	N	206	D	3.66	-	85.0				
	223	20	40	R	R	N	135	D	0.19	-	95.0				
	224	-	41	R	R	N	241	D	0.19	-	78.0				
	225	-	45	R	R	N	249	D	0.19	49.4	82.0				
	Mean		**47**	**R**			**183**		**1.07**	**58.7**	**87.4**				**>50**
102	226	-	41	R	R	Y	234	D	1.14	-	0.1	T	Y	-	0
	227	-	44	R	-	Y	188	D	1.17	17.9	0.0				

Table 5 Continued

BC_1 Family	BC_2F_1	Individual BC_2F_1 Plant Data										BC_2F_2 Progeny Data			
		2*n*	Dy†	Pl	Sd	Aw	Ht	Mr	% INT‡	% PS	% SS	Ht§	Aw	Sd	Mean % SS¶
	228	20	41	R	R	Y	201	D	1.14	15.2	2.1	T	Y	-	0
	229	-	43	R	R	N	178	D	0.57	-	0.6	T	Y	-	0
	230	-	45	R	R	Y	224	D	0.38	-	0.1	T	Y	-	0
	231	-	43	R	R	Y	229	D	0.95	51.5	1.5	T	Y	-	0
	232	-	42	R	R	N	226	D	0.76	11.5	4.5	T	Y	-	0
	233	-	42	R	R	N	173	D	0.76	4.0	0.1				
	234	-	44	R	R	Y	211	D	1.14	22.1	3.0	T	Y	-	0
	235	20	45	R	R	Y	224	D	0.97	10.0	1.3	T	Y	-	0
	247	-	43	R	R	N	170	D	0.76	-	1.0	T	Y	-	0
	Mean		**43**	**R**	**R**		**206**	**D**	**0.88**	**18.9**	**1.3**				**0**
107	237	-	44	R	R	Y	221	D	0.38	-	0.1	T	Y	-	0
	238	-	44	R	R	N	203	D	1.16	41.6	5.5	T	SEG	-	0
	239	-	43	R	R	Y	170	D	0.76	13.4	1.3	T	Y	-	0
	240	-	43	R	R	N	203	D	0.58	35.1	3.4	T	SEG	-	0
	241	-	46	R	R	N	218	D	0.95	-	0.3	T	SEG	-	0
	242	20	45	R	-	N	216	D	0.76	-	0.0				
	243	-	44	R	R	Y	196	D	0.77	8.6	0.5	T	Y	-	0
	244	20	43	R	R	N	216	D	0.57	0.0	0.1	T	Y	-	0
	Mean		**44**	**R**	**R**		**191**	**D**	**0.74**	**19.7**	**1.4**				
NR481	**Mean**	**20**	**57**	**R**	**R**	**Y**	**206**	**D**	**0.00**	**88.3**	**94.2**				
	LSD(.05)		6.1				36.6		2.68	15.8	8.4				
	ANOVA#		**				NS		NS	**	**				

† Dy, Pl, Sd, Aw, Ht, Mr, PS, SS are days to flowering, plant color, seed color, awns, height (cm), midrib, pollen stainability, and seed set respectively
‡ % INT is introgression, the percent of the *S. macrospermum* genome detected via AFLP markers in the respective plant
§ HT in the BC_2F_2 generation potentially segregated for dwarfing genes, S is short, T is tall, and SEG is segregating
¶ Seed set was not measured for BC_2F_2 progeny from plants 201-211 as these were field evaluated in Weslaco, TX, however seed was harvested from each plant and no sterile plants were found. All other BC_2F_2 evaluation was carried out in the greenhouse.
# Analysis of variance between mean values for families and check, not individuals

Chromosome numbers for plants within family 101 were $2n = 20$ for 14 of the 15 plants analyzed and one plant had 21 chromosomes. Two plants each from families 102 and 107 also had $2n = 20$ chromosomes (Table 5). BC_2F_1 progeny ($2n = 20$) were produced without embryo rescue from parents that had 36, 37, and 38 chromosomes. Whereas the restitution nucleus conferred survivability to the rescued BC_1F_1 embryos, it appears that it was selected against when embryos were not rescued and seeds were produced. Of those surveyed, 95% of BC_2F_1 plants had 20 chromosomes.

All BC_2 individuals were tall, had red plant and seed color, and a dry midrib similar to the recurrent *S. bicolor* parent (NR481), except the BC_2s in family 101 in which three individuals had white seed color, two individuals had juicy midribs, and one was short (102cm) (Table 5). These traits are recessively inherited and should not be expressed in a population of BC_2F_1 individuals whose pollen parent (NR481) is tall, red seeded, has a dry midrib, and has not segregated for these traits. Pollen contamination from a different genotype was impossible because other genotypes were not grown in the greenhouse during that time. These phenotypic classes are shared by BTx623, which as discussed earlier was the pollen parent of this specific BC_1F_1; therefore, self-pollination could explain the recessive phenotypes. Alternatively, it is possible that *S. macrospermum* introgression is causing the unexpected phenotypes. The wild species has red seed color, dry midrib, and tall plant height, but genetic control of these traits is not necessarily the same between the species. *Sorghum macrospermum* introgression acting in a dominant manner could explain the phenotypes in the BC_2F_1s, but the traits would be expected to segregate in selfed progeny, which did not occur (Table 5). If,

however, some backcross progeny actually resulted from self pollination (BC_1F_2), then it is possible that homozygous introgression is causing the phenotypes. Unfortunately, with the available data this possibility cannot be separated from normal segregation of BTx623 alleles that could also cause the phenotypes in selfed progeny. It had been noted that their female parent, BC_1F_1 101, occasionally set seed on panicles that had not been directly pollinated with NR481 pollen. It was initially thought that these resulted from open pollination from adjacent NR481 plants, but given the phenotypes, this is not possible. Further complicating the matter, fertile pollen was never observed for any BC_1F_1 plants which makes self-pollination unlikely. Thus, it is unknown whether these "BC_1F_2" individuals were produced from self-pollination or were the result of some form of asexual reproduction. Asexual reproduction could operate in the absence of functional sexual reproduction. Schertz and Stephens (1965) observed that diploid and triploid plants were produced 14 days after hot-water emasculation from non-pollinated panicles. They concluded that in the absence of pollination there is an occasional doubling of an egg cell that parthenogenically resulted in diploid seeds, in effect natural doubled haploids. If "BC_1F_2" plants were in fact naturally produced doubled haploids, they would be expected to be 100% homozygous. Segregation was observed in selfed progeny of 9 of 13 analyzed BC_2F_1 individuals (Table 5), making this possibility highly unlikely.

Diploid gametes (n = 20) could alternatively be produced via failed cytokinesis of the dyads during the second stage of meiosis (Singh, 2003). As an example, a PMC possessing 36 chromosomes with 10 II and 16 I at metaphase I could produce two dyad

cells with 10 and 26 chromosomes, assuming the univalents segregated as a restitution nucleus. If cytokinesis failed during meiosis II, the sister chromatids would separate, and following megagametogenesis form an egg cell with 20 chromosomes. If this cell developed into an embryo parthenogenically, it would not necessarily be 100% homozygous since the chromosomes underwent recombination during meiosis I resulting in the sister chromatids being genetically different. This $2n = 20$ progeny plant could not be differentiated from a selfed plant, and since fertile pollen was very rarely observed, this forms the best hypothesis for reproduction matching the present data. This hypothesis indicates that BC_2F_1 progeny produced from BC_1F_1 101 are a mix of possible pedigrees: backcross derived BC_2F_1s; possibly selfed BC_1F_2s; and very likely parthenogenic diploid progeny. As separation of all individuals into these classes is not possible, this generation will still be referred to as BC_2F_1.

BC_2F_2 progeny were evaluated for visual expressions of introgression in both the field and greenhouse. Overall, BC_2F_2 progeny deriving from family 101 had adequate seed set and segregated for traits polymorphic between BTx623 and NR481, such as seed color and plant height. This significant variability in the population made identifying phenotypic evidence of introgression virtually impossible. No conclusive phenotypic signs of introgression were evident in this family. BC_2F_2 plants in families 102 and 107 showed one obvious sign of introgression: male-sterility. Female fertility was unaffected as backcross seed set was normal. Partial male sterility in the BC_2F_1 plants in these families was hypothesized to be caused by *S. macrospermum* introgression acting in a dominant manner, and the plants were presumed to be

heterozygous for any introgression. BC_2F_2 plants were expected to segregate for male-sterility, but lack of segregation suggests that the BC_2F_1 plants were homozygous for such introgression (Table 5). Again, this is not possible if the plants were actually the result of backcrossing. But, as seen in family 101, some form of reproduction, likely asexual, is occurring other than backcrossing. Selfing again seems unlikely since stainable pollen was rarely seen, but F_2 progeny from 3 of the 16 BC_2F_1s segregated for awns indicating they were not 100% homozygous. These progeny could not be naturally produced doubled haploids, but very well could have been produced parthenogenically as explained for family 101 above. Given their apparent homozygosity for introgression, all BC_2F_1 plants in families 102 and 107 were likely produced exclusively asexually.

Molecular Marker Analysis of Introgression

The amount of the *S. macrospermum* genome that was introgressed into the BC_2 generation was evaluated using AFLP markers. In total, 32 primer combinations produced 528 AFLP markers unique to *S. macrospermum*. The total amount of the *S.*

macrospermum genome detected in the BC_2F_1 generation was 26% (138 of 528 unique *S. macrospermum* markers). Most introgression bands (82%) were found in single individuals, while 5% were shared by between 6 and 14 BC_2F_1s. Each family possessed three types of introgression: unique to that family; shared between two families; and shared by all three families (Figure 3). Estimates for introgression on an individual basis ranged widely from 0-18.6% (Table 5), although the amount of introgression did not significantly differ on a family mean basis (0.75% - 1.07%).

