THESIS

FERTILITY AND CHROMOSOME COMPOSITION OF WHEAT X JOINTED GOATGRASS BACKCROSS PROGENY

Submitted by

Craig T. Beil

Department of Bioagricultural Sciences and Pest Management

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Master's Committee:

Advisor: Philip Westra
Co-Advisor: Patrick Byrne

Scott Haley
Dale Shaner

ABSTRACT

FERTILITY AND CHROMOSOME COMPOSITION OF WHEAT X JOINTED GOATGRASS BACKCROSS PROGENY

Interest in the development of genetically engineered (GE) wheat (*Triticum aestivum* L.) through the use of transgenic technologies has increased concern surrounding gene movement from wheat to wild plant populations. Crop-weed hybridization and subsequent generations of recurrent backcrossing to the weedy species could lead to the introgression of advantageous genes from a crop into a weed population. Despite these same risks being present with traditionally bred crops, transgenic technology makes it possible for single genes or blocks of genes to be inserted into a genome of interest as a compact unit. Movement of a single chromosomal segment, containing a novel transgene, into a weedy species' genome could lead to the expression of the novel transgenic trait. Introgression of genes improving tolerance to biotic and abiotic stresses could increase the competitive ability of wild plants and lead to new management challenges. Interspecific gene flow has long been proposed as a mechanism for evolution in natural plant populations and the cases of crop to weed gene flow is an extension of this theory.

Gene flow between bread wheat and jointed goatgrass (*Aegilops cylindrica* Host.) is of concern as the latter species is an agricultural weed in the western United States. Wheat (2n=6x=42, genomes ABD) and jointed goatgrass (2n=4x=28, genomes CD) share the common diploid ancestor *Aegilops tauschii* Coss., the donor of the D genome. Wheat and jointed goatgrass's genetic relatedness and similar development phenology allow these two species to hybridize at a low frequency, providing a bridge for gene flow from wheat to the weedy relative.

Although hybridization of wheat and jointed goatgrass is an important barrier to overcome for gene escape to occur, it does not guarantee that a novel gene from wheat will become introgressed into jointed goatgrass's genetic background. Introgression of crop genes is dependent on stabilizing the foreign gene sequence into the new genome, which only occurs after recurrent backcrossing to the wild species and persistence of the new genotype. The goal of this research, which consisted of two studies, was to assess the risk of gene introgression from wheat to jointed goatgrass. The rates at which repeated backcrosses occur via natural pollination by jointed goatgrass will need to be quantified in order to fully assess the risk of gene introgression from wheat to jointed goatgrass. The first study was designed to quantify the rate of backcrossing of wheat x jointed goatgrass first generation backcross progeny (BC_1) in the field, as a rate of backcrossing of BC_1 plants to jointed goatgrass alone has not been determined under field conditions. The second study sought to develop and utilize a genomic *in situ* hybridization (GISH) procedure to identify A- and B-genome chromosomes separately from C- and D-genome chromosomes in the wheat x jointed goatgrass second generation backcross progenies (BC_2) that were generated from the work done in chapter one. These two chapters work together in assessing the biological risk of releasing wheat varieties with novel genes.

The first chapter examined the rate of backcrossing of BC_1 progeny to jointed goatgrass as this is the breeding direction required for introgression of novel crop genes into a weedy species. Field trials were conducted in Colorado during the 2010-11 and 2011-12 growing seasons and were supplemented with greenhouse trials to determine female fertility and self-fertility of wheat x jointed goatgrass BC_1 plants. Backcrossing rates in the field were determined with germination evaluations of spikes collected from 14 BC_1 plants from 2011 and 6 BC_1 plants from 2012 pollinated by jointed goatgrass. Pollination by jointed goatgrass led to the production

of 43 BC_2 plants in 2010-11 and 1 BC_2 plant in 2011-12. Mean backcrossing rates were estimated at 0.648% in 2010-11 and 0.024% in 2011-12. Median backcrossing rates were estimated at 0.041% in 2010-11 and 0.0% in 2011-12. Female fertility rates of BC_1 plants were evaluated on emasculated and hand pollinated spikes from 66 plants, and resulted in a mean observed female fertility rate of 0.03%. With each successive backcross to jointed goatgrass there is a greater number of C- and D-genome bivalent chromosome pairings during meiosis, resulting in increased fertility of BC_1 plants compared to wheat x jointed goatgrass hybrid plants. Because of the increase of potential bivalent chromosome pairings, male fertility has been observed in BC_1 plants. With increased female and male fertility observed in BC_1 plants, the potential exists for BC_1 plants to self-pollinate. Self-fertility of BC_1 plants was estimated on spikes from 17 plants using a single bagged spike (to prevent cross-pollination) in addition to 37 plants used in a whole bagged plant method with mean observed self-fertility of rates of 0.0% and 0.004%, respectively. These results demonstrate the first direct account of a mean backcrossing rate of BC_1 plants pollinated by jointed goatgrass under field conditions. With partial self-fertility restored in the BC_2 generation and shown to increase in subsequent selfing generations, BC_2 individuals could propagate by seed via self-pollination even if there is no jointed goatgrass in the vicinity for pollination. Isolated patches of BC_2 individuals would mark the beginning of a genetically unique jointed goatgrass population with the acquisition of novel genes from wheat and stable introgression occurring in the BC_2S_2 generation. Thus, the ability of BC_1 plants to backcross to jointed goatgrass in the field would make it highly likely that a wheat gene conferring a selective advantage could introgress into a jointed goatgrass population. It is possible that transgenic wheat cultivars will be released in the future and determining the

best management practices of these transgenic cultivars is necessary to minimize novel gene introgressions from wheat into weedy species.

There are a number of documented cases of gene flow from crops to wild relatives. However, little information is available about the long-term persistence of crop genes in wild populations. Ecological consequences of GE wheat deployment are difficult to predict and assess, yet research examining the retention of DNA sequences by genomes in wheat x jointed goatgrass backcross progeny could provide new insights into strategies to minimize the risk of gene escape from GE wheat. Gene movement from wheat to jointed goatgrass can occur via three mechanisms: gene recombination through homologous chromosome pairing, translocation, and chromosome addition. Among these three mechanisms of gene movement, gene transfer from wheat to jointed goatgrass by gene recombination (via the D genome) would be more common than gene transfer by translocation or chromosome retention. The D-genome chromosomes of wheat can pair with the D-genome chromosomes of jointed goatgrass, forming bivalents and allowing for crossing over to occur during meiosis. Jointed goatgrass's lack of the A and B genomes present in wheat has led scientists to speculate that novel transgenes inserted into the A or B genomes of wheat would minimize the escape and introgression of a transgene from GE wheat. Since the stable transfer of genes from the A or B genomes of wheat to jointed goatgrass requires that homologous chromosomes pair with each other during meiosis, the A- and B-genome chromosomes of wheat will likely be lost during the backcrossing of the hybrid to jointed goatgrass. This has made chromosomes of the A and/or B genomes of wheat prime candidates for the location of transgene insertion. Identification of differences in chromosome retention or translocation of wheat genome sequences into jointed goatgrass may provide insight into the safety of GE wheat.

The second chapter sought to develop and utilize a GISH procedure for identifying chromosomes by genome in wheat x jointed goatgrass backcross progenies. GISH has become a widely applicable cytogenetic technique for examining genome structure in interspecific hybrids and polyploid species. Direct labeling of genomic DNA from the diploid species *T. urartu* and *Ae. speltoides* was used to produce fluorescently labeled probes to visually identify chromosomes and chromosome segments belonging to the A and B genomes, respectively. Whole genomic probes showed extensive cross hybridization across all genomes when hybridized individually or hybridized with multiple probes. This resulted in fluorescent hybridization signals that were nearly homogeneously distributed among all chromosomes leading to an inability to karyotype chromosomes by genome. Varying concentrations of unlabeled genomic DNAs were unsuccessful in limiting cross-hybridization of repetitive DNA sequences to non-targeted chromosome regions. Multi-color GISH proved to be an unreliable technique for differentiating multiple genomes simultaneously in wheat x jointed goatgrass backcross progeny. This study showed that DNA sequences shared between *Triticum* and *Aegilops* will bind non-specifically labeled DNA sequences to non-specific chromosome regions and lead to challenges in karyotyping chromosomes by genome. The development and utilization of a GISH procedure that overcomes the cross-hybridization between closely related genomes observed in this study has the potential to provide insight into differences in chromosome addition or translocation of wheat sequences by genome into wheat x jointed goatgrass backcross progeny which may provide insight into the occurrence or ease of transgene movement from GE wheat to jointed goatgrass.

It is timely to address the issue of gene movement from wheat into a weedy relative as much research is still needed to fully assess the risks associated with potential gene flow from

wheat to jointed goatgrass before commercial release of GE wheat varieties. Here we have considered the rate at which wheat x jointed goatgrass BC_1 plants backcross to jointed goatgrass for a second generation in the field and their genetic makeup. Despite many of the same risks being possible with traditionally bred wheat cultivars, the environmental risks associated with gene flow of GE crops must be thoroughly addressed to prevent undesirable consequences for both agricultural and natural ecosystems. Since gene flow from wheat to jointed goatgrass can occur via hybridization and recurrent backcrossing, an effective stewardship program should accompany the release of wheat cultivars containing novel traits to ensure the longevity of this technology. The conclusions drawn from this report are critically important as new genetic and breeding techniques become available for wheat improvement.

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LITERATURE REVIEW

Jointed goatgrass (*Aegilops cylindrica* Host.) is an agricultural weed in the western United States. This winter annual grass is not native to the U.S., and has become a competitive weed in agricultural settings since its introduction as a contaminant of winter wheat (*Triticum aestivum* L.) seed in the late 19th century (Mayfield, 1927). This weed is predominantly found in fall-sown wheat where it infests 2 million ha, negatively impacting yields by competing for resources, reducing harvest efficiency, and lowering harvested grain quality as a contaminant in wheat grain. In the United States, jointed goatgrass has been reported in 32 states and continues to expand its range, spreading at a rate of 20,000- 50,000 hectares per year due to movement of jointed goatgrass seed by combines, grain trucks, and contaminated wheat seed (Donald and Ogg, 1991; Washington State University, 2009; USDA, 2013). These two species are similar in plant size and height, flowering time, spikelet arrangement along the rachis of the spike, vernalization response, and germination timing (Donald and Ogg, 1991). Their genetic relatedness and similar phenology allow these two species to hybridize, providing a bridge for gene flow from wheat to the weedy relative (Gaines et al., 2008; Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Morrison et al., 2002; Perez-Jones et al., 2010; Seefeldt et al., 1998; Wang et al., 2001; Zemetra et al., 1998).

Interest in the development of herbicide-resistant and environmental stress-tolerant genetically engineered (GE) wheat cultivars has focused attention on the possibility of introgression of wheat genes into jointed goatgrass populations. Pollen-mediated gene flow leading to crop-weed hybridization and subsequent generations of recurrent backcrossing to the weedy species could lead to the introgression of beneficial genes from a crop into a weed population (Ellstrand and Hoffman, 1990). Despite these same risks being present with

traditionally bred crops, transgenic technology makes it possible for single genes or blocks of genes to be inserted into a genome of interest as a compact unit. Movement of a single wheat chromosomal segment, containing a novel transgene, into a jointed goatgrass genome could lead to the expression of the novel transgenic trait. Although there are a number of documented cases for gene flow from crops to wild relatives (Ellstrand et al., 1999), little information is available about the long-term persistence of crop genes in wild populations or the impact of fitness-related genes on weedy species' population dynamics. Within the next decade, we may begin to see an increase in the number of crops that possess yield-enhancing traits that were identified in distantly related (tertiary) germplasm and incorporated using transgenic technologies. These yield enhancing traits will be novel due to the absence or rarity of these genes in wild plant populations. Introgression of genes from a crop species to a weedy species facilitating improved tolerance to biotic and abiotic stresses such as herbivory, pathogens, drought or salinity could increase the competitive ability of the weedy species and lead to new management challenges (Darmency, 1994; Warwick et al., 1999). Applied transgenic technology has been used to improve drought tolerance in corn (*Zea mays*) (Castiglioni et al., 2008) and it is likely this same technology could be used to improve drought tolerance in wheat cultivars. Semi-arid areas, like the western U.S., draw particular concern as introgression of a drought tolerance transgene from improved wheat cultivars into jointed goatgrass could result in a more adapted and competitive species, allowing it to expand its range, invade new habitats, and increase its competitiveness with domesticated and native species (Arnold, 1992; Snow and Palma, 1997; Stewart et al., 2003).

FREQUENCY AND FERTILITY

Bread wheat (2n=6x=42, genomes ABD) and jointed goatgrass (2n=4x=28, genomes CD) share the common ancestor *Ae. tauschii* Coss., the donor of the D genome (Kimber and Sears, 1987). Despite wheat and jointed goatgrass both being predominantly self-pollinating species, when these two species grow in close proximity and their flowering times overlap, cross-pollination leading to hybridization of these two species can occur (Perez-Jones et al., 2010; Zemetra et al., 1998). The difference in ploidy between wheat and jointed goatgrass - wheat is a hexaploid and jointed goatgrass is a tetraploid - does not prevent the production of F_1 hybrid plants. The occurrence and rate of hybridization is critically important and the F_1 is often the most studied generation of the introgression process as it serves as the first barrier of crop-to-weed gene flow. Guadagnuolo et al. (2001) observed an average hybridization rate of 1% in two populations and a hybridization rate of 7% in another population. In eastern Colorado, the average frequency of hybridization between imazamox resistant (IR) wheat and jointed goatgrass, when grown side by side, was 0.1% with a maximum observed hybridization rate of 1.6% (Gaines et al., 2008). When crop-weed hybridization occurs, domesticated crops typically serve as the pollen donor and the weedy species as the pollen recipient due to the higher volume of pollen being produced from the larger population of crop plants in relation to the lower volume of pollen produced by the smaller population of weedy plants (Ladizinsky, 1985). Despite this assertion, it is possible for jointed goatgrass to serve as the pollen parent during initial hybridization of these two species. The use of the wheat-specific chloroplast simple sequence repeat marker WCt-3 showed that 13.6% of the hybrid plants analyzed from a grower's field had wheat as the female parent, while the remaining plants had jointed goatgrass as the female parent (Hansen et al., 2010). In another study, 14.2 % of the hybrid plants examined had

wheat as the female parent while the other 85.8% of tested hybrid plants had jointed goatgrass as the female parent (Gandhi et al., 2006). Although hybridization is an important barrier to overcome for gene flow to occur, it does not guarantee that a novel gene will become fully introgressed into a weedy species' genetic background. Introgression of wheat genes is dependent on stabilizing the foreign gene sequence into the new genome, which only occurs after recurrent backcrossing to the wild species and persistence of the new genotype. Therefore, the rates at which backcrossing occur via pollination by jointed goatgrass after hybridization with wheat must be quantified in order to truly gauge the risk of introgression between these two species.

The lack of homologous chromosome partners for the A-, B-, and C-genome chromosomes in hybrid plants, (the wheat parent provides A-, B-, and D-genome chromosomes and the jointed goatgrass parent provides C- and D-genomes chromosomes), leads to unbalanced chromosome numbers and low fertility in the hybrid plants (Mallory-Smith et al., 1996). Wheat x jointed goatgrass hybrids were initially thought to be sterile due to the lack of chromosome pairings during meiosis with the exception of the D-genome chromosomes (Donald and Ogg, 1991; Johnston and Parker, 1929; Priadcencu et al., 1967). Hybrids are self-infertile due to male sterility but have shown low levels of female fertility (Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Snyder et al., 2000; Wang et al., 2001; Zemetra et al., 1998). Wheat x jointed goatgrass hybrids have been successfully backcrossed in the greenhouse with either wheat or jointed goatgrass as the recurrent parent (Mallory-Smith et al., 1996). Mean rates of female fertility of hybrids plants have been observed between 0.2% and 2.2% based on manual pollinations in the greenhouse with either wheat or jointed goatgrass pollen (Mallory-Smith et al., 1996; Schoenenberger et al., 2006; Wang et al., 2001; Zemetra et al., 1998). Since either

parent can serve as the pollen donor during the first backcross generation, it is important to understand the frequency of hybrid backcrossing to jointed goatgrass as this is the breeding direction required for the introgression of genes to the weedy species following initial hybridization. In a study where hybrids were pollinated in the field, Gandhi et al. (2006) observed 86.7% of BC_1 plants were backcrossed with wheat while 5.4% of BC_1 plants were backcrossed in the direction of jointed goatgrass. Seed set rates of hybrid plants in field studies have been comparable to backcrossing seed set rates observed in greenhouse studies. Field trials have found mean backcrossing rates between 0.42% and 3.8% with either wheat or jointed goatgrass serving as the pollen donor (Econopouly et al., 2011; Morrison et al, 2002; Snyder et al., 2000). This demonstrates that the seed set observed in greenhouse experiments was not an artifact of controlled emasculation and hand pollination but that BC_1 seedlings can be germinated from the seeds produced on hybrid spikes pollinated in the field.

With each successive backcross to jointed goatgrass there is a greater number of C-genome bivalent chromosome pairings during meiosis to complement the already established D genome. This increase in chromosome pairings results in increased male and female fertility of BC_1 plants compared to wheat x jointed goatgrass hybrid plants. Mean rates of female fertility of BC_1 plants have been estimated between 4.4% and 7.5% based on manual pollinations in the greenhouse with jointed goatgrass pollen (Mallory-Smith et al., 1996; Wang et al., 2001; Zemetra et al., 1998). A mean rate of BC_1 male fertility was estimated at 1.8% when BC_1 plants were used to pollinate emasculated jointed goatgrass plants (Wang et al., 2001). With increased female and male fertility observed in BC_1 plants, the potential exists for BC_1 plants to self-pollinate. A mean rate of BC_1 self-fertility has been observed at 0.06% (Snyder et al., 2000; Wang et al., 2001). Schoenenberger et al. (2006) observed the vast majority of their BC_1 plants

were completely self-sterile, with the exception of two individual plants producing self-seed at rates of 0.16% and 5.21%. The production of second generation backcross (BC_2) progeny serves as an important step in the introgression of wheat genes into the jointed goatgrass genome. Wang et al. (2001) observed BC_2 female fertility at 18.0% when pollinated by jointed goatgrass and male fertility of 8.9% when pollinating emasculated jointed goatgrass plants. The increase in both female and male fertility resulted in an observed self-fertility rate of 6.9% for the BC_2 generation (Wang et al., 2001). Wang et al. (2001) continued with backcrossed selfing generations and recorded self-fertility rates of 55.6% and 78.9% for the BC_2S_1 and BC_2S_2 generations, respectively. With partial self-fertility restored in the BC_2 generation and increasing in subsequent generations (Wang et al., 2001; Zemetra et al., 1998), BC_2 individuals could propagate by seed via self-pollination even if there is no jointed goatgrass in the vicinity for pollination. Isolated patches of BC_2 individuals would mark the beginning of a genetically unique jointed goatgrass population with the acquisition of novel genes from wheat.

The ability of BC_1 plants to backcross a second time to jointed goatgrass in the field has previously been examined in the Pacific Northwest (Mallory-Smith et al., 1999). In these field trials, the mean frequency of seed set on the BC_1 plants was 2.5% with individual plant seed set frequencies ranging from 0.2% to 5.2% for the 1996-97 growing season. In the 1997-98 growing season, the mean frequency of seed set on BC_1 plants was 7.5% with individual plants ranging from 1.6% to 20.3% seed set (Mallory-Smith et al., 1999). While these results constitute the first account of BC_1 plants' ability to produce seed when backcrossed to jointed goatgrass in field trials they do not allow conclusions concerning fertility of the seeds or the actual production or emergence of the BC_2 generation through the use of germination trials. The rates at which repeated backcrosses occur via natural pollination by jointed goatgrass and the fertility of these

backcrossed seeds will need to be quantified in order to fully assess the risk of gene introgression from wheat to jointed goatgrass. The longevity and usefulness of new wheat cultivars with novel traits are largely dependent on the means by which they are implemented. Adequate risk assessment and management studies need to be conducted to provide an accurate assessment of the risk of gene flow. Although backcrossing rates for BC_1 plants have been determined under greenhouse conditions, mean backcrossing rates recorded using hand pollination techniques do not simulate natural conditions in the plants' habitat and exclude factors such as pollen vectors, pollen production, anthesis date variability, flowering opening, and environmental variability (Ellstrand and Hoffman, 1990). Quantifying the rate of BC_2 production using jointed goatgrass as the sole pollen donor to pollinate BC_1 plants in a field study is necessary to fully assess the risk and occurrence of introgression. Field studies have shown that the rates of backcrossing to jointed goatgrass vary by both year and site (Econopouly et al., 2011; Snyder et al., 2000). The levels of gene flow from crops to weed species appears to depend on a multitude of spatiotemporal factors, while also being idiosyncratic, varying among individuals, populations, and years (Ellstrand and Hoffman, 1990). This evidence suggests that environmental conditions play an important role in the level of outcrossing that occurs. Multiple evaluations of backcrossing rates across varying environments in fields are necessary in order to determine the range of outcrossing rates, while identifying environments and conditions that lead to lower or higher rates of outcrossing. By determining the rates of backcrossing during multiple growing seasons, it may be possible to identify environmental conditions that present the greatest risk for gene introgression while also providing strategies that will decrease the chances of introgression of wheat gene sequences into jointed goatgrass populations.

PARENTAGE AND CHROMOSOME COUNTS

Wheat and jointed goatgrass are both members of the Triticeae tribe. The main genetic characteristic of this plant group is a basic chromosome number of seven (Monte et al., 1993). Wheat is a hexaploid (2n=6x=42, genomes ABD) and jointed goatgrass is a tetraploid (2n=4x=28, genomes CD). The F_1 hybrids of wheat x jointed goatgrass are pentaploid with 35 chromosomes (2n=5x=35, genomes ABCDD); 21 chromosomes (ABD) coming from wheat and 14 chromosomes (CD) coming from jointed goatgrass. These hybrids are phenotypically intermediate of wheat and jointed goatgrass. The sexual compatibility of these two species raises concerns for potential gene flow as the hybrids have the potential to serve as a bridge for gene movement from wheat to the weedy relative.