Eleven of the BC_2F_1s from family 101 (44%) did not have detectable levels of introgression, while two had the highest levels (3.7% and 18.6%). The total amount of introgression detected within family 101 was high (22.9%), although most derived from the two outstanding individuals. Introgression was detected in all BC_2F_1 individuals within families 102 and 107, but the range was narrow, from 0.38%-1.17% (Table 5). The total amount of introgression detected in families 102 and 107 were 3.4% and 1.5%, respectively. A majority of the introgression markers detected in families 102 and 107

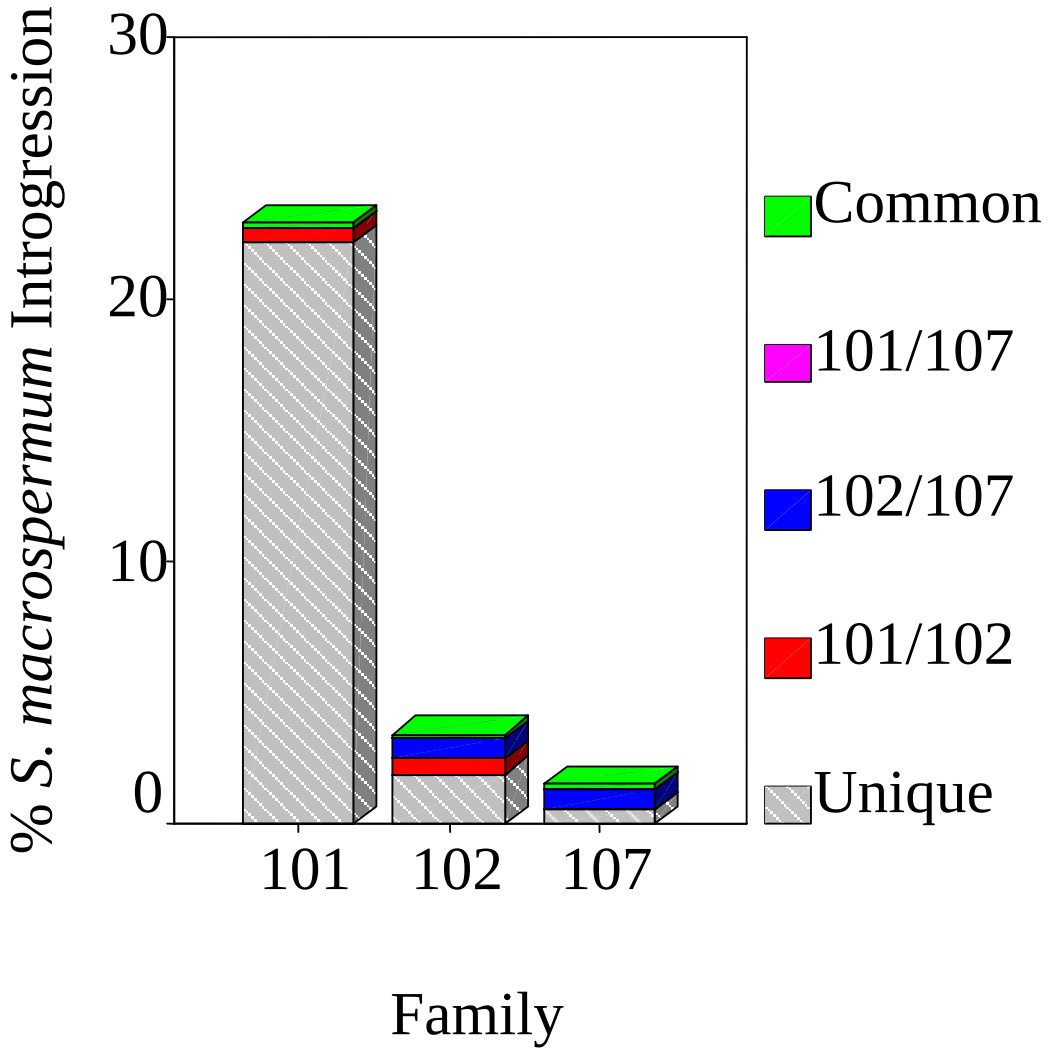

Figure 3. Graph depicting *S. macrospermum* introgression, as detected using AFLP markers, of BC_2F_1 individuals summed by family. Stacked bars represent introgression that is unique to a family, shared by two families, or common to all three families.

(9/16 and 7/8, respectively) were present in multiple (4 to 6) individuals within the family, indicating that common introgression sequences were inherited. Thus, inheritance of introgression in these two families does not appear to be random. This data in combination with the phenotypic male-sterility that is expressed by all individuals in these two families indicates there was selection of gametes carrying a common block of introgression. In contrast, almost half of individuals within family 101 had no detectable introgression and few markers were present in multiple family members (4/117, excluding individuals 206, 209, and 222). Common introgression was found between the three excluded individuals, but overall introgression in the family appeared random.

The two individuals that were distinctly different from the rest were BC_2F_1s 209 and 222, both of which were from family 101 and had 18.6% and 3.7% of the *S. macrospermum* genome detected within their DNA. Selected SSR markers were run on these DNA samples to confirm introgression. Two different SSRs confirmed independent introgression of *S. macrospermum* DNA in these plants. Txp482 confirmed introgression in BC_2F_1 209 but was absent in BC_2F_1 222, while the opposite confirmation occurred with Txp523. Txp482 and Txp523 are located on SBI-01 of the genetic map by Menz et al. (2002) at approximately 31cM and 28cM, respectively (http://sorgblast3.tamu.edu). SSR markers surrounding these two locations showed that introgression had not occurred in both plants. This indicates that if the introgressed SSR sequences are on SBI-01, they are part of a small introgression segment. Alternatively, the *S. macrospermum* SSR sequence may not have been homoeologous to SBI-01, and

thus be on another *S. bicolor* chromosome, or it was not introgressed into the *S. bicolor* genome at all and be located on a whole *S. macrospermum* addition chromosome.

Molecular Cytogenetic Analysis

GISH was used on somatic chromosome spreads to visualize *S. macrospermum* introgression. Recombinant chromosomes were found in both the BC_1 and BC_2 generations. BC_1F_1 109 ($2n = 38$) is clearly composed of 20 *S. bicolor* chromosomes and 18 *S. macrospermum* chromosomes and shows a single chromosome with evidence of recombination. The unique morphology of the recombinant chromosome, the only chromosome to contain a satellite region (Kim et al., 2005b), identifies the introgression to be on the short arm of SBI-01. This BC_1 was not fertile, but it does show recombinant chromosomes present in the BC_1 generation.

Multiple types of *S. macrospermum* introgression were found in the BC_2 generation. BC_2F_1 209 (18.6% introgression) ($2n = 20$) visibly shows two *S. macrospermum* chromosomes and 18 *S. bicolor* chromosomes in its genome (Figure 4, A). Visualization of the *S. bicolor* genome reveals that the *S. macrospermum* chromosomes are non recombinant (Figure 4, B). The *S. bicolor* chromosomes, evidenced by the CEN38 probe, are 10 from the A subgenome and 8 from the B_1 subgenome. This plant is an alien substitution line; two B_1 *S. bicolor* chromosomes have been replaced with two *S. macrospermum* chromosomes. The introgression detected by molecular markers, including Txp482, is primarily located on two *S. macrospermum* alien substitution chromosomes. The cytogenetic evidence cannot disprove the existence