Due to the low fertility of wheat x jointed goatgrass hybrids, emasculation and manual pollination of hybrid plants in a greenhouse in order to create a large enough population of BC_1 plants for analyses can be impractical for some research facilities. Instead, researchers may choose to use isolated field plots with hybrid plants surrounded by jointed goatgrass to force the production of a first generation backcross in the direction of jointed goatgrass. Since much of the research being done on wheat gene introgression is in wheat growing areas, and with viable wheat pollen having been observed as far as 1,000 m from its pollen source (Virmani and Edwards, 1983), it can be difficult to position experimental field plots in complete isolation from wheat pollen. This potential lack of isolation allows a small possibility of experimental hybrid plants being pollinated by wheat pollen instead of jointed goatgrass. If the hybrids are backcrossed by wheat, then the genetic background of backcross progeny would become wheat-like. If the backcross progeny's genetic background is not moving in the direction of jointed goatgrass then the risk of introgression to a weedy species cannot be accurately assessed.

Therefore, it is important to know whether hybrids are backcrossed to wheat or jointed goatgrass when using field-produced BC_1 plants.

Researchers have found it difficult to determine the pollen parent of field derived BC_1 plants strictly using the number of chromosomes of an individual plant because of the range of chromosome numbers that have been observed in the BC_1 population (Wang et al., 2000). Although hybrid plants always have 35 chromosomes, its gametes can have a wide range of chromosome numbers outside the typical halving of its genetic information. This is due to differences in transmission of chromosomes from the A, B, and C genomes since they are present as univalent chromosomes and thus do not separate as homologous chromosome pairs. This leads to chromosome numbers of BC_1 plants being highly variable. Backcrossing of wheat x jointed goatgrass hybrids for several generations with jointed goatgrass would theoretically return the backcrossed plants chromosome number to that of jointed goatgrass (2n=4x=28). The restoration of 28 chromosomes would arise as the 7 C-genome chromosomes retained from the hybrid form bivalents with the newly introduced C-genome chromosomes with each successive backcross to jointed goatgrass, and the univalent A- and B-genome chromosomes are gradually selected against during unsuccessful meiotic division all while the 14 D-genome chromosomes are retained. Zemetra et al. (1998) observed chromosome counts for BC_1 plants with a range of 30-49, while another study recorded BC_1 plants to have an average of 40.9 and 44.7 chromosomes in 2000 and 2001, respectively (Hansen et al., 2010). The chromosome number of BC_1 plants whose pollen parent was jointed goatgrass ranged from 32 to 49, while the chromosome number of BC_1 plants whose pollen parent was wheat varied from 38 to 56 (Hansen et al., 2010). In another study the chromosome number of BC_1 plants whose pollen parent was jointed goatgrass ranged from 34 to 49, while BC_1 plants whose pollen parent was wheat varied

from 40 to 56 (Wang et al., 2002). The overlap in the chromosome numbers of the two types of BC_1 plants shows how the paternity of field derived BC_1 plants cannot be determined simply by counting the total number of chromosomes.

When extra chromosomes are present in a plant they are most often transmitted to progeny through female gametes (Schulz-Schaeffer and Haller, 1988). In the case of BC_1 plants having 56 chromosomes, 35 of these chromosomes would have come from the hybrid (maternal) as a result of an unreduced gamete and 21 of the chromosomes would have come from wheat (paternal). This would result in a BC_1 plant with seven C-genome chromosomes. In the case of the BC_1 plants having 49 chromosomes, 35 of these chromosomes would have come from the hybrid (maternal) as a result of an unreduced gamete and 14 of the chromosomes would have come from jointed goatgrass (paternal). This would result in a BC_1 plant with 14 C-genome chromosomes (Hansen et al., 2010).

It has been shown that plant phenotype and morphology are non-informative in determining the pollen donor due to the variation of phenotypes observed in BC_1 plants of both wheat and jointed goatgrass paternity. Therefore, one cannot determine paternity of the BC_1 generation based on a number of observable plant characteristics: coleoptile color, leaf width, presence or absence of hairs on the leaf margin, number of hairs on the leaf margin, number of veins in the leaf, spike appearance, spike length, plant height, growth habit, or number of spikes produced on the BC_1 plant (Snyder et al., 2000). Molecular markers are also an unreliable means for determining the pollen parent of BC_1 plants since a molecular marker unique to either wheat or jointed goatgrass could exist in both types of BC_1 plants and thus not provide information about the paternity of these plants (Wang et al., 2002).

A likely method of determining the paternity of a BC_1 plant is to count the number of C-genome chromosomes present in the plant's mitotic cells during metaphase. The C genome is unique to jointed goatgrass with the hybrid (2n=5x=ABCDD) only having one set of seven C-genome chromosomes. It has been proposed that BC_1 individuals with zero to seven C-genome chromosomes would have originated from a hybrid plant that was backcrossed with wheat while a BC_1 individual with seven to fourteen C-genome chromosomes could have originated from a hybrid plant that was backcrossed with jointed goatgrass (Wang et al., 2002). If a BC_1 plant had seven C-genome chromosomes, then its pollen donor would still be ambiguous. Genomic *in situ* hybridization (GISH) has been shown to be a powerful tool in identifying C-genome chromosomes (Linc et al., 1999; Wang et al., 2000; Wang et al., 2002). Wang et al. (2002) were able to show that the differences between the two BC_1 paternal types resulted in differences in the number of C-genome chromosomes. Hansen et al. (2010) identified the number of C-genome chromosomes from field derived BC_1 plants ranged from 3 to 14.

USING TRANSGENIC TECHNOLOGIES

The commercialization of biotech crops in 1996 began with 1.7 million hectares planted to biotech crop varieties (James, 2012). For sixteen consecutive years, land planted with biotech crops has increased. In 2012 the area planted to biotech crops covered 170.3 million hectares and were used by farmers in 28 countries worldwide (James, 2012). It is important to note that the majority of these, 20 of the 28 countries, were considered developing third-world countries (James, 2012). For the first time, developing countries grew more, 52% of the global biotech crops in 2012, than industrial countries and are expected to continue to exceed industrial countries in total hectares in coming years (James, 2012). Farmers in the United States

cultivated 8 different biotech crops including: maize, soybean, cotton, canola, sugarbeet, alfalfa, papaya, and squash (James, 2012). The dramatic increase in the number of transgenic plants and requests for field trial permits for transgenic plant development has been followed with increasing concern about the potential negative effects of commercial scale cultivation of GE crops.

Transgenic or GE refers to gene transfer via recombinant DNA methods in a laboratory. The development of recombinant DNA techniques allows scientists to insert any number of novel genes into a plant genome of interest and may include DNA regulatory sequences that alter gene expression. Genes of interest can be taken from plants, microbes, animals, or be uniquely synthesized. Because of the novelty a transgene can confer, many commercially important plants are being considered for breeding improvements via transgenic technologies.

Many of the applications of genetic engineering will likely benefit crop development and yield when grown within the borders of an agricultural setting; yet genetic engineering and commercial production in some plant species could lead to undesirable consequences for both agricultural and natural ecosystems (Ellstrand and Hoffman, 1990; Ellstrand et al., 1999; Stewart et al. 2003). Questions of whether transgenic crops could lead to serious, irreversible environmental problems have generated considerable debate. On one side, agronomists see that the phenotypes of transgenic cultivars are similar to the phenotypes of plant cultivars that are being selected for via traditional breeding methods (Miller and Conko, 2005). Since all grains grown commercially in the United States and elsewhere have been genetically improved by one technique or another, some feel GE crops are not inherently unfamiliar or risky (Miller and Conko, 2005). "It is the combination of phenotype and use that determines the risk of agricultural plants, not the process or breeding techniques used to develop them" (Miller and

Conko, 2005). Many individuals on this side of the argument feel transgenic technologies may even be safer than many classical techniques used in crop improvement, which include mutation breeding and wide-cross hybridization (Miller and Conko, 2005). These classical breeding techniques can result in uncharacterized modifications anywhere along the genome resulting in new and unknown genes, proteins, and secondary metabolites being introduced into the food chain (Miller, 1994; Miller and Conko, 2005). It is argued that transgenic technology is a more precise, more predictable plant improvement technique (Miller and Conko, 2005). In contrast, some argue that the ability to insert and express an unlimited number of genes or alleles from unrelated organisms makes transgenic technology a new and potentially dangerous technique that should be subjected to stricter regulations (Miller and Conko, 2005). One of the major concerns is that widespread cultivation of transgenic crops could speed the evolution of highly competitive weeds or pesticide-resistant insects (Dale et al., 2002). This risk applies to traditionally bred crops as well, but the highly publicized release of new GE cultivars has focused attention on this technology and its potential consequences.

RISK OF GENE MOVEMENT

Interspecific hybridization leading to gene flow has long been proposed as a mechanism for evolution in natural populations (Anderson, 1949). Gene flow between cultivated species and their weedy or wild relatives is not unique to wheat and jointed goatgrass. Plant breeders have used interspecies hybridization to exploit genetic diversity and to identify novel genes for crop improvement. However, sexual compatibility and gene flow between a domesticated species and a wild relative when not controlled is cause for concern. Twelve of the world's 13 most widely cultivated crops are known to hybridize with a related wild species (Ellstrand et al.,

1999) including canola (*Brassica napus* L.) (Brown and Brown, 1996; Jorgensen and Andersen, 1994), sorghum (*Sorghum bicolor*) (Arriola and Ellstrand, 1996), corn (*Zea mays*) (Doebley, 1984), rice (*Oryza sativa*) (Langevin et al., 1990), sunflower (*Helianthus* spp.) (Linder et al., 1998; Whitton et al., 1997), and cotton (*Gossypium* spp.) (Brubaker et al., 1993). Cases of gene flow from crops to their wild relatives provide examples of micro-evolution that may have important applied implications.

The concern of enhanced weediness of plant species has received considerable attention as more and more crops are being modified through the use of transgenic technologies with enhanced novel traits. Warwick et al. (1999) see the potential of transgenic crops to change populations of plants in three primary ways. The first is the escape and propagation of transgenic plants as volunteers in a field where the cropping system has changed, making these volunteers an unwanted, weedy plant (Warwick et al., 1999). The possibility of transgenic wheat becoming a weed in agricultural and disturbed sites does not seem to be a great risk unless a transgenic trait is able to turn wheat from an annual to a perennial; without this change wheat may struggle to survive outside of cultivation (Warwick et al., 1999). The second way transgenic plants could change plant populations is through the hybridization and subsequent introgression of transgenes into wild or weedy populations (Warwick et al., 1999). If the newly introgressed crop genes confer a fitness change in the weedy species, it would result in an alteration of natural gene frequencies. The third is genetic changes in populations of peripheral or unrelated species as a result of changes to the environment or agricultural practices where transgenic plants are released (Warwick et al., 1999).

In principle, plant cultivars developed by traditional breeding techniques pose the same risk of gene escape as transgenic varieties. In practice, it is unclear whether plants carrying

novel transgenic genes pose the same environmental risks as conventionally-bred cultivars with the same or similar phenotypes. Regardless of the method used for plant improvement, the risk of gene escape is dependent on the biology of the crop donating the gene, the wild plant receiving the gene, and the introduced gene. A crop that has undergone little domestication from its wild progenitor would provide the greatest risk of gene escape as it is still highly genetically similar to its wild relative resulting in minimal reproductive and genetic barriers (Ellstrand and Hoffman, 1990). Examining the wild plant, the greatest risk is associated with plants that are already common, highly weedy, and closely related to a domesticated crop species. The impact of gene escape will also depend on the nature of the gene. Because gene expression is dictated by a number of genes in an individual's genetic background through epistatic and pleiotropic interactions, a novel gene's expression in a wild plant population is very hard to predict as a gene may not behave in the same way as it moves across different genetic backgrounds. Even a gene conferring a fitness advantage will most likely not be retained in a wild population. Targeted introgression in plant breeding has shown that introgression of a specific gene is often associated with an assembly of linked alleles. We would thus expect a similar degree of linkage when transgenes introgress from crops to wild relatives. A transgene could thus be prevented from introgression into a wild population if it lowers the fitness or is tightly linked to a gene that lowers fitness of the weedy species (Stewart et al., 2003). Many of the traits that are the target of current plant breeding efforts have the potential to confer a significant fitness advantage if expressed in a wild plant. These traits may include but are not limited to tolerances to drought, salinity, pathogens, insects, heat, or frost. Yet, many of the traits that plant breeders have selected for in crops (e.g., dwarfing, absence of dormancy, and non-shattering inflorescences) are traits that provide little or no competitive advantage in the wild. An approach for containing

gene spread would be to create a tandem construct of tightly linked alleles to reduce the fitness of a weedy species that may acquire the novel transgene. This approach, termed transgenic mitigation (TM) is based on the premise that even mildly harmful TM traits would be eliminated from weed populations as weedy plants compete strongly among themselves (Daniell, 2002).

The most likely opportunity for the escape of a crop's engineered genes is through crop-weed hybridization (Ellstrand and Hoffman, 1990). Without hybridization, gene flow cannot occur. In order for the engineered trait to persist in a weedy population the hybrid must persist or the hybrid must backcross to the weed, allowing the engineered trait to enter the wild population via introgression under positive selection (Ellstrand and Hoffman, 1990). In cases where a crop is the result of a naturalized weed and the two species are sexually compatible and capable of pollen exchange, the escape of novel genes is inherently risky. Thus, the true danger lies less in the type of breeding technique used to incorporate a novel gene or at what frequency it occurs, but rather in the biology of the transferred gene and how it is expressed in the wild population.

The risk of gene introgression and possible invigoration of the weedy species is increased in proportion to the degree of sexual compatibility between the crop and its weedy relatives, as sexual compatibility increases the likelihood of the transfer of genes also increases. The ability of wheat and jointed goatgrass to hybridize successfully has been attributed to the fact that they share the same D genome ancestor, even though at least 12 *Aegilops* species have been able to form natural hybrids with hexaploid wheat with only some of them having a common genome with wheat (Van Slageren, 1994). The relative risk of gene escape is dependent on the level of cross-compatibility of the species involved and is thought to decrease across species that are more distantly related. A genetic barrier to introgression could lower the risk of transgene

escape from crops to wild relatives, whereas the lack of such a barrier could lead to substantial introgression and a high occurrence of transgene movement and persistence in a wild population. Genetic barriers that arise during meiosis because of the differences in ploidy and genomes between wheat (2n=6x=42, genomes ABD) and jointed goatgrass (2n=4x=28, genomes CD) may help to prevent the introgression of alleles from wheat to this wild relative. Despite the differences in ploidy and genomes, these two species are able to hybridize allowing for the possibility for novel genes to move from wheat to jointed goatgrass. The three proposed mechanisms of gene movement from wheat to jointed goatgrass are gene recombination through homologous chromosome pairing, translocation, and chromosome addition. The D-genome chromosomes of wheat pair with the D-genome chromosomes of jointed goatgrass, forming seven bivalents in the hybrids during meiosis. Therefore, if a novel gene is on the D genome of wheat, it can move to jointed goatgrass by homologous chromosome pairing and gene recombination (Perez-Jones et al., 2006a; Perez-Jones et al., 2006b). While it has been assumed that recombination between chromosomes from different genomes of *Triticeae* species does not occur, there is some evidence showing the potential for inter-genomic gene transfer (Cifuentes et al., 2006). The translocation of a chromosome segment is the second method of gene transfer that could occur from wheat to jointed goatgrass. It has been suggested that the occurrence and subsequent risk of gene flow from wheat to jointed goatgrass could be reduced by inserting transgenes or choosing novel genes that are located on the A- or B-genome chromosomes of wheat since these genomes are absent in jointed goatgrass. However, a transgene located on the A or B genome of wheat could be transferred to the C or D genomes of jointed goatgrass through a spontaneous translocation event. The occurrence of a translocation event, in which a portion of an A- or B-genome chromosome of wheat broke off and became attached to a jointed goatgrass

chromosome, was reported in a BC_2S_2 plant using genomic *in situ* hybridization (Wang et al., 2000). The third method of possible gene transfer from wheat to jointed goatgrass is whole chromosome retention. Wheat x jointed goatgrass backcross progenies with more than 28 chromosomes are retaining extra A- and/or B-genome chromosomes from wheat (Wang et al., 2000). If these extra chromosomes are homologous pairs, this extra pair of homologous chromosomes despite being from the A or B genomes of wheat are expected to be stable in jointed goatgrass over generations. Among the three mechanisms of gene movement, gene transfer from wheat to joined goatgrass by gene recombination (via the D genome) would be more common than gene transfer by translocation or chromosome addition. Lin (2001) tested the transferability of genes inserted into two different wheat genomes (B and D) into jointed goatgrass genomes (C and D). One wheat line was transformed with the glufosinate resistant *Bar* gene in the B genome and another line was transformed with the glufosinate resistant *Bar* gene in the D genome. Manually produced BC_1 plants were generated using jointed goatgrass as the recurrent parent. When the BC_1 plants were tested for the presence of the *Bar* gene, two-thirds of BC_1 plants produced from the D genome transformed line carried the resistance gene, whereas none of the BC_1 plants from the B genome transformed line carried the resistance gene (Lin, 2001). These results suggest that the transfer of transgenes located on the A or B genomes of wheat to jointed goatgrass is reduced compared to the D genome during sexual hybridization and backcrossing. The extent of this reduction is currently unknown and difficult to hypothesize. The persistence of foreign genes in a native population as a result of introgression will depend on whether the novel alleles are neutral, detrimental, or beneficially expressed in the genomic background under the environmental conditions of the population receiving the alleles. Gene movement under natural pollination conditions with minimal selection should be closely studied

in order to identify regions of the crop genome that have a lower rate of movement and are thus less likely to be transferred into new genomes. As more genomic and introgression information is gathered, transgenes should be targeted to areas that are least likely to move into a wild population to serve as a barrier to introgression.

The relative economic importance of a crop species may be an important indicator of its risk of gene flow. Since the most likely chance for the escape of a crop's novel genes is through crop-weed hybridization (Ellstrand and Hoffman, 1990), the opportunity for such hybridization to occur depends on the presence of a crop and weed that are genetically related and present together in both time and space. Crops with higher economic importance tend to be cultivated under larger areas. The larger the area dedicated to the cultivation of an economically important crop the greater the probability of a hybridization event with a weedy relative. The higher the number of hybrids in the field and the higher the number of wild plants present in the same field for backcrossing, the greater the chance that a novel gene in a domesticated species could move into a wild population. If a crop carrying a novel trait is to be released in the same geographic region as a sexually compatible wild relative, steps need to be taken to reduce the chances for crop-weed hybridization and subsequent backcrossing leading to introgression.

There are several strategies that can be used to reduce the risk of novel gene escape from cultivated crops into related wild species. One approach is spatial isolation of the two species in an attempt to prevent hybridization. If the crop and the weed species of concern are both highly selfing species, then isolation by distance may be a plausible approach to prevent their mating. Under certain cropping systems, cultural practices may provide sufficient reduction of crop-weed hybridization. If crops are planted so that their flowering times do not overlap with a weedy relative, then hybridization is prevented by restricting pollen flow at the critical time. The

development of wheat varieties tolerant to a variety of chemical herbicides would provide a means for controlling jointed goatgrass populations in agricultural fields. If herbicides are used in rotations it would preserve the herbicide resistance traits while also minimizing the risk of hybridization and backcrossing by controlling the number of jointed goatgrass plants present in a wheat field. We cannot assume that current methods and cultural practices are sufficient to contain engineered genes or that their effects on ecosystems will be minuscule. Nor can we assume that the hazards created by crop-weed hybridization are unique only to genetically engineered species.

Herbicide-resistant wheat has been developed using mutation breeding followed by selection of newly created genetic diversity with a broad spectrum herbicide of interest. Although this provides a means to control jointed goatgrass and other troublesome weed species, it provides the opportunity for the development of herbicide resistant jointed goatgrass via gene flow and by selecting individuals with genetic variation that confers resistance to the herbicide. The release of wheat cultivars resistant to Imazamox (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid) raised a major concern as introgression of this herbicide resistance gene into populations of jointed goatgrass would potentially increase weediness. Two herbicide-resistant hybrids produced by natural hybridization between an herbicide-resistant wheat variety and jointed goatgrass were identified in an imazamox-resistant wheat field (Seefeldt et al., 1998). The hybrid plants survived an imazamox application in the field, and produced seven BC_1 plants that were found to also be herbicide resistant. The sexual transfer of an herbicide resistance gene from wheat to jointed goatgrass in the field would reduce the life span of the herbicide resistant wheat cultivar and the herbicide as a tool in managing jointed goatgrass and other weeds. Since the

commercialization of biotech crops in 1996, herbicide tolerance continues to be the dominant biotech trait planted in farmers' fields. In 2011, herbicide tolerance in soybean, maize, canola, cotton, sugarbeet, and alfalfa covered 59% of the 160 million hectares of GE crops grown globally (James, 2011).