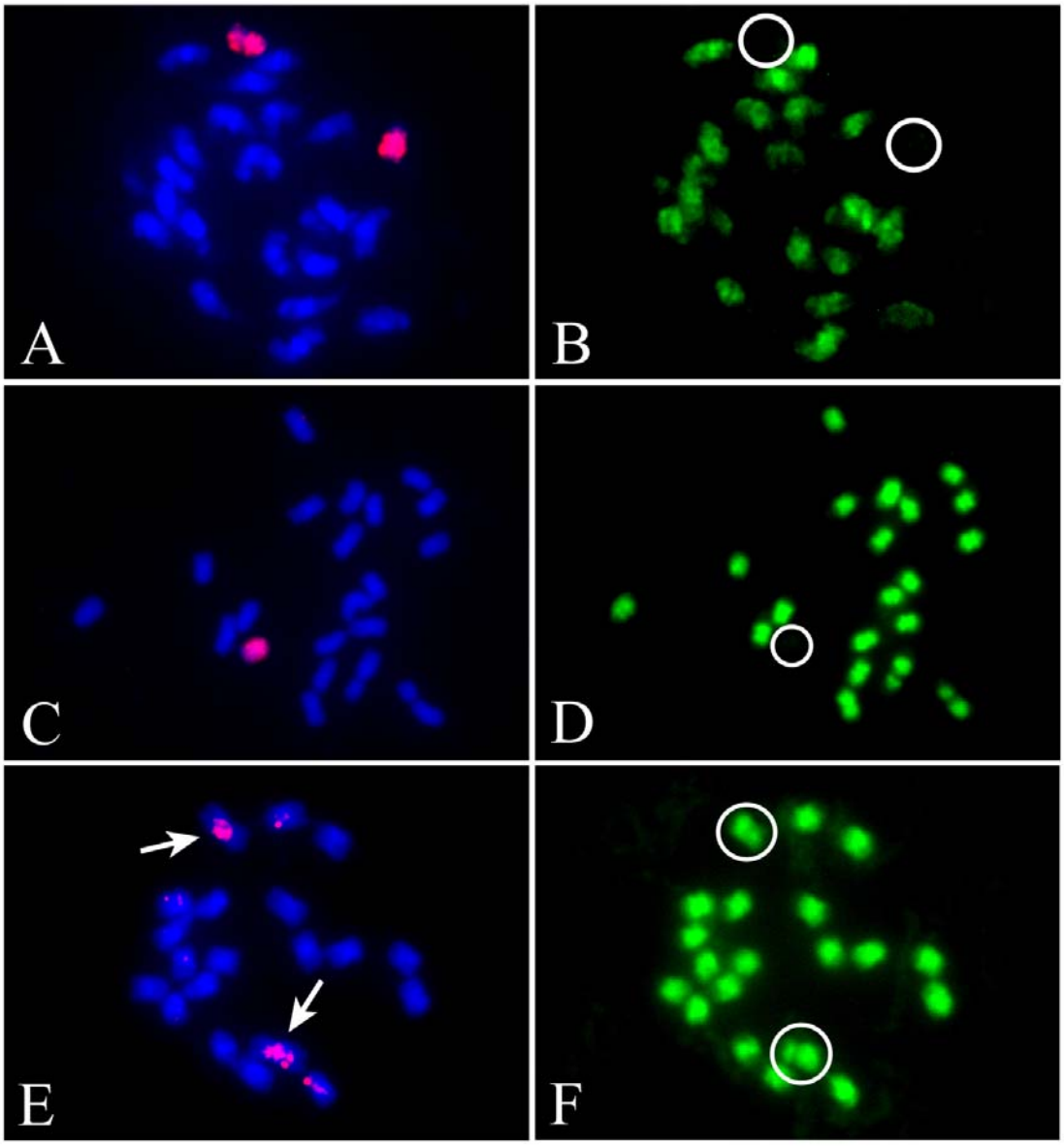

Figure 4. Genomic *in situ* hybridization of somatic chromosome spreads from introgression BC_2F_1 generation. (A, C, E) Chromosomes hybridized with *S. macrospermum* GISH probe (red) and stained with DAPI (blue). (B, D, F) Chromosomes hybridized with *S. bicolor* GISH probe (green). (A) BC_2F_1 209 ($2n = 20$) showing two chromosomes with significant *S. macrospermum* hybridization (red), (B) lack of *S. bicolor* hybridization (circles) indicates they are non recombinant whole *S. macrospermum* chromosomes. (C) BC_2F_1 222 ($2n = 21$) showing one chromosome with significant *S. macrospermum* hybridization (red), (D) lack of *S. bicolor* hybridization (circle) indicates it is a non recombinant whole *S. macrospermum* chromosome. (E) BC_2F_1 244 ($2n = 20$) showing two chromosomes with *S. macrospermum* hybridization sites (arrows) which also show (F) *S. bicolor* hybridization (circles) indicating these are recombinant chromosomes with *S. macrospermum* introgression.

of small introgression blocks within the *S. bicolor* genome. This type of introgression has been used extensively in wheat breeding where alien substitution is well tolerated by the genome (Jiang et al., 1994; Jones et al., 1995; Jauhar and Chibbar, 1999). Seed set was slightly lower than the check but still reasonably high (72%). Morphologically this plant appeared to be in the range of that for segregation between BTx623 and NR481; therefore, no phenotypic trait can presently be assigned to the alien chromosomes, although analysis of progeny likely will. It is surprising that the plant tolerates this level of alien substitution as *S. bicolor* trisomic lines have been recovered (Schertz, 1966) but monosomic lines have not, presumably due to their lethality. This indicates that homoeologous chromosomes from the *S. macrospermum* genome sufficiently compensate for the missing *S. bicolor* chromosomes which results in relatively normal plant function.

GISH using *S. macrospermum* DNA as probe revealed that BC_2F_1 222 (3.7% introgression) ($2n = 21$) had one whole *S. macrospermum* chromosome along with 20 *S. bicolor* chromosomes (Figure 4, C). GISH using *S. bicolor* as probe showed that the *S. macrospermum* chromosome is non recombinant (Figure 4, D). This plant is an alien addition line containing a single *S. macrospermum* chromosome along with the full *S. bicolor* genome. The introgression in this plant as detected using molecular markers is most likely all located on a single *S. macrospermum* alien addition chromosome; however, the presence of small introgression blocks cannot be disproven. Txp523, which detected introgression in this plant, most likely is homoeologous to a sequence on the *S. macrospermum* chromosome. This plant displays no deleterious effects of the

introgression in that seed set was high (85%) and the plant was vigorous. One potential phenotype influenced by introgression was the presence of normal and shriveled endosperm seeds produced by selfing. The approximate ratio of normal to shriveled seed was not different from a 3:1 ratio ($X^2 = 1.12^{ns}$). This would be consistent with reduced seed size for progeny inheriting two copies of the alien chromosome. This presumes, however, that normal segregation of an alien chromosome occurs through both gametes. The fitness of gametes carrying an extra chromosome is normally reduced; thus, the transmission rate of an alien chromosome would likely also be low. It is possible that this phenotype is controlled by the transmission of an alien chromosome, but this hypothesis needs cytological verification.

SSR markers Txp482 and Txp523 were detected in BC_2F_1s 209 and 222, respectively, but neither marker was present in both plants. This indicates that the alien addition chromosome in 222 is different from both substitution chromosomes in 209. AFLP data is consistent with this hypothesis because only three introgression markers are shared out of 98 present in BC_2F_1 209 and 19 present in 222. Both SSR markers map to chromosome 1 in the *S. bicolor* genome, which may indicate that the two detected *S. macrospermum* chromosomes are both homoeologous to SBI-01, perhaps the related chromosomes from subgenomes A_m and B_{1m} (Kuhlman et al., in review). The introgression estimate for 209 is much higher than 222. Introgression estimates were based on AFLP markers which are mostly dominant; therefore, being homozygous for an introgression marker does not increase the introgression estimate. Thus, it would be unlikely for BC_2F_1 209 to contain two homologous *S. macrospermum* substitution

chromosomes and still have a five fold increase in estimated introgression. Neither *S. bicolor* nor *S. macrospermum* karyotypes show that broad of range for chromosome size; therefore, inheritance of larger homologous chromosomes does not explain the increased introgression (Wu, 1990; Kim et al., 2005a). BC_2F_1 209 most likely contains two different *S. macrospermum* substitution chromosomes, both of which are different from the addition chromosome in BC_2F_1 222.

GISH using *S. macrospermum* DNA as probe revealed BC_2F_1s 228 and 244 ($2n$ = 20, 20; 1.1% and 0.57% introgression, respectively) both contain two chromosomes with *S. macrospermum* introgression. The introgression chromosomes also show hybridization with the *S. bicolor* probe (Fig. 3, F) and strong hybridization with CEN38; therefore, they are members of the A subgenome. Using morphology to identify somatic chromosomes, the introgression sites appear to be located on SBI-01 homologous chromosomes. These two plants are examples of introgression backcrosses, and they contain *S. macrospermum* DNA introgressed into the *S. bicolor* genome. These two plants show phenotypic evidence of introgression like all members of their respective families (102 and 107). Individuals 228 and 244 had low selfed seed set (2.1% and 0.1%, respectively) and all their BC_2F_2 progeny were completely male-sterile. Backcross seed set was normal. This strongly supports the hypothesis that these plants, and possibly all plants in these families, are homozygous for the introgression that they contain.

Sixty-six percent of the AFLP introgression bands in BC_2F_1 244 are common to BC_2F_1 228. In fact, 17 of 19 BC_2F_1 plants from families 102 and 107 share some

common introgression with BC_2F_1 244. A portion of the introgression block present in BC_2F_1 244 seems to have been preferentially transmitted to most progeny deriving from BC_1F_1s 102 and 107. None of the 25 BC_2F_1 progeny from BC_1F_1 101 share any of the introgression block found in BC_2F_1 244. This molecular evidence along with the suggestion that both 228 and 244 have introgression blocks on homologous SBI-01 chromosomes strongly supports the hypothesis that inheritance of this introgression block was not random. It appears that strong selection was operating to transmit portions of this introgression block to apparently all BC_2F_1 progeny in these two families.

BC_2F_1 206 ($2n = 20$; 1.72% introgression) contains common introgression with BC_2F_1 209. Seven of its 9 introgression AFLP markers are also detected in BC_2F_1 209. Although not analyzed with GISH, this individual likely contains a recombinant introgression block homologous to a portion of one of the alien substitution chromosomes present in 209.

Summary

Introgression breeding utilizing the tertiary gene pool species *S. macrospermum* has resulted in the recovery of $2n = 20$ chromosome backcrosses that contain wild species introgression. BC_1F_1s were successfully recovered using the female hybrid gamete in combination with embryo rescue. Chromosome numbers were high and sterility was a problem; however, viable BC_2F_1 seed was set from backcrossing on 20% of the BC_1 plants. It is unclear what proportion of the BC_2F_1 individuals were produced

through sexual backcrossing versus parthenogenesis of 20 chromosome egg cells, but both likely occurred.

Molecular markers verified that BC_2F_1 individuals contained *S. macrospermum* introgression and measurements were between 0 and 18.6%. Molecular cytogenetic techniques, FISH and GISH, revealed that the introgression in the BC_2F_1 plants was of three types: alien substitution; alien addition; and alien introgression lines. Male-sterility was the only obvious phenotypic trait observed that is likely caused by the introgression DNA.

Family differences were apparent in this germplasm. BC_1F_1 101 and its BC_2 progeny showed the highest levels of fertility compared with families 102 and 107. BC_2s from this family were the only examples of alien substitution and addition lines observed. It is unknown whether the mixed pedigree of BC_1F_1 101 is the cause of the increased fertility but it is reasonable to hypothesize. The family may have possessed a mix of alleles that facilitated recovery of alien addition and substitution lines as well as buffered the deleterious effects of recovered introgression. Such a hypothesis would suggest that using a complex highly heterozygous population in introgression breeding may maximize the amount of recovered introgression as well as reduce the associated fertility problems.

The germplasm produced from this investigation opens up a vast number of potential future research projects not limited to the following suggestions. Characterize the phenotypic effect of the alien substitution and addition chromosomes in various genetic backgrounds. In crosses with *S. bicolor* trisomic or monosomic lines, attempt to

induce homoeologous recombination with the alien addition and substitution chromosomes. Analyze BC_3F_1 and BC_3F_2 progeny of alien introgression lines from families 102 and 107 to determine if the sterility was caused by homozygous introgression blocks. Make field selections of short agronomically acceptable BC_2F_2 progeny from family 101 and characterize for phenotypic effects.

The true value of using *S. macrospermum* in an introgression breeding program will be known only if valuable introgression is characterized. One severe limitation of this research was that the *S. bicolor* recurrent parent used, acc. NR481, is very poor agronomically, and thus field evaluation of introgression is difficult. If valuable introgression is found, it would need to be moved into a more acceptable agronomic background before being used in a breeding program. One immediate need is for the *iap iap* genotype to be backcrossed into an elite germplasm line that can serve as the recurrent parent in future introgression projects.

The current research shows that the wild species *S. macrospermum* is now available to plant breeders and researchers for the improvement of cultivated sorghum. Using this research as a starting point, the true value of *S. macrospermum* genetic diversity can be determined.

CHAPTER V

EFFECTS AND GENETIC MAP POSITION OF THE *IAP* LOCUS

Introduction

Sorghum is an important food and feed crop with worldwide grain production in 2005 of 56,957,314 metric tons which ranks fifth among cereal grains (FAOSTAT data, 2006). The U.S. accounted for approximately 10,000,000 metric tons of that crop which was valued at $715,000,000 (USDA 2006). Plant breeders continually make progress in improving the crop for traits like yield potential, disease and insect resistance, as well as abiotic stresses. Their efforts are ultimately limited by the amount of genetic variation available to them for the desired trait. Identifying valuable new sources of germplasm is a key component to supplying plant breeders with the genetic variation they need.

Plant breeders have mainly used germplasm within the primary gene pool to improve the crop (Duncan et al., 1991). There have been limited efforts to utilize the secondary gene pool, which includes *S. halepense* and *S. propinquum* (Wooten, 2001; Cox et al., 2002; Dweikat, 2005). Until recently, hybridization between sorghum and the 19 wild species in the tertiary gene pool was impossible. One reason for reproductive isolation was pollen-pistil incompatibilities between the species, wherein wild species pollen tube growth was inhibited in the *S. bicolor* stigma resulting in failure of fertilization of the gametes (Hodnett et al., 2005). By utilizing germplasm homozygous for the *iap* allele (Laurie and Bennett, 1989), incompatibilities were

reduced thus allowing for the production of interspecific hybrids between sorghum and species in the tertiary gene pool (Price et al., 2006).

The *Iap* gene was identified while attempting to produce sorghum x maize hybrids (Laurie and Bennett, 1989). The authors discovered a single accession, NR481, which allowed maize pollen tubes to grow through the stigmas and style of sorghum into its ovary. Based on Hogenboom's (1973) theory of incongruity, the *Iap* gene likely controls a barrier mechanism operating in the pistil for which divergent species, like maize and wild sorghums, lack corresponding penetration genes. The manifestation of the *Iap* barrier is inhibited pollen tube growth for pollen which does not contain penetration genes. Thus, their pollen is unable to overcome the *S. bicolor* pistil barrier resulting in failed fertilization. *Sorghum bicolor* does posses the corresponding penetration genes resulting in its pollen being completely functional on its pistils. Under this hypothesis, the *iap* allele is a mutant nonfunctional allele which when homozygous results in the failed operation of the pistil barrier. Pollen of wild species does not require corresponding penetration genes for normal pollen tube growth and successful fertilization. This type of interspecific incompatibility is likely not related to the S-RNase mediated self-incompatibility mechanisms operating in the Poaceae (Heslop-Harrison, 1982), but it is more similar to the crossability genes found in wheat (*Kr*) and maize (*Ga-S* and *TcB*). To study the gene function and sequence homology to other crossability genes, the genetic location of the *Iap* locus must first be determined.

Research on the introgression breeding potential of one tertiary species, *S. macrospermum*, has shown promising results. Genetic recombination between the

parental genomes in a *S. bicolor* x *S. macrospermum* hybrid was observed, and subsequently 2*n* = 20 interspecific backcrosses revealed the presence of *S. macrospermum* introgression (Kuhlman et al., in review). The availability of this wild species for sorghum improvement is no longer hypothetical, and efforts are progressing on determining the functionality of recovered introgression. These rely on using *S. bicolor* germplasm homozygous for *iap* to recover the initial interspecific cross. One limitation was the reliance on a single source of *S. bicolor* with the *iap iap* genotype. Results also indicated that using complex populations (*iap iap*) may increase recovered introgression by maximizing *S. bicolor* genetic factors which increase recoverability of introgression (Kuhlman et al., in review). To efficiently move the *iap* allele into elite sorghum germplasm and heterozygous breeding populations, molecular markers linked to the *Iap* locus would be beneficial.

The objectives of this research were to confirm the phenotypic reaction and inheritance of the *iap* allele, map its genetic location, and identify suitable molecular markers for use in marker assisted selection.

Materials and Methods

Plant Material

The accession used by Laurie and Bennett (1989) to identify the *Iap* locus, acc. NR481 (*iap iap*), was used as the control to develop the maize pollen tube growth phenotype used in this research. BTx623 was used as the negative control since interspecific sorghum hybrids are difficult to produce with this parent and maize pollen

tube growth is minimal since its genotype is *Iap Iap*. NR481 was crossed onto BTx623ms3 (an unreleased backcross version of BTx623 segregating for the male-sterility allele *ms3*) and the hybrid was self-pollinated to create an F_2 population segregating for both the *iap* and *ms3* alleles. Sterile plants (*ms3 ms3*) were phenotyped for pollen tube growth and used as a mapping population to identify the genetic map location of *Iap*.

A separate near-isogenic line population segregating for the *ms3* allele was used to determine its genetic map location in order to prove selection of *ms3 ms3* individuals in the F_2 *Iap* mapping population did not affect the results. The recurrent parent, 31945-2-2, was crossed with male-sterile QL36*ms3*. The hybrid was selfed and male-sterile (*ms3 ms3*) F_2 progeny were backcrossed to the recurrent parent. Five cycles of backcrossing were completed. Fertile and sterile plants in the BC_5F_2 generation were sib-crossed; the resulting progeny rows were selected for 1:1 segregation of male-fertility and male-sterility. Bulk fertile by male-sterile crosses produced a backcross segregating population, fertile (*Ms3 ms3*) and male-sterile (*ms3 ms3*), that is near-isogenic to 31945-2-2. This population was created by Dr. David Jordan, fertility phenotypes and isolated DNA was kindly provided.

Pollen Tube Growth Evaluation

Maize pollen was used to determine the pollen tube growth reaction of individual plants as described by Laurie and Bennett (1989). Hand-emasculated or genetic male-sterile sorghum florets were dusted with freshly collected maize pollen, cv. DK66-80,

one day after emasculation or anthesis. Twenty-four hours post pollination, florets were harvested and placed into vials and fixed in 3:1 (95% ethanol : glacial acetic acid) for a minimum of one week; then the florets were transferred into 70% ethanol for long term storage at -20ºC. Pistils were excised from the florets prior to processing. Pistils were processed using a modified version of the protocol described by Kho and Baer (1968) which was recently described (Hodnett et al., 2005). Briefly, pistils were softened overnight in 0.8*M* NaOH, stained with 0.025% (w/v) aniline blue in 0.1*M* K_2PO_4 for approximately 30 minutes in the dark. Twelve intact pistils were mounted on a glass slide in 50% 0.1*M* K_2PO_4 buffer and 50% glycerol under a 24x50 mm coverslip. Slides were observed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany). Fluorescence of the callose in the pollen tubes was induced using 390 to 420-nm light from a mercury lamp with a 450-nm emission filter. Each stigma was counted for number of maize pollen grains that germinated and then the pollen tubes were quantified that reached the axis, top of the style, bottom ¼ of the style, and the ovary. The phenotype used to identify *iap iap* genotype plants is discussed in the results section.

Genetic Mapping

The genetic map location of the *Iap* and *Ms3* loci were determined by using AFLP and SSR markers from the Menz et al. (2002) genetic map. The F_2 population used to map *Iap* consisted of 15 *iap iap* genotypes and 14 *Iap* __ genotypes. The near-isogenic line population used to map *Ms3* consisted of 49 *ms3 ms3* (sterile) and 47 *Ms3*

ms3 (fertile) individuals. Genomic DNA from the *Iap* population was extracted from leaf tissue using the FastDNA Spin Kit (MP Biomedicals, Solon, OH). Genomic DNA from the near-isogenic line population was provided by Dr. David Jordan. AFLP markers, using both *EcoRI*/*MseI* and *PstI*/*MseI* restriction enzyme combinations, were generated using a modified protocol from Vos et al. (1995). The AFLP template, preamplification, and selective amplification reactions of the *EcoRI*/*MseI* and *PstI*/*MseI* fragments were as described by Klein et al. (2000) and Menz et al. (2002), respectively. IRD-labeled SSR primers, obtained from LI-COR (LI-COR Inc, Lincoln, NE), were used in amplification reactions as previously described (Klein et al., 1998). Bulk segregate analysis was used to identify putative genetic map locations of both loci (Michelmore et al., 1991), and markers were then run on the populations as individuals to generate genotypic data for linkage analysis. The AFLP and SSR amplification products were analyzed on a LI-COR model 4200 dual-dye automated DNA sequencing system. Electrophoresis conditions were as described by Klein et al. (2000). Digital gel images were scored by hand and data was formatted for linkage analysis in Mapmaker/Exp v3.0b (Whitehead Institute, Cambridge, MA). Genotypic data was analyzed using a minimum LOD of 3.0 and the ‘group’ and ‘compare’ commands were used to determine the best order of the loci.