IN SITU HYBRIDIZATION

Ecological consequences of using GE wheat are difficult to predict and assess, yet research looking at movement of genetic information by genome and chromosome retention in wheat x jointed goatgrass backcross progeny could provide new insights into strategies to minimize the risk of gene escape from wheat. As mentioned in the previous section (Risk of Gene Movement), the three proposed mechanisms of gene movement from wheat to jointed goatgrass are gene recombination through homologous chromosome pairing, translocation events, and chromosome addition. Among these three mechanisms of gene movement, gene transfer from wheat to joined goatgrass by gene recombination (via the D genome) is expected to be more common than gene transfer by translocation or chromosome addition. Since the stable transfer of genes from the A or B genomes of wheat to jointed goatgrass requires that their chromosomes pair with each other during meiosis, the A- and B-genome chromosomes of wheat will likely be lost during the backcrossing of the hybrid to jointed goatgrass as only C- and D-genome chromosomes are introduced. This has led scientists to venture that novel transgenes inserted into the A or B genomes of wheat would minimize the escape and introgression of a transgene from GE wheat and made them prime candidates for transgene insertion. Pairing patterns of durum wheat (*Triticum durum* L.) x *Aegilops geniculata* hybrids showed that A-genome sequences were more likely to be transferred following hybridization and backcrossing

of these two species (Cifuentes et al., 2006). The identification of differences in chromosome addition or translocation of wheat sequences by genome into jointed goatgrass's genomes may provide insight into the occurrence or ease of transgene movement from GE wheat to jointed goatgrass.

The identification of individual chromosomes in plants has traditionally been based on morphological characteristics such as arm length ratios based on centromere position and heterochromatin and euchromatin banding patterns. Although these techniques have been successful in karyotyping chromosomes of many species, these techniques fail to differentiate chromosomes that are relatively uniform in size and heterochromatin banding patterns. *In situ* hybridization (ISH) protocols using fluorescently labeled DNA probes have proven to be a widely applicable cytogenetic technique in chromosome and genome research. ISH was first applied to plant chromosomes in wheat using rye repetitive DNA sequences (Rayburn and Gill, 1985). Probes using repetitive DNA sequences give species-specific hybridization patterns that allow chromosomes to be karyotyped (Kubaláková et al., 2005), alien chromosomes to be identified (Lapitan et al., 1986), and/or provide insight into the evolutionary relationships among species (Raskina et al., 2002). Despite the successes of ISH, the isolation and characterization of DNA banding patterns can be time consuming and uncertain when a high number of chromosomes or a wide range of unknown chromosomes are present. Another approach for chromosome identification using ISH is the use of labeled total genomic DNA as a probe to identify chromosomes from different genomic backgrounds. This technique was termed genomic *in* situ hybridization (GISH) (Schwarzacher et al., 1989) and has become an extensively used technique for the detection of alien chromosomes of distantly related species (Le et al., 1989; Mukai and Gill, 1991; Schwarzacher et al., 1992) as well as detection of chromosome

translocation events (Molnár-Láng et al., 2000; Schwarzacher et al., 1992; Wang and Zhang, 1996; Wang et al., 2000). GISH has become a versatile research tool because of its ability to characterize genomes and chromosomes in allopolyploid species and its ability to have multiple target sequences of chromosomal DNA identified simultaneously using multiple fluorescently labeled genomic probes (Han et al., 2003; Mukai et al., 1993; Sanchez-Moran et al., 1999; Shishido et al., 1998).

Polyploidy has been considered one of the most common occurrences associated with plant evolution, making polyploidy the rule not the exception (Soltis and Soltis, 1999). Polyploids are a significant force in plant evolution with approximately 70% of angiosperms being polyploids (Masterson, 1994). Bread wheat (AABBDD) has no direct hexaploid wild progenitor. Rather, it possesses three sets of homoeologous chromosomes originating from three wild diploid, grass species (Kilian et al., 2009). The D genome of bread wheat originated from the wild diploid *Ae. tauschii* through alloploidization with the domesticated, tetraploid *T. dicoccon* (AABB). The A and B genomes of durum wheat were the result of hybridization between the wild diploid *T. urartu* (AA) and a wild diploid B-genome donor, frequently reported to belong to the *Sitopsis* section of *Aegilops* (Kilian et al., 2011). Despite intensive research, the origin of the B genome of wheat still remains unknown although the genome of *Ae. speltoides* is the most closely related to the B genome of wheat when compared with other *Sitopsis* species (Raskina et al., 2002). Families of analogous, sometimes identical, nucleotide sequences are present in the DNA of polyploid organisms, and in some cases constitute a substantial proportion of the total genomic DNA of the individual. The wheat genome is comprised of 83% repeated sequences (Flavell et al., 1974). These repeated sequences can lead to false positives with GISH by binding non-specifically labeled sequences to non-specific DNA regions. This is referred to

as non-specific or cross-hybridization and is an important limitation of GISH. Discrimination of chromosomes by genome is easier between genomes of different and distant genera than between genomes within the same genus. Because total genomic DNA includes a wide range of multiple-copy sequences, it is unlikely to be genome or chromosome specific in a polyploid species. In fact, non-specific hybridization is greater for probes made from large DNA samples, due to their likelihood of including repetitive elements. To ensure specific labeling of the chromosomes by the genomic DNA complement, labeled dispersed repetitive sequences and homoeologous sequences need to be excluded or prevented from hybridizing to chromosomes while still allowing for the detection of single or low-copy DNA sequences.

Wheat and jointed goatgrass are both members of the tribe Triticeae and genomic DNA sequences have been found to be common between groups and genera within the Triticeae tribe (Flavell et al., 1981). When parental species share similar families of dispersed repeats, unlabeled genomic DNA or blocking DNA is often used in excess to prevent homoeologous chromosomes from being fluorescently labeled via cross-hybridization. The addition of an excess amount of unlabeled, blocking DNA from other species not used as a probe can increase the specificity of the labeled probe(s). Results from interspecific hybrids have shown that the addition of blocking DNA is not essential to differentiate the parental chromosomes from distantly related species (e.g. *Secale africanum* and *Hordeum chilense* (Schwarzacher et al., 1989)), but is essential when species are closely related (e.g. *H. vulgare* and *H. bulbosum* (Schwarzacher et al., 1992)). Using the blocking technique, genomic DNA from one species remains unlabeled and is allowed to hybridize to the chromosomal DNA sequences that are common between the blocking DNA and the DNA used as the labeled probe. The unlabeled blocking DNA hybridizes to the common DNA sequences along the chromosome spreads, while

the low copy genome specific sequences on the chromosome remain available as sites for probe hybridization. This technique works to block fluorescently labeled repetitive DNA from binding to non-specific sites along chromosomes while allowing species specific sequences to hybridize to their respective chromosomes or chromosome regions. However, if dispersed repeats of the parental sequences are too similar or extensive homogenization of DNA sequences between the parental genomes has occurred, the chromosomes belonging to the parental complements of the interspecific hybrids or allopolyploid species can no longer be discriminated reliably by GISH. This has been observed in *Brassica* amphidiploid species where the A and C genome components in *Brassica napus* could not be clearly distinguished from one another using GISH due to extensive homoeology between these two genomes (Snowdon et al., 1997). RFLP analysis has confirmed the proximity of rye (*Secale cereale*) and crested wheatgrass (*Agropyron cristatum*) to the three genomes of *T. aestivum* (Monte et al., 1993) making them potential candidates for blocking homoeologous sequences between the three genomes of wheat. GISH methods have been able to specifically label both rye and barley chromatin in a wheat background using total wheat DNA for blocking repetitive sequences (Islam-Faridi and Mujeeb-Kazi, 1995; Mukai and Gill, 1991). The use of blocking DNA allowed for the discrimination of chromosomes of wheatgrass origin from wheat, despite these genomes being closely related (Kosina and Heslop-Harrison, 1996).

To discriminate the A, B, and D genomes of wheat from the C and D genomes of jointed goatgrass, wheat genomic DNA cannot be used as a probe in wheat x jointed goatgrass backcross progeny to detect the movement or retention of the A or B genomes of wheat because of the shared D genome between wheat and jointed goatgrass. Wang et al. (2000) worked around this limitation by using DNA from *T. durum* (AABB) as their labeled probe and unlabeled jointed

goatgrass DNA as blocking DNA at a ratio of 1:500 (probe: blocking DNA). This was effective in suppressing the repetitive DNA sequences between *T. durum* and *Ae. cylindrica* in order to visualize A- and B-genome chromosomes in wheat x jointed goatgrass BC_2S_2 progeny. Wang et al. (2000) were able to show that BC_2S_2 plants with 29 chromosomes had one chromosome that fluoresced when hybridized with a *T. durum* probe, BC_2S_2 plants with 30 chromosomes had two chromosomes that fluoresced when hybridized with a *T. durum* probe, and BC_2S_2 plants with 31 chromosomes had three chromosomes that fluoresced when hybridized with a *T. durum* probe. This confirmed that extra chromosomes from the A or B genomes of wheat were being retained in wheat x jointed goatgrass backcross individuals with more than 28 chromosomes. Because of the genomic probe used, they were unable to determine whether the extra chromosomes were from the A or B genomes of wheat. Wang et al. (2000) also used *Ae. markgrafii* genomic DNA (CC) as a labeled probe, and unlabeled wheat DNA (AABBDD) as blocking DNA at a ratio of 1:100 (probe : blocking DNA) to visualize chromosomes of the C-genome. The wheat DNA served to block common DNA sequences while allowing the genome DNA sequences specific to *Ae. markgrafii* to fluorescently label the C-genome chromosomes. Wang et al. (2000) successfully showed seven fluorescently labeled C-genome chromosomes in wheat x jointed goatgrass hybrids. The same GISH procedure was performed on BC_2S_2 individuals and showed that all the BC_2S_2 individuals had 14 C-genome chromosomes including individuals with 29 and 30 chromosomes (Wang et al., 2000). GISH using each diploid progenitor of wheat and jointed goatgrass separately as probes would be a useful technique for detecting chromosome movement on an individual genome level. Few techniques are known for simultaneous identification of the four genomes that may be present in wheat x jointed goatgrass backcross progeny.

Analyses have shown that both the C and D genomes of *Ae. cylindrica* are very similar to the C and D genomes of its two diploid progenitors, *Ae. caudata* and *Ae. tauschii* (Friebe et al., 1992; Linc et al., 1999). This would allow genomic DNA from *Ae. caudata* and *Ae. tauschii* to serve as highly specific, probes for the identification of C- and D- genome chromosomes separately in wheat x jointed goatgrass backcross progeny. The genomic DNA of *T. monococcum* was shown to hybridize strongly with the A- genome of *T. durum*, enabling discrimination of its A- and B- genome chromosomes (Kosina and Heslop-Harrison, 1996). While Wang et al. (2000) were able to discriminate the A and B genomes of *T. durum* from the C and D genomes of *Ae. cylindrica* as well as the C genome of *Ae. markgrafii* from the A, B, and D genomes of wheat. Coupled together, these experiments would suggest that a technique using total genomic DNA from each of the four genomes that may be present in wheat x jointed goatgrass backcross progeny could be used as labeled probes in *in situ* hybridization experiments to differentiate each of the genomes.

In wheat fields there is a higher density of wheat pollen relative to jointed goatgrass pollen. This increases the likelihood that F_1 hybrids will be backcrossed to wheat rather than to jointed goatgrass. Using GISH it was determined that only 7 of 88 (8%) of BC_1 plants had more than 7 C-genome chromosomes (Hansen et al., 2010), an indication that the hybrids were backcrossed with jointed goatgrass leading to possible introgression.