Results and Discussion

Pollen Tube Growth Phenotype and Segregation of *Iap*

Laurie and Bennett (1989) identified the *Iap* locus as controlling a barrier to maize pollen tube growth in sorghum stigmas. They classified plants into two groups: those that allowed maize pollen tube growth to the base of the style (*iap iap*) and those that inhibited maize pollen tube growth in the stigma (*Iap* __). In their research NR481 (*iap iap*) allowed maize pollen tube growth in virtually all stigmas observed. Only a single plant was found that had incomplete classification: 2 of 10 stigmas allowed maize pollen tube growth to the base of the style. F_1 hybrids (*Iap iap*) and all other accessions (*Iap Iap*) tested allowed only short maize pollen tubes to develop. The pollen tube growth phenotype was consistent and showed little environmental variation. Classification reportedly was very obvious, either all stigmas allowed maize pollen tube growth or they did not.

In this study, the maize pollen tube growth phenotype was much less consistent. Maize pollen tube growth observed in the control genotype NR481 (*iap iap*) was less than that reported by Laurie and Bennett (1989) in all environments studied. Out of 575 stigmas analyzed, 44% had maize pollen tube growth into the stigma axis and 29% had growth to the bottom ¼ of the style. All NR481 plants would have had incomplete classification according to the phenotype reported by Laurie and Bennett (1989) because all had some stigmas that did not have maize pollen tube growth to the bottom ¼ of the style. Genotypic variation was ruled out as a source of variation as this specific accession came directly from the previous authors. Temperature, light intensity, and

timing of pollination have all been shown to affect pollen tube growth rate (Campbell et al., 2001; Sun et al., 1991), however, no environmental factors were identified that adequately explained the reduced pollen tube growth. In the present study, none of 303 stigmas from control negative genotypes, BTx623 and the F_1, had maize pollen tube growth into the stigma axis. Thus, the negative phenotype does not show variation and classification based on presence of maize pollen tube growth into the style can be confidently assigned. The maize pollen tube growth phenotype used in the present research requires observation of twenty-four stigmas and plants are classified as positive if pollen tubes are observed in the bottom ¼ of the style and negative if maize pollen tubes are not observed beyond the stigma branches. A false negative phenotype is tentatively assigned if growth is observed into the stigma axis but fails to reach the bottom ¼ of the style. False negative plants most likely have the ability to allow maize pollen tube growth but are affected by environmental variation, making their expression of the phenotype incomplete. This phenotypic classification is more likely to produce error in the form of truly *iap iap* plants classified as *Iap* __, based on lack of maize pollen tube growth, rather than falsely assigning an *iap iap* genotype to a truly *Iap* __ plant.

Segregation of the maize pollen tube growth phenotype, as controlled by a recessive allele at a single locus, does not differ from expected ratios in BC_1F_1 (1:1) and BC_1F_2 (1:7) generations (X^2 = 0.84ns and 1.78ns, respectively) (Table 6). Segregation in the F_2 generation is different from a 3:1 ratio (X^2 = 4.47*), but when likely false negative genotypes, those showing maize pollen tube growth into the stigma axis are included as

Table 6. Segregation of the maize pollen tube growth phenotype as controlled by the *Iap* gene in multiple populations and environments

Genotype	Env†	N	PTG (+) (*iap iap*)‡	PTG (-) (*Iap* __)	$X^{2\#}$
NR481 (*iap iap*)	FA 05	10	10	0	
	SU 05	4	3	1§	
	SP 05	21	18	3§	
F_1 (BTx623*ms3*/NR481) (*Iap iap*)	FA 05	8	0	8	
	SU 05	2	0	2	
	SP 05	6	0	6	
BC_1F_2 ((BTx623*ms3*//)NR481)-F_1)	FA 05	104	8	96 (5)	(1:7) 1.78^{ns}, 0.02^{ns}
F_2 ((BTx623*ms3*/NR481)-F_1)	SU 05	125	20	105 (9)	(1:3) 4.47*, 0.13^{ns}
BC_1F_1 ((ATx623)//NR481)	SP 05	19	7	12 (1)	(1:1) 0.84^{ns}, 0.21^{ns}

† Environments were in the greenhouse in Fall 2005 (FA 05) and Spring 2005 (SP 05) and field grown in Summer 2005 (SU 05).
‡ PTG (+) and (-) are positive and negative pollen tube growth phenotypes. Parenthesis in PTG (-) phenotype are number of false negative classifications.
§ One plant in SU 05 and 3 plants SP 05 are false negative classifications
# Critical region of $X^2_{.05}$ is 3.84, parenthesis contain expected segregation ratios. The first and second X^2 values test the observed segregation ratios with false negative phenotypes classified as PTG (-) and PTG (+), respectively.

iap iap genotypes, segregation ratios do not differ from expected (X^2 = 0.13ns). The existence of false *Iap* __ genotypes is clear as 11% of NR481 plants did not have maize pollen tube growth (Table 6). The segregation of the pollen tube growth phenotype agrees with the conclusion that it is controlled by a single genetic locus, previously identified as *Iap* (Laurie and Bennett, 1989).

Genetic Map Location of *Iap*

Sixty-four *PstI*/*MseI* AFLP primer combinations produced 362 polymorphic markers between NR481 and BTx623ms3. Twelve of those appeared to differentiate the two bulk pools, *iap iap* and *Iap* __. Upon running the markers on the full mapping panel, three were linked to the *Iap* locus. Two of the markers had been previously mapped and the third was subsequently scored in the RI population used to create the Menz et al. (2002) genetic map. All three markers mapped to a 22cM region on SBI-02. Adjacent AFLP and SSR markers were then targeted and a genetic map of the region was created with the genotypic data (Figure 5). Three AFLP markers were closely linked to the *Iap* locus. These were Txa6647 (2.1cM) (Figure 6) and Txa4079 (2.7cM) flank *Iap,* and Txa13074 (0.0) shares the same position. The closest SSR markers are Txp63 (10.8cM) and Txp50 (11.4cM) which flank the *Iap* locus (Figure 5). This positions the *Iap* locus at between 17.0 and 18.2cM on SBI-02 on the Menz et al. (2002) genetic map. Primer combinations and marker fragment size for linked markers are given in Table 7. Since selection was practiced in the F_2 mapping population, only