Once wheat x jointed goatgrass backcross progeny are produced, the parental origin of the progeny's chromosomes, the stability of its karyotype, and whether there are any translocations or other chromosomal reorganizations will be important information in assessing the risk of transgene introgression. Cytogenetic analysis of backcross progeny will provide information as to the rate of A- and B-genome chromosome loss with additional backcrossing or

selfing. If the A- and B-genome chromosomes are lost as fertility is restored then concerns over the transfer of novel genes from wheat to jointed goatgrass can be minimized by placing the genes in the A or B genomes of wheat. If A- and B-genome chromosomes are capable of translocation events onto the C- and D-genome chromosomes, research showing which chromosomes these translocation events most often occur will provide a better understanding of how to reduce the occurrence of transgene movement.

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CHAPTER 1

Fertility of Wheat (*Triticum aestivum* L.) x Jointed Goatgrass (*Aegilops cylindrica* Host.) First Generation Backcross Progeny

INTRODUCTION

Jointed goatgrass (*Aegilops cylindrica* Host.) is an agricultural weed in the western United States. This grass is not native to the U.S., and has become a competitive weed in agricultural settings since its introduction from Eurasia as a contaminant of winter wheat (*Triticum aestivum* L.) seed in the late 19th century (Mayfield, 1927). This weed is predominantly found in fall-sown wheat where it infests 2 million ha of winter wheat and is spreading at a rate of 20,000-50,000 ha per year due to movement of jointed goatgrass seed by combines, grain trucks, and contaminated wheat seed (Donald and Ogg, 1991; Washington State University, 2009; USDA, 2013). The two species are similar in plant size and height, flowering timing, spikelet arrangement along the rachis of the spike, vernalization response, and germination timing (Donald and Ogg, 1991). Their genetic relatedness and similar development phenology allow these two species to hybridize, providing a bridge for gene flow from wheat to the weedy relative (Gaines et al., 2008; Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Morrison et al., 2002; Perez-Jones et al., 2010; Seefeldt et al., 1998; Wang et al., 2001; Zemetra et al., 1998).

Interest in the development of herbicide-resistant and environmental stress-tolerant, genetically engineered (GE) wheat cultivars has focused attention on the possibility of transfer of wheat genes into jointed goatgrass populations. Pollen-mediated gene flow leading to crop-weed hybridization and subsequent generations of recurrent backcrossing to the weedy species could lead to the introgression of advantageous genes from a crop into a weed population (Ellstrand

and Hoffman, 1990). Despite these same risks being present with traditionally bred crops, transgenic technology makes it possible for single genes or blocks of genes to be inserted into a genome of interest as a compact unit. Movement of a single wheat chromosomal segment, containing a novel transgene, into a jointed goatgrass genome could lead to the expression of the novel transgenic trait. Although there are a number of documented cases for gene flow from crops to wild relatives (Ellstrand et al., 1999), little information is available with regard to the long-term persistence of crop genes in wild populations or the impact of fitness-related genes on weedy species' population dynamics. Within the next decade, we will continue to see an increase in the number of GE crops that possess many yield-enhancing traits that are absent or rare in populations of wild relatives. Introgression of genes improving tolerance to biotic and abiotic stresses such as herbivory, pathogens, drought or salinity could increase the competitive ability of weeds and lead to new management challenges (Darmency, 1994; Warwick et al., 1999). Applied transgenic technology has been used to improve drought tolerance in maize and it is likely this same technology could be used to improve drought tolerance in wheat cultivars (Castiglioni et al., 2008). Semi-arid areas, like the western U.S., draw concern as introgression of a drought tolerance transgene from improved wheat cultivars into jointed goatgrass could result in a more adapted and competitive species, allowing it to expand its range, invade new habitats, and increase its competitiveness with domesticated and native species (Arnold, 1992; Snow and Palma, 1997; Stewart et al., 2003).

Bread wheat (2n=6x=42, genomes ABD) and jointed goatgrass (2n=4x=28, genomes CD) share the common ancestor *Ae. tauschii* Coss., the donor of the D genome (Kimber and Sears, 1987). Despite wheat and jointed goatgrass both being highly self-pollinating species, when these two species are grown in close proximity and their flowering times overlap, cross-

pollination leading to hybridization of these two species can occur (Gaines et al., 2008; Seefeldt et al., 1998). The difference in ploidy between wheat and jointed goatgrass (wheat is a hexaploid and jointed goatgrass is a tetraploid) does not serve as a genetic barrier to prevent the production of F_1 hybrid plants. Guadagnuolo et al. (2001) observed an average hybridization rate of 1% in two populations and a hybridization rate of 7% in another population. In eastern Colorado, the average frequency of hybridization between imazamox resistant (IR) wheat and jointed goatgrass, when grown side by side, was 0.1% with a maximum observed hybridization rate of 1.6% (Gaines et al., 2008). When crop-weed hybridization takes place, domesticated crops typically serve as the pollen donor and the weedy species as the female parent (Ladizinsky, 1985). Although hybridization is the first requirement for gene flow to occur from wheat to jointed goatgrass, it does not guarantee that a novel gene will become fully introgressed into the weedy relative's genetic background. The introgression of wheat genes is dependent on stabilizing these gene sequences into the new, foreign genome of jointed goatgrass, which only occurs after recurrent backcrossing to jointed goatgrass and persistence of the new genotype. Therefore, the rates at which backcrossing occurs via pollination by jointed goatgrass after hybridization with wheat must be quantified in order to gauge the risk of introgression of wheat gene sequences into jointed goatgrass.

The lack of homologous chromosome pairs for the A-, B-, and C-genome chromosomes in hybrid plants, (the wheat parent provides A-, B-, and D-genome chromosomes and the jointed goatgrass parent provides C- and D- genome chromosomes), results in the low fertility observed in hybrid plants (Mallory-Smith et al., 1996). Because of the lack of chromosome pairing during meiosis, wheat x jointed goatgrass hybrids are self-infertile due to complete male sterility but have shown low levels of female fertility (Guadagnuolo et al., 2001; Mallory-Smith et al., 1996;

Wang et al., 2001; Zemetra et al., 1998). Wheat x jointed goatgrass hybrids can only set viable seed if fertilized with either wheat or jointed goatgrass pollen. Mean rates of female fertility of hybrid plants have been observed between 0.2% and 2.2% based on manual pollinations in the greenhouse (Mallory-Smith et al., 1996; Schoenenberger et al., 2006; Wang et al., 2001; Zemetra et al., 1998). Seed set rates on hybrid plants in field studies have been comparable to backcrossing seed set rates observed in greenhouse studies. Field trials have found backcrossing rates between 0.42% and 3.8% with either wheat or jointed goatgrass serving as the pollen donor (Econopouly et al., 2011; Morrison et al., 2002; Snyder et al., 2000). This demonstrates that the seed set on hybrid plants observed in greenhouse experiments was not an artifact of controlled emasculation and hand pollination but that BC_1 seedlings can be germinated from the seeds produced on hybrid spikes pollinated in the field.

With each successive backcross to jointed goatgrass there is a greater number of potential C- and D-genome bivalent chromosome pairings that can occur during meiosis. This results in increased seed set on BC_1 plants compared to wheat x jointed goatgrass hybrid plants due to both increased female fertility and increased male fertility. Rates of female fertility of BC_1 plants have been estimated between 4.4% and 7.5% based on manual pollinations in the greenhouse with jointed goatgrass pollen (Mallory-Smith et al., 1996; Wang et al., 2001; Zemetra et al., 1998). A rate of BC_1 male fertility was estimated at 1.8% when BC_1 plants pollinated emasculated jointed goatgrass plants (Wang et al., 2001). Backcrossing rates found by using hand pollination do not simulate natural conditions in the plants' habitat and exclude factors such as pollen vectors, natural seed production, anthesis date variability, flower opening, and environmental variability (Ellstrand and Hoffman, 1990). Although hand pollination is useful

for assessing the ability of two species to cross or the fertility of a species, it is not an accurate measure of the backcrossing ability of two species in nature.

With increased female and male fertility observed in BC_1 plants, the potential exists for BC_1 plants to self-pollinate. Nevertheless, self-pollination rates are very low as shown by two previous reports. Schoenenberger et al. (2006) observed that almost all of their BC_1 plants were completely self-sterile, with only two individual plants producing self-seed at a rate of 0.16% and 5.21%, while BC_1 self-fertility was observed at 0.06% by Wang et al. (2001). A rate of backcrossing of BC_1 plants to jointed goatgrass alone has not been determined under field conditions. The rates at which repeated backcrosses occur via natural pollination by jointed goatgrass will need to be quantified in order to fully assess the risk of gene introgression from wheat to jointed goatgrass. Using jointed goatgrass as the sole pollen donor in a field study is necessary as this is the direction of backcrossing required to restore the weedy genome after the initial interspecific hybridization event. The second generation backcross (BC_2) serves as an important step in the transfer of wheat genes to the jointed goatgrass genome. With partial self-fertility restored in the BC_2 generation (Wang et al., 2001), these individuals could propagate by seed via self-pollination even if there is no jointed goatgrass in the vicinity. If these BC_2 individuals acquired genes from wheat not already present in jointed goatgrass, isolated patches of advanced backcross, self-pollinating jointed goatgrass plants would mark the beginning of a genetically unique jointed goatgrass population with the acquisition of novel genes from wheat.

A field study has not been conducted for seed production on BC_1 plants in the central Great Plains, an important region for dry land wheat production that is also infested with jointed goatgrass. Field studies in Colorado have shown that the rates of outcrossing in jointed goatgrass varied both by year and site (Econopouly et al., 2011). This evidence suggests that

environmental conditions play an important role in the level of outcrossing that occurs. Multiple evaluations across varying environments are necessary in order to determine the range of outcrossing rates, while identifying environments and conditions that are liable to produce higher rates of outcrossing and thus increase the opportunity for gene movement from wheat to a weedy species. The rate of backcrossing will be informative for understanding how frequently BC_1 plants produce viable seeds under natural field conditions when pollinated by jointed goatgrass, which acts as the mechanism for transfer of wheat genes into the jointed goatgrass genome.

In this study, two years of field trials and greenhouse studies were conducted as a component of assessing the risk of gene introgression from wheat to jointed goatgrass. The objectives of this research were (1) to estimate the backcrossing rate of BC_1 x jointed goatgrass under field conditions, (2) to estimate female fertility of BC_1 plants by hand pollination with jointed goatgrass to determine the level of female fertility in our population, and (3) to estimate the level of self-fertility of BC_1 plants under greenhouse conditions to validate the backcrossing rates observed in the field trials.

MATERIALS AND METHODS

Progeny/Experimental Material Development

Hybrid seed was produced in the greenhouse in 2007 using the hard red winter wheat cultivar 'Above' (Haley et al., 2003) and jointed goatgrass seed collected in Platner, CO. Since wheat spikelet florets are larger than those of jointed goatgrass, wheat was used as the female parent in order to ensure full emasculation while preventing self-pollination of the wheat plant. Florets of the wheat plants were hand-emasculated and manually pollinated with jointed goatgrass pollen to produce hybrid seed following the method of Zemetra et al. 1998. Seed

collected from the wheat spikes was planted in 5-cm square pots containing potting soil (Fafard #2 SV, Conrad Fafard, Inc., Agawam, MA 01001) and germinated in the greenhouse. The resulting hybrid plants were transplanted into a field plot at the Colorado State University Agricultural Research, Development, and Education Center (ARDEC) in Fort Collins, CO, during the 2007-08 growing season (Econopouly et al., 2011). Jointed goatgrass from Paoli, CO, was seeded over the entire plot, both within and between rows, to force close proximity of the hybrid plants with jointed goatgrass pollen. Since hybrid plants are self-infertile due to male sterility (Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Wang et al., 2001; Zemetra et al., 1998), viable seed on these hybrid plants could only have been produced through an outcrossing event. Spikes were collected from over 100 hybrid plants, and a random subset of the spikes from these hybrids were planted into 27 x 53-cm flats containing potting soil in the summer of 2010 and again in 2011. Greenhouse conditions consisted of day- and night-time temperatures of approximately 24/18˚C. Plants were germinated under natural light conditions supplemented with 400W sodium halide lamps to provide a consistent 16-h photoperiod. Seedlings that germinated from the hybrid spikes were the result of a backcrossing event with jointed goatgrass and were identified as first generation backcross (BC_1) plants.

FIELD TRIALS

Backcrossing Rates

Field trials were conducted to estimate the rate at which BC_1 plants backcross to jointed goatgrass when jointed goatgrass serves as the predominant pollen source under field conditions. The experimental plots were planted at ARDEC in Fort Collins, CO, during the 2010-11 and 2011-12 growing seasons. Minimum and maximum temperature, relative humidity, wind gust

speed and precipitation amounts were obtained from the Colorado Agricultural Meteorological Network (Colorado State University, 2010) for the growing season (Figure 1.1).

Sixty-five BC_1 plants that germinated from the seed set on the hybrid spikes as a result of backcrossing to jointed goatgrass at ARDEC during the 2007-08 growing season were transplanted into a research plot in a grid pattern with 0.6 m spacing between plants in all directions in the fall of 2010. Jointed goatgrass seed collected from Paoli, CO, was planted within and between rows of BC_1 plants over the entire plot to serve as the dominant pollen source. A border of Triticale (x *Triticosecale*) was planted along the perimeter of the plot to act as a wind and external pollen buffer for the research area. In order to determine if environmental variation affects the rate of a second generation of backcrossing, 45 BC_1 plants that germinated from the hybrid spikes that were backcrossed to jointed goatgrass at ARDEC during the 2007-08 growing season were transplanted into a research plot at ARDEC in a grid pattern with 0.9 m spacing in one direction and 1.4 m spacing between plants in the other direction in the fall of 2011. Again, jointed goatgrass seed collected from Paoli, CO, was planted within and between rows of BC_1 plants over the entire plot to serve as the dominant pollen source. Due to a later planting date for 2011 fall-planted BC_1 plants, and the high percentage of winter injury and plant death observed during the 2010 growing season, 0.12 m medium clear plastic plant humidity domes (The Garden Cloche, Fort Worth, TX) were placed over each BC_1 plant and securely anchored with metal stakes into the ground in an attempt to increase survivorship during early establishment. BC1 plants and jointed goatgrass seed were irrigated just after planting to aid in establishment in 2010-11 and in 2011-12.

In both seasons, jointed goatgrass tiller density for the experimental plot was determined by measuring the area around four randomly selected BC_1 plants, cutting all the jointed goatgrass

at the end of the growing season, and counting the tillers by hand. Anthesis dates for both BC_1 plants and jointed goatgrass were recorded based on the presence of exposed anthers of the florets on the spike of the main tiller to determine if adequate overlap of flower maturity was observed between BC_1 plants and jointed goatgrass. Distance of the nearest wheat field to the experimental plot was determined for each growing season.

As the chromosome compliment of wheat x jointed goatgrass backcross progeny approach that of jointed goatgrass due to repeated backcrossing, many of these backcross progeny are expected to show strong jointed goatgrass-like phenotypes. Since seed shattering is a common characteristic of jointed goatgrass, perforated plastic pollination bags were tied around each BC_1 plant as they began to dry in the field in order to collect the spikes of BC_1 plants that may be prone to shattering. The BC_1 plants were collected by cutting all the tillers at ground level. The plastic pollination bags were removed from the plants once indoors, where spikes were clipped, counted, bagged, and labeled in envelopes for each BC_1 plant to keep a record of plant identity. Spikes from each BC_1 plant were stored at room temperature for a minimum of 16 weeks for post-ripening before germination trials in order to ensure non-dormancy of seeds (Fandrich and Mallory-Smith, 2006). It has been shown that as the duration of after-ripening increases, jointed goatgrass seed germinates earlier, at faster rates, and to greater final percentages compared to non after-ripened seed (Fandrich and Mallory-Smith, 2006).

GREENHOUSE TRIALS

Female Fertility

The female fertility of BC_1 plants was determined by manually pollinating emasculated BC_1 spikes with jointed goatgrass pollen. The BC_1 plants used for this experiment were a subset of BC_1 plants that germinated from the hybrid spikes collected at ARDEC during the 2007-08 growing season but not used in the field trials (mentioned previously). Again, plants that emerged from the seeds collected from the hybrid spikes were assumed to be BC_1 plants and were vernalized at approximately 3˚C with a 10-h photoperiod under fluorescent lights for 8 weeks. After vernalization, plants were transplanted into 5-cm square pots with potting soil and transplanted again into 15.5-cm diameter circular pots with potting soil, when tillering began, with a slow release plant fertilizer (Osmocote 14-14-14). Anthesis and stigma maturity of individual florets of the BC_1 plants were not synchronous along the length of the spike. The asynchrony of anthesis forced emasculation of the spikes in 2 to 3 stages with 2 to 3 days between each stage. Multiple manual pollinations with jointed goatgrass spikes were made to overcome the asynchrony of stigma development. Emasculated and pollinated spikes were kept under closed waxed pollination bags to prevent pollen contamination. Plants were grown in the greenhouse with day- and night-time temperatures of approximately 24/18˚C. Plants were grown under natural light conditions supplemented with 400W sodium halide lamps to provide a consistent 16-h photoperiod.

Self-Fertility

Self-fertility of BC_1 plants was examined by allowing BC_1 plants grown in the greenhouse to self-pollinate. Self-pollination was examined using two different methods. The

first method involved covering single spikes of BC_1 plants with wax pollination bags. This method guaranteed viable pollen produced on a spike would be the sole pollen source available to any receptive stigmas of the same spike, ensuring self-pollination. The second method used perforated plastic pollination bags to cover all the spikes of a BC_1 plant. This method prevented external pollen from pollinating the plant but allowed greater pollen availability for BC_1 plants that produced multiple spikes while allowing florets with asynchronous maturity the opportunity to be self-pollinated by another floret, thus allowing self-pollination to occur as a whole-plant event rather than a single-spike event.

GERMINATION STUDY

Controlled germination studies were conducted as the primary means of quantifying the rate of backcrossing in the field trial as well as female fertility and self-fertility in the greenhouse trials. The number of spikes and spikelets per spike produced were counted and recorded for each plant from both the field and greenhouse trials. Natural germination conditions were simulated by allowing seeds to remain in the spikelet. Intact spikes and shattered spikelets were planted in 27-by-53-cm flats with potting soil. Non-shattered spikelets of a single spike were disarticulated and planted in a single row, ordered from the basal spikelet to the tip spikelet to allow for identification of germination location along the length of the spike. Both shattered and non-shattered spikelets were planted on a 3-cm-deep layer of potting soil in 12 rows, 4 cm apart, covered with an additional 2 cm of potting soil. Flats were placed in a greenhouse with conditions consisting of day- and night-time temperatures of approximately 24/18˚C. Plants were grown under natural light conditions supplemented with 400W sodium halide lamps to provide a consistent 16-h photoperiod. A row of jointed goatgrass spikelets was planted within

each flat to serve as a positive control of the growing conditions. Flats were watered daily and left in the greenhouse for a minimum of four weeks to allow for delayed germination. Fandrich and Mallory-Smith (2005) found that the time needed for germination of both the primary and secondary position of each spikelet was 10 d. Germination of both primary and secondary seed was observed in this study, confirming the adequacy of the four weeks used.

At the end of the germination period the number of seeds that germinated and had seedling emergence were recorded for each plant and given an identification number. Seedlings were transplanted into 5-cm square pots with potting soil. Plants were visually assessed before and after vernalization in order to confirm backcross identity visually. The frequency of backcrossing (BC_2 germination-Field), female fertility (BC_2 germination-Greenhouse), and selfing (BC_1S_1 germination-Greenhouse) were defined as $=Y/n$, where Y is the number of germinated seedlings and n is the number of florets. Since there are typically 1 to 3 viable florets per spikelet (Donald and Ogg, 1991; Van Slageren, 1994), with over 99% of spikelets producing only one or two seeds (Donald and Zimdahl, 1987), the total number of spikelets was multiplied by two to give n, as per Snyder et al. (2000).

RESULTS

FIELD TRIALS

Backcrossing Rates

The dates for anthesis of field-grown BC_1 plants varied among individuals and between the two growing seasons. The first day of anthesis for BC_1 plants was not synchronous for all plants and occurred intermittently from 5 to 23 June in 2011 and from 22 May to 7 June in 2012. The degree of overlap in flowering time for BC_1 plants and jointed goatgrass varied for the two

growing seasons: first anthesis for jointed goatgrass was also not synchronous and in 2011 the first date of anthesis for jointed goatgrass plants occurred in two main flushes. Half of the field had jointed goatgrass plants that showed their first anthesis on 7 June and the remaining half of the field of jointed goatgrass showed its first flush of anthesis on 11 June, while in 2012 anthesis for jointed goatgrass showed similar variation of first anthesis. Several areas of jointed goatgrass in the field showed first anthesis on 6 June while other sections showed first anthesis on 11 June, with the latest quadrants of jointed goatgrass reaching first anthesis on 14 June. BC_1 plants continued with floral receptiveness for several weeks after first anthesis due to asynchronous spike heading of BC1 plants with an abundant number of tillers. Overlap in floral development of both BC_1 plants and jointed goatgrass allowed for the level of outcrossing between these two species to be observed via the rate of backcrossing for both growing seasons.

Environmental variation was observed between the 2010-11 and 2011-12 growing seasons in Fort Collins, CO (Figure 1.1). During June, the month of flowering, the mean minimum relative humidity was 22.2% for the 2010-11 growing season and 12.1% for the 2011-12 growing season. The mean maximum and mean minimum temperatures were 27.1/9.3 ˚C for 2010-11 and 32.1/11.5 ˚C for 2011-12. The cumulative precipitation in June for the 2010-11 growing season was 64.5 mm, whereas the cumulative precipitation in June for the 2011-12 growing season was 3.8 mm. The average maximum daily wind gust in June for the 2010-11 season was 7.9 m/s with a minimum of 3.9 m/s and a maximum of 16.3 m/s. The average maximum daily wind gust in June for the 2011-12 growing season was 9.6 m/s with a minimum of 5.4 m/s and a maximum of 15.3 m/s. During the 2010-11 growing season, from planting in September 2010 through harvest in July 2011, the experimental plot received a total of 336.0 mm of rain. The 2011-12 growing season received 25% less rain with a total of 254.3 mm from

planting to harvest. The average jointed goatgrass tiller count during anthesis was 3,662 tillers per m^2 for the 2010-11 growing season and 815 tillers per m^2 for the 2011-12 growing season.