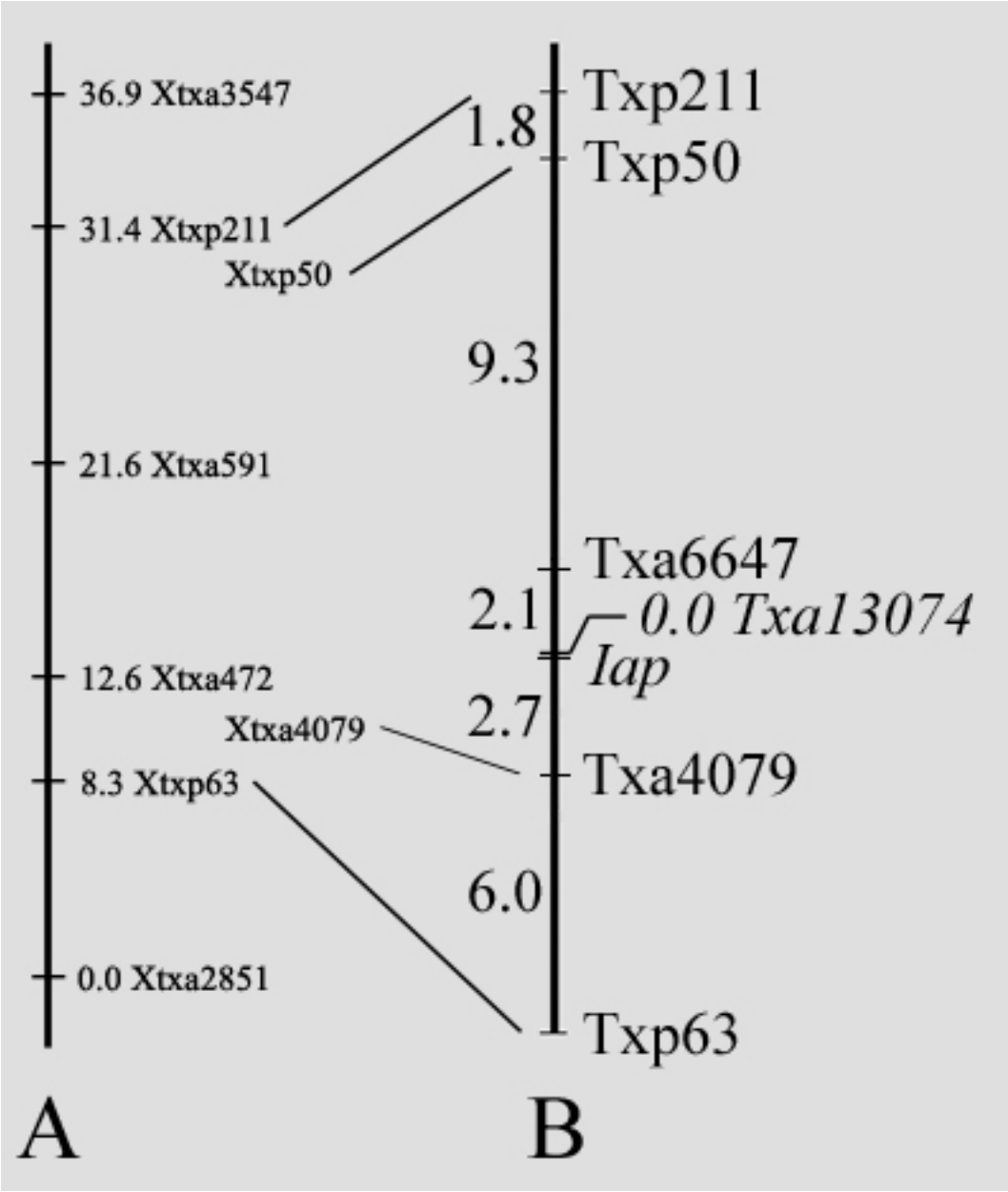

Figure 5. Genetic map of a region of *S. bicolor* Chromosome 2 (SBI-02). (A) Published genetic map (Menz et al., 2002) corresponding to (B) a map created using 29 F_2 individuals, phenotyped for maize pollen tube growth, to determine the location of the *Iap* gene.

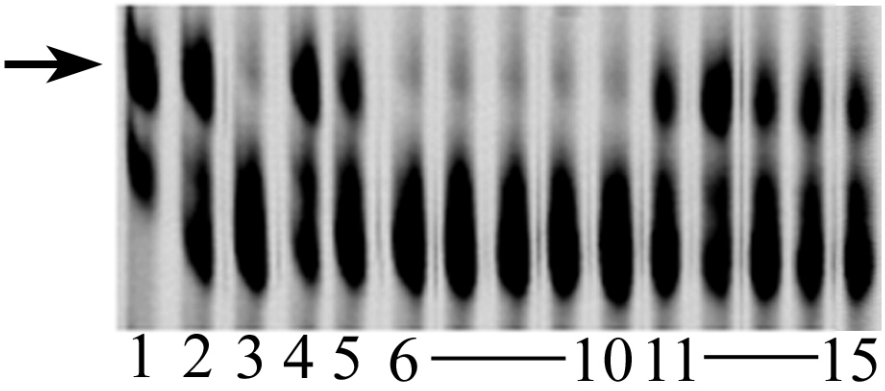

Figure 6. Gel image of AFLP marker Txa6647 (arrow). Lanes 1-15 are (1) IS3620C, (2) BTx623, (3) NR481, (4) BTx623ms3, (5) BTx623ms3/NR481, (6-10) F_2 individuals that allow maize pollen tube growth (*iap iap*), and (11-15) F_2 individuals that inhibit maize pollen tube growth (*Iap* __).

Table 7. Forward and reverse primer sequences (5'-3') for SSR and AFLP markers identified as linked to the *Ms3* and *Iap* loci

Marker Name	F-primer	R-primer	Size (bp)
Txp425	AAGGCCTAAAACTTGTTGAACG	TCACTCATCTCCATCATTGTCA	186
Txp426	GCGTATGAATCTTCGTTTTATTCA	CCATCATTTTGATGAAATGCAC	250
Txp427	CACGAGGGCAGTGTGGAC	GCATCCCGTACAGCTTCAG	117
Txp63	CCAACCGCGTCGCTGATG	GTGGACTCTGTCGGGGCACTG	204
Txp50	TGATGTTGTTACCCTTCTGG	AGCCTATGTATGTGTTCGTCC	299
Txp211	TCAACGGCCAATGATTTCTAAC	AGGTTGCGAATAAAAGGTAATGTG	216
Txa6647	M+CCC	P+CTC	310
Txa4079	M+CTG	P+CGT	268
Txa13074	M+CGA	P+AGA	65

Additional information regarding markers is available at http://sorgblast3.tamu.edu

male-sterile (*ms3 ms3*) individuals were used for ease in phenotyping; however, there was concern that this may have affected the map location of the *Iap* gene.

Genetic Map Location of *Ms3*

A separate population, near-isogenic for *ms3*, was used to genetically map the location of *Ms3*. The *Ms3* locus has been reported to be linked approximately 7 map units from an awn gene (Doggett, 1970). Presence or absence of awns is normally simply inherited but multiple genes are capable of control (Rooney, 2000). Two linkage maps report the location of awn loci on SBI-03 (Tao et al., 2000; Hart et al., 2001). A survey of SSR markers in the corresponding region was done. Linkage of three SSR markers to the *Ms3* loci was found in the near-isogenic line population. Txp427, Txp425, and Txp426 are linked at 5.6cM, 13.6cM, and 16.8cM to the *Ms3* loci, respectively (Figure 7). These genetically mapped markers are located on SBI-03 (http://sorgblast3.tamu.edu). This positions the *Ms3* locus at approximately 179-185cM on SBI-03 in the Menz et al. (2002) genetic map. SSR markers flanking *Ms3* on the other side of Txp427 were not polymorphic in the near-isogenic line population. The linked SSR markers were run on the 29 F_2 *Iap* mapping individuals (all male-sterile *ms3 ms3*) and the same map order of loci was obtained, confirming the location of *Ms3* to SBI-03 (data not presented). The presence of the *Ms3* locus on a different chromosome than *Iap* makes unlikely the possibility that segregation distortion, from selection of *ms3 ms3* individuals, affected the map position of *Iap*.

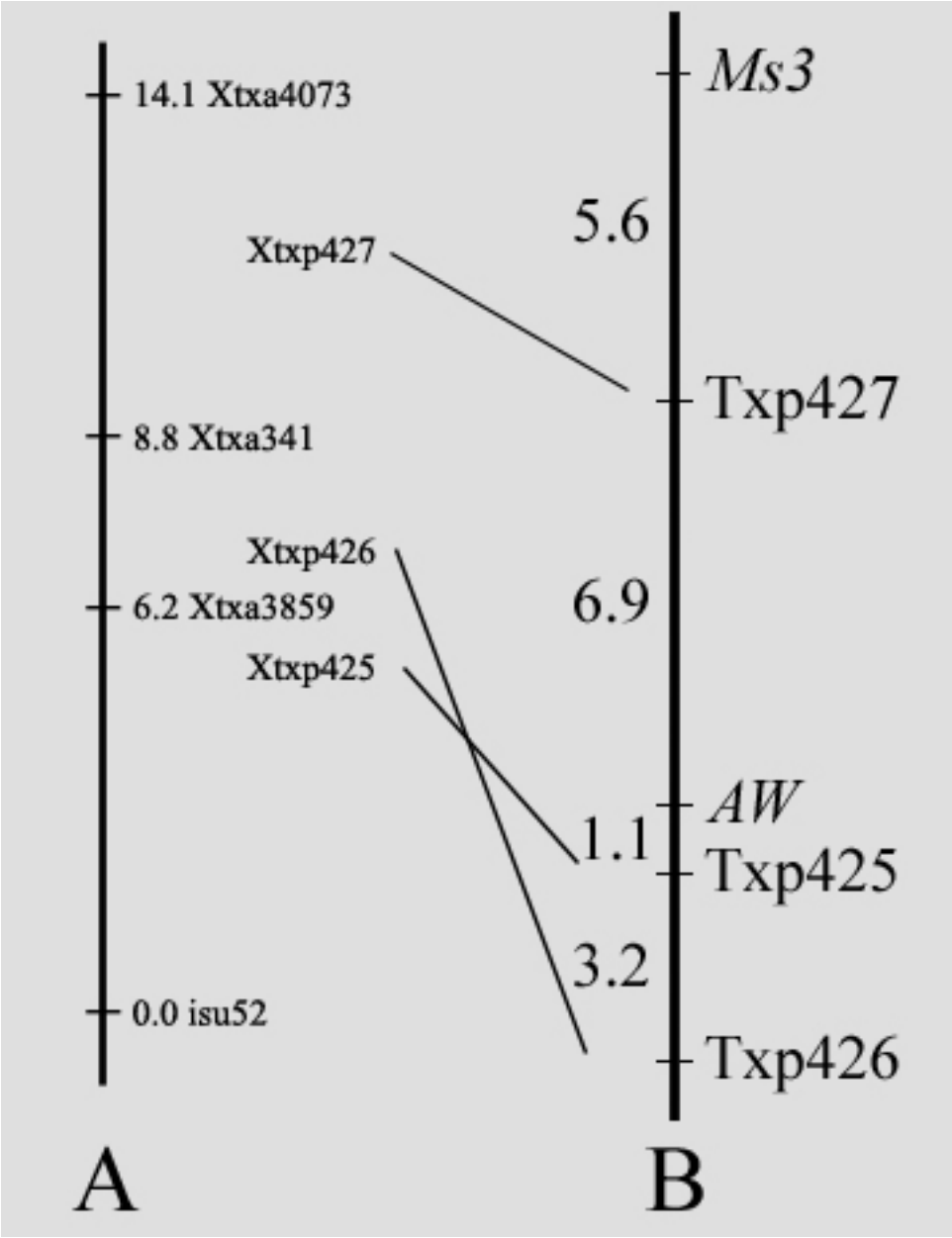

Figure 7. Genetic map of a region of *S. bicolor* chromosome 3 (SBI-03). (A) Published genetic map (Menz et al., 2002) corresponding to (B) a map created using 96 backcross near-isogenic progeny, phenotyped for male-sterility and awns (*AW*), to determine the location of the *Ms3* gene.

Summary

This research confirms that maize pollen tube growth in sorghum pistils is controlled by a single locus. The phenotype observed by Laurie and Bennett (1989) for segregation of the *iap* allele is likely subject to environmental variation, and a modified phenotype provided suitable discrimination. The map position of the *Iap* locus is approximately 17.0-18.2cM on SBI-02. AFLP and SSR markers, suitable for marker-assisted selection, were closely linked to *Iap*. This information will allow marker-assisted backcrossing of the *iap* allele into complex breeding populations as well as multiple elite backgrounds. The map position provided can also be a starting point for fine mapping of *Iap*, required for gene cloning.

CHAPTER VI

DEVELOPMENT OF SORGHUM GENETIC STOCK TX3361

Introduction

Sorghum is an important food, feed, and forage crop with worldwide grain production in 2005 of 56,957,314 metric tons which ranks fifth among cereal grains (FAOSTAT data, 2006). Production in the U.S. accounted for approximately 10,000,000 metric tons of that crop which was valued at $715,000,000 (USDA 2006). Production limitations for sorghum in the U.S. include abiotic stresses such as drought and biotic stresses like insect and disease pressure. Plant breeders continually make progress in improving the crop for these and many other traits, including yield potential, but are ultimately limited by the amount of genetic variation available for the desired trait. Without genetic variation for a trait, genetic improvement is not possible. Identifying valuable new sources of germplasm is a key component to supply plant breeders with the genetic variation they need for improvement. Wild species can be valuable sources of novel genetic variation for improvement of yield, disease and insect resistance, and abiotic stresses (Goodman et al., 1987; Jiang et al., 1994; Jones et al., 1995; Jauhar and Chibbar, 1999).

Plant breeders have improved sorghum by utilizing germplasm within the primary gene pool, namely the species *S. bicolor* (Duncan et al., 1991). The secondary gene pool contains *S. propinquum* and *S. halepense* and both can be crossed with

sorghum; however, only a few serious improvement efforts have utilized these genetic resources (Wooten, 2001; Dweikat, 2005). The tertiary gene pool consists of the remaining 17 species within the genus *Sorghum* and, until recently, hybrids between *S. bicolor* and any of these species had never been recovered despite numerous efforts (Karper and Chisholm, 1936; Ayyanger and Ponnaiya, 1941; Garber, 1950; Endrizzi, 1957; Tang and Liang, 1988; Wu, 1990; Sun et al., 1991; Huelgas et al., 1996).

Hodnett et al. (2005) determined that one cause of reproductive isolation with the tertiary gene pool was pollen-pistil incompatibilities. Pollen tube growth of wild species was inhibited in the stigma and style of sorghum preventing successful fertilization. The reproductive barriers proved to be strong but not complete as Price et al. (2005b) finally recovered one interspecific hybrid between cytoplasmic male-sterile (CMS) sorghum and *S. macrospermum*. The efficiency of producing this hybrid improved dramatically by using a *S. bicolor* genotype homozygous for the *iap* allele. The *Iap* locus controls a pistil barrier that prevents foreign species pollen tube growth; whereas, the recessive genotype (*iap iap*) allows pollen tube growth of maize as well as wild sorghum species (Laurie and Bennett, 1989; Price et al., 2006). Utilizing *iap iap* germplasm allowed the facile recovery of interspecific hybrids with *S. macrospermum*, as well as never before produced hybrids with *S. angustum* and *S. nitidum* (Price et al., 2006).

Recent research on *S. bicolor* x *S. macrospermum* interspecific hybrids showed that allosyndetic recombination occurred between the genomes and that introgression was recovered in backcross progeny (Kuhlman et al., in review). *Sorghum macrospermum* is the only tertiary gene pool species characterized for use by sorghum

breeders, but future research will likely reveal that other species can be utilized by using *iap iap* germplasm. One limitation cited was that the *iap iap* genotype used to make the initial cross was agronomically very poor (Kuhlman et al., in review). The *Iap* gene was identified in *S. bicolor* accession 'NR481' (Laurie and Bennett, 1989) which is tall, has a pigmented testa, and is extremely susceptible to lodging. Its potential for use in an introgression program is limited because any genetic variation from wild species transferred will be in a very poor genetic background. The objective of this research was to backcross the *iap iap* genotype into elite Texas A&M germplasm for use as the recurrent parent in an introgression breeding program.

Materials and Methods

Plant Material

NR481, an unreleased line homozygous for the *iap* allele (Laurie and Bennett, 1989), was used as the donor parent and genetic male-sterile BTx623ms3 as the elite recurrent parent. Plant height was measured in centimeters from the soil surface to the tip of the mature panicle. Plants were also characterized for plant color, seed color, awns, days to 50% anthesis, lodging, and stable segregation of male-sterility (*ms3*). Breeding, selection, and evaluation was conducted at College Station and Weslaco, TX.

Pollen Tube Growth Evaluation

Maize pollen was used to determine the pollen tube growth reaction of individual plants as described by Laurie and Bennett (1989). Hand-emasculated or genetic male-

sterile sorghum florets were dusted with freshly collected maize pollen, cv. DK66-80, one day after emasculation or anthesis. Twenty-four hours post pollination, florets were harvested and placed into vials and fixed in 3:1 (95% ethanol : glacial acetic acid) for a minimum of one week; then they were transferred into 70% ethanol for long term storage at -20ºC. Pistils were excised from the florets prior to processing. Pistils were processed using a modified version of the protocol described by Kho and Baer (1968) which was recently described (Hodnett et al., 2005). Briefly, pistils were softened overnight in 0.8*M* NaOH, stained with 0.025% (w/v) aniline blue in 0.1*M* K_2PO_4 for approximately 30 minutes in the dark. Twelve intact pistils were mounted on a glass slide in 50% 0.1*M* K_2PO_4 buffer and 50% glycerol under a 24x50 mm coverslip. Slides were observed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany). Fluorescence of the callose in the pollen tubes was induced using 390 to 420-nm light from a mercury lamp with a 450-nm emission filter. Each stigma was counted for number of maize pollen grains that germinated and then the pollen tubes were quantified that reached the axis, top of the style, bottom ¼ of the style, and the ovary. Plants showing maize pollen tube growth into the bottom ¼ of the style in any of 24 stigmas was given a positive phenotype and considered *iap iap*.

Results and Discussion

Tx3361, a proposed sorghum genetic stock, was developed and is under review for release by the Sorghum Improvement Program of the Texas Agricultural Experiment

Station in College Station, TX. This line is being considered for release because of its improved agronomic performance and *iap iap* genotype.

Tx3361 was derived from the backcross BTx623ms3 x (BTx623ms3 x NR481). NR481 is 2-dwarf in height, has a red pericarp, red plant color, pigmented testa, awns, and is highly susceptible to lodging. BTx623ms3 is an unreleased derivative of BTx623 which segregates for the genetic male-sterility allele, *ms3*. BTx623 is an elite parental line developed by the Texas Agricultural Experiment Station which has been commonly used to make commercial hybrids. It is 3-dwarf in height, has a white pericarp, and red plant color. Individuals from the BC_1F_1 population were self-pollinated and selected for 3-dwarf height, white pericarp, no awns, absence of pigmented testa, and reduced lodging in the field in College Station, TX 2005.. The BC_1F_2 families were grown in a greenhouse, hand emasculated, and tested for maize pollen tube growth. Selected *iap iap* individuals were self-pollinated and progeny-rows were grown at College Station, TX in 2006. Lines were evaluated for lodging, height, awns, and segregation of the *ms3* allele. Selected male-fertile and sterile plants (BC_1F_3) within *ms3* segregating rows were sib-mated. Individual sib crosses were grown at Weslaco, TX in 2006 and evaluated for stable backcross segregation of *ms3*, lodging, height, maturity, and maize pollen tube growth to confirm their *Iap* locus genotype (Figure 8). The selected line, designated as Tx3361, was bulk sib-mated between male-sterile and fertile plants to produce breeder's seed of the proposed genetic stock.

The observed expression of the *iap iap* genotype based on maize pollen tube growth to the base of the style was lower than that reported by Laurie and Bennett

(1989) but was similar to that reported by Kuhlman et al. (in review). Maize pollen tube growth in Tx3361 was similar to NR481 in all tested environments. Tx3361 has short plant height, improved lodging resistance, early maturity, white seed color, non-pigmented testa, and is backcross segregating for male-sterility (Table 8). This genetic stock can be used as a female parent to obtain interspecific hybrids with exotic sorghum species and possibly species beyond the genus *Sorghum*. Any recovered introgression will be in a more favorable genetic background for further evaluation and breeding. Seed of Tx3361 will be maintained by the Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474.