The production of BC_2 seed as a result of pollination by jointed goatgrass was evaluated in the germination studies using spikes collected from 14 plants from the 2010-11 growing season and 6 plants from the 2011-12 growing season. The mean number of spikelets produced per BC_1 plant was 236.8 for 2010-11 and 353.7 for 2011-12 corresponding to an average of 473.6 florets per plant in 2010-11 and 707.3 florets per plant for 2011-12. Forty-three BC_2 seedlings emerged from a total of 3,315 spikelets (6,630 florets) in the germination study, which gave a mean backcrossing rate of 0.648% for the 2010-11 growing season. One BC_2 seedling emerged from a total of 2,122 spikelets (4,244 florets) in the germination study giving a mean backcrossing rate of 0.024% for the 2011-12 growing season. The average backcrossing rate for the two growing seasons was 0.404%. There was no correlation between the first date of anthesis of BC1 plants and backcrossing rate for individual BC_1 plants.

The frequency of BC_2 seedling germination per BC_1 plant did not follow a normal distribution (Figure 1.2). Median backcrossing rates were estimated at 0.041% in 2010-11 and 0.0% in 2011-12. Seven of the 14 BC_1 plants tested during the 2010-11 growing season did not produce seed that germinated into BC_2 seedlings; this resulted in these individuals having an observed backcrossing rate of 0.0%. The highest number of BC_2 seedlings derived from by a single BC_1 plant was 17, from a BC_1 plant grown during the 2010-11 growing season that produced 49 spikes and 429 spikelets (backcrossing rate of 1.98%). However, the highest backcrossing rate observed for a single BC_1 plant was 2.19%. Five of the 6 BC_1 plants tested during the 2011-12 growing season did not give rise to any BC_2 seedlings. The single BC_2 germination event recorded for the 2011-12 growing season was observed on a BC_1 plant that

produced 38 spikes with a total of 311 spikeletes (622 florets) resulting in a backcrossing rate of 0.16% for this individual plant. Due to the small sample size and the variation in backcrossing rates, large stand errors were observed for each growing season making 95% confidence intervals non-descriptive for each growing season.

GREENHOUSE TRIALS

Female Fertility

The level of female fertility of BC_1 plants was tested using 66 BC_1 plants grown in the greenhouse during the summer of 2011. The mean number of spikelets emasculated and manually pollinated per BC_1 plant was 24.7, corresponding to an average of 49.4 florets per plant. A single plant germinated from 1,632 spikelets (3,264 florets) in the germination studies which gave a mean observed female fertility rate of 0.03%.

Self-Fertility

To validate the backcrossing rates observed in the field trials, the level of self-fertility of BC_1 plants was tested using two different self-pollination methods. For the individually bagged spike method, spikes were collected from 17 plants. The mean number of spikelets planted in the germination trials per BC_1 plant was 20.7 which corresponded to an average of 41.4 florets per BC_1 plant. Zero seedlings emerged from spikes from the bagged method giving a mean observed self-fertility rate of 0.0%. For the whole bagged plant method, spikes were collected from 37 plants. The mean number of spikelets planted in the germination trials per BC_1 plant was 316.1 which corresponded to an average of 632.3 florets per BC_1 plant. One seedling

emerged from spikes from the bagged plant method giving a mean observed self-fertility rate of 0.004%.

DISCUSSION

The production of BC_2 seed on first generation backcross progeny, with jointed goatgrass as the recurrent pollen donor, has previously been reported at rates of 4.4% to 7.5% (Mallory-Smith et al., 1996; Wang et al., 2001; Zemetra et al., 1998). Although these studies showed the possibility of producing BC_2 progeny after two generations of backcrossing jointed goatgrass to wheat x jointed goatgrass hybrids, these rates are based on emasculation and manual pollination techniques in a greenhouse and thus may not be representative of field conditions. Field trials examining the rate of backcrossing across multiple growing seasons are thus needed in order to understand the risk of introgression of wheat genes to jointed goatgrass under natural conditions as environmental factors will dictate the rate of gene flow. Field studies with hybrids have shown that the rates of backcrossing to jointed goatgrass vary both by year and site (Econopouly et al., 2011; Snyder et al., 2000). The levels of gene flow from crops to weed species appear to depend on a multitude of spatiotemporal factors, while also being idiosyncratic, varying among individuals, populations, and years (Ellstrand and Hoffman, 1990). In order to provide the most accurate rate of backcrossing of BC_1 plants to jointed goatgrass, field trials were undertaken to quantify the rate of backcrossing for two growing seasons in Colorado. This is the first demonstration that under field conditions BC_1 plants can produce BC_2 seedlings when backcrossed to jointed goatgrass for a second consecutive generation. The field trials gave an observed mean rate of backcrossing between BC_1 plants and jointed goatgrass of 0.648% in 2010-11 and 0.024% in 2011-12. From the two years of field trials, 44 BC_2 seedlings were

produced from 20 BC_1 plants used in the germination trials. This equated to each BC_1 plant producing just over two BC_2 plants. This suggests that a population of BC_1 plants can double its size during the second generation of backcrossing to jointed goatgrass. Previous studies have reported that only two generations of backcrossing wheat x jointed goatgrass hybrids to jointed goatgrass are required to markedly increase male and female fertility to a level where self-fertility is realized (Wang et al., 2001; Zemetra et al., 1998). With partial self-fertility restored in the BC_2 generation, these progeny can now set seed via self-pollination even if there is no jointed goatgrass in the vicinity. Thus, the production of BC_2 seed in the field could allow the persistence of subsequent generations of wheat x jointed goatgrass progeny in the environment, providing a possible mechanism for introgression of wheat genes into jointed goatgrass populations.

The backcrossing rates from the two years of field trials reported here fall within the range of previously reported rates (0-22.3%) of backcrossing to first generation backcross progeny (Wang et al., 2001; Zemetra et al., 1998) but represent the lower end of this range. Several factors may have led to the lower observed mean backcrossing rate observed in this study. In the field study, dates of first anthesis were not identical between jointed goatgrass and BC_1 plants which led to differences in the duration of anthesis overlap between jointed goatgrass and BC_1 plants. The dates of anthesis for jointed goatgrass were more consistent and began in a narrower time frame than the dates of anthesis for BC_1 plants. Asynchrony between stigma receptivity of the BC_1 plants and pollen shed from the jointed goatgrass plants would result in fewer pollination events and thus low seed set.

Previous reports of backcrossing rates have been performed as greenhouse studies using manual pollination techniques. These types of studies do not rely on natural means of pollination

as they optimize the synchrony of stigma receptiveness and optimize the chances of fertilization by minimizing the distance the pollen has to travel from pollinator to pollen receptor. Thus, emasculation and manual pollination techniques may not accurately estimate the rate of seed production as it would occur in the field. The reported backcrossing rates from greenhouse experiments are most often labeled as 'female fertility rates'. Since they optimize pollination during stigma receptiveness, they are a more accurate representation of the maximum floret fertility of BC_1 plants. The highest backcrossing rates observed in past greenhouse studies (7.5%) may not only be the result of optimized pollination timing and placement in the greenhouse but also due to their use of embryo rescue techniques (Mallory-Smith et al., 1996). Embryo rescue germination techniques would result in values higher than would be observed in studies designed to simulate natural germination conditions, as attempted in this study. Although the rates observed in emasculation and pollination trials provide evidence of the possibility of backcrossing for a second generation, there may be some disparity regarding how these values correlate to backcrossing rates as they would occur in the field.

The field experiments were designed for jointed goatgrass to be the only available pollen source. Pollination of BC_1 plants by jointed goatgrass would produce the second generation of backcrossing in the direction of the wild parent. In our greenhouse study when attempting manual pollinations of BC_1 plants, delivering mature pollen from jointed goatgrass to BC_1 plants during stigma receptivity served as a limiting factor in providing maximum pollination opportunity for all florets. Despite multiple pollination attempts per emasculated spike, some florets may have only been receptive during one manual pollination attempt as floret maturity was not synchronized along the length of the spike. BC_1 plants that were emasculated and manually pollinated by jointed goatgrass in the greenhouse trials gave a mean female fertility

rate of 0.03%. Wang et al. (2001) observed a mean BC_1 female fertility of 4.4%. Our lower observed female fertility rate may have been a direct result of limited pollen. Backcrossed seed set also varies by the availability of pollen as well as the distance between pollen source and the maternal parent. A higher backcrossing rate was observed in the 2010-11 field trial than was recorded for female fertility in our greenhouse trial. This can be explained by a larger amount of pollen being available in the field where jointed goatgrass was planted within and between rows of BC_1 plants which would allow for multiple pollinations to occur during stigma receptivity. This was not the case in the greenhouse trial, where the pollination bags limited the pollen availability to the number of direct applications of a single spike. The availability of pollen may also explain why the backcrossing rate for 2011-12 was significantly lower than that for 2010-11. The 2010-11 growing season had significantly higher jointed goatgrass tiller density during anthesis when compared to 2011-12. It has been shown that wheat cultivars often require several pollination events to stimulate the recipient flower to allow for pollen growth on the stigma (Fritz and Lukaszewski, 1989). The probability of such repeated events is certainly higher within a field with thousands of flowers producing pollen than a field with fewer flowers for pollination. The spikes of *Aegilops* species and bread wheat require 3 to 5 days to complete flowering (Boguslavsky, 1979; Peterson, 1965), and the duration of flowering is longer for *Aegilops* species, compared to wheat, because of asynchronous tillering (Van Slageren, 1994). This continuous flowering period of *Aegilops* increases the chances of cross pollination with cultivated wheat. During 2011-12, both BC_1 plants and jointed goatgrass were much shorter (data not shown) compared to the 2010-11 growing season. The between-row planting of jointed goatgrass were also farther from the BC_1 plants during the 2011-12 season. Any one of these variables, such as, - greater distance, fewer plants, or shorter plants- would make it more difficult

to backcross jointed goatgrass to BC_1 plants. All of these variables together may have resulted in a much lower observed backcrossing rate in 2011-12 than in the 2010-11.

Variation in backcrossing rates can also be due to differences in environmental conditions. Seed set on hybrid plants has been shown to be variable based on environmental variation across both years and locations (Econopouly et al., 2011; Snyder et al., 2000) due to differences in pollen dispersal. Environmental conditions, including but not limited to day and night temperature, wind speed and direction, water availability, humidity, and light can influence pollen dispersal (Hegde and Waines, 2003; Jarosz et al., 2003, 2005). Wheat has been shown to have increased cross-pollination frequency during times of drought and high temperatures (Briggs et al., 1999; Hegde and Waines, 2003) by increasing glume opening (Hegde and Waines, 