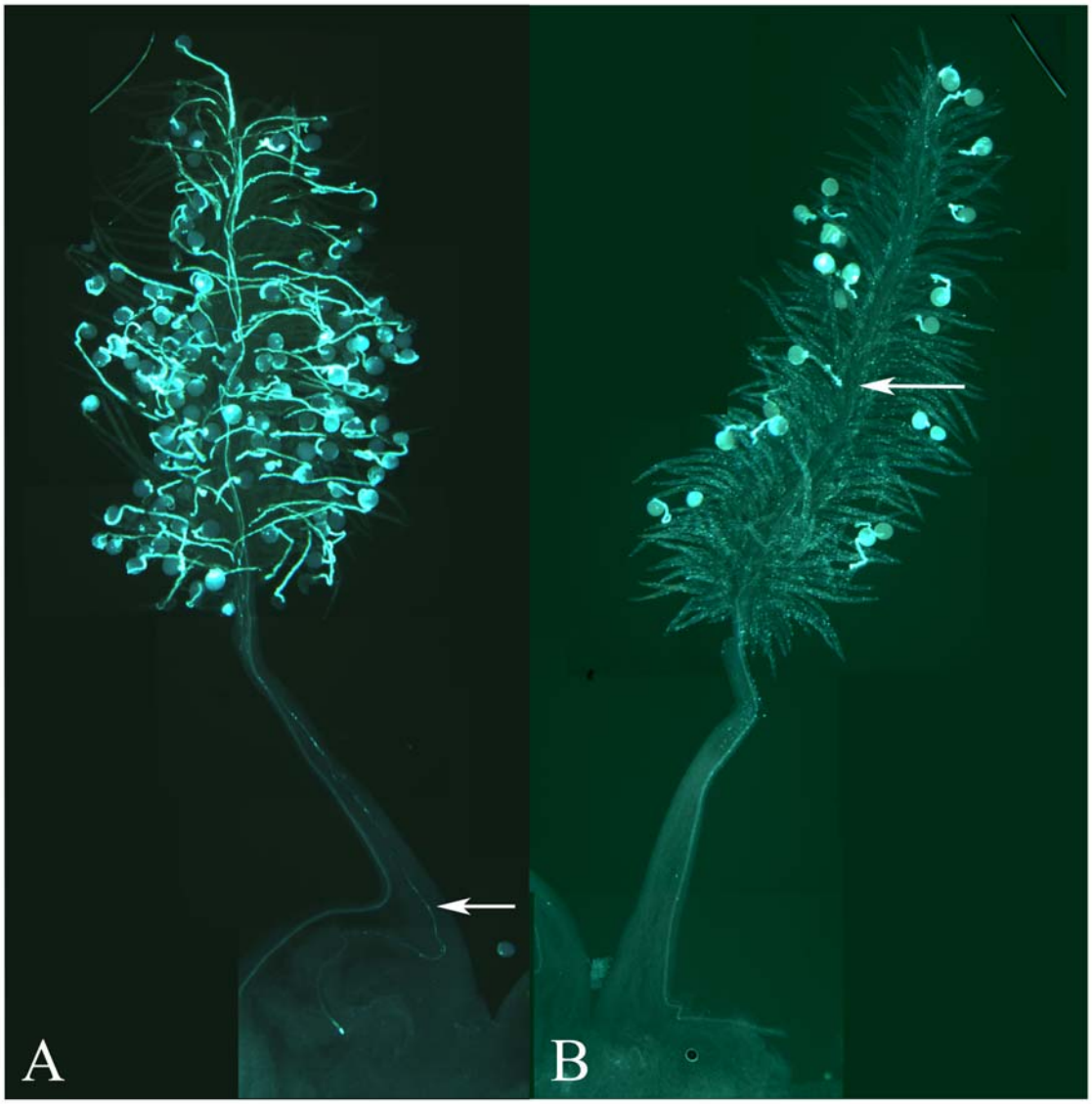

Figure 8. Maize pollen tube growth in sorghum stigmas. (A) Tx3361 pistil showing maize pollen tube growth, arrow shows maize pollen tube growing through the base of the style into the ovary, (B) Sorghum pistil showing no maize pollen tube growth, arrow shows maize pollen tube failing to enter the stigma axis.

Table 8. Agronomic traits of the two parents and the proposed genetic stock evaluated in Weslaco, TX 2006

	NR481	BTx623ms3	Tx3361	$LSD_{(.05)}$
Dwarf Loci[1]	___ *dw2* ___ ___[†]	*dw1 Dw2 dw3 dw4*	*dw1 Dw2 dw3 dw4*	
Pericarp Color[2]	R	W	W	
Awns[3]	Y	N	N	
Pigmented Testa[3]	Y	N	N	
ms3 backcross segregation[3]	N	Y	Y	
Maize PTG[4]	22.5%^{A}	0.0%^{B}	15.3%^{A}	11.0%
Iap Locus	*iap iap*	*Iap Iap*	*iap iap*	
Height (cm)	234^{A}	137^{B}	137^{B}	19.1
Exsertion (cm)	21^{A}	9^{B}	11^{B}	7.1
Lodging[5]	5.7^{A}	0.6^{B}	1.8^{B}	1.5
Days to 50% Anthesis	49^{C}	65^{A}	53^{B}	3.5

[1] Dwarf Loci: represents the homozygous allele at each dwarfing locus

[†]'NR481' has 2 loci that are homozygous recessive but only the genotype at *Dw2* is known

[2] Pericarp color: R = red, W = white

[3] Awns, pigmented testa, and *ms3* backcross segregation: Y = yes, N = no

[4] Frequency of sorghum pistils with maize pollen tube growth to the base of the style

Values are means, different letters within rows indicate significantly different means $\alpha = .05$

[5] Lodging: 0 – 9 scale, 0 = 0-10%, 9 = 90-100% lodging

CHAPTER VII

CONCLUSIONS

Prior to this research, knowledge of the introgression breeding potential of tertiary gene pool species in *Sorghum* was, for all practical purposes, absent. In this investigation, one tertiary gene pool species, *S. macrospermum*, was used to create interspecific hybrids with *S. bicolor* in order to study the possibility of gene transfer. It was determined that allosyndetic recombination occurred at a moderate level (2.6 II per PMC) in the interspecific hybrids and that at least 80% of the *S. bicolor* genome has potential for recombination with *S. macrospermum*. It was also found that the A subgenome of *S. bicolor* undergoes recombination 2.5 times more frequently than the B_1 subgenome, which means that introgression into A subgenome chromosomes is more likely. Based on genomic associations, *S. macrospermum* is hypothesized to have the genomic formula AAB_1B_1YYZZ with subgenomes Y and Z unknown.

Backcrosses using the interspecific hybrid as the female parent, in combination with embryo rescue, resulted 15 BC_1F_1 progeny. Their chromosome numbers ranged from 35 to 70 and the plants were male-sterile. However, three BC_1F_1s had enough female fertility to produce BC_2F_1 seed. Ninety-five percent of the BC_2F_1 plants tested had 20 chromosomes, and one plant was $2n = 21$. *Sorghum macrospermum*

introgression (0-18.6%) was detected in 75% of BC_2F_1 progeny; in total 26% of the *S. macrospermum* genome was detected in the BC_2 generation. Male-sterility was the only phenotypic effect that could be confidently hypothesized to be caused by introgression. Two families had significant male-sterility and had only the recurrent parent (NR481) in their pedigree. The third family was a mixed pedigree involving BTx623 and it had near normal levels of male-fertility and seed set. Molecular cytogenetic techniques showed that three types of germplasm had been created: alien addition lines; alien substitution lines; and introgression lines. The alien addition line (3.5% introgression) contained a single non-recombinant *S. macrospermum* chromosome plus all 20 *S. bicolor* chromosomes. The alien substitution line (18.5% introgression) had two non-recombinant *S. macrospermum* chromosomes plus 18 *S. bicolor* chromosomes. Two introgression lines (1.1% and 0.6% introgression) were confirmed to have two recombinant, likely homologous, chromosomes that contained *S. macrospermum* introgression sites plus 18 non-recombinant *S. bicolor* chromosomes. This research demonstrated that recovery of backcross progeny is possible and that *S. macrospermum* introgression occurs.

The *Iap* gene controls a reproductive barrier in sorghum pistils that inhibits wild species pollen tube growth, thus preventing fertilization. Germplasm homozygous for the *iap* allele allows pollen tube growth and was used in this research to create the interspecific hybrid. The *Iap* locus was mapped to chromosome 2 (SBI-02) and positioned at 17.0-18.2cM on the Menz et al. (2002) genetic map. Flanking AFLP

markers were identified at 2.1 and 2.7cM, a third AFLP marker (0.0cM) shared the same map position as *Iap*. The closest flanking SSR markers were at 8.7 and 11.4 cM.

The original *iap iap* genotype germplasm used to create interspecific hybrids is agronomically very poor. A genetic stock, Tx3361, was created that is *iap iap*, but has improved agronomic characteristics such as short plant height, white seed color, non-pigmented testa, reduced lodging, no awns, early maturity, and is backcross segregating for male-sterility (*ms3*). This genetic stock is proposed for release from the Texas Agricultural Experiment Station and can be used as a recurrent parent in an introgression breeding program.

The research reported herein shows that the tertiary gene pool species *S. macrospermum* can be used by plant breeders. These results should serve as a baseline from which to improve for sorghum introgression breeding programs and may also be useful for research involving other members of the tertiary gene pool. The present research opens the door for multiple future research projects. Using similar molecular cytogenetic techniques, the identities of the unknown *S. macrospermum* subgenomes

may be uncovered in crosses with other *Eusorghum* species. The phenotypic effect of the alien substitution and addition chromosomes can be characterized in various genetic backgrounds. Homoeologous recombination may be able to be induced in crosses with sorghum monosomics or trisomics. Field selections can be made of short agronomically acceptable BC_2F_2 progeny from family 101 for characterization of introgression effects. Using marker-assisted selection, a highly heterogeneous breeding population (*iap iap*) can be created which may increase the survivability of interspecific hybrids or increase the amount of recoverable introgression. Finally, a sorghum introgression breeding program needs to be established which can complete the suggested research as well as explore the limits of gene transfer with other species. There is an immense amount of potential that can be accomplished based on these results and such a program could have a major impact on sorghum research.

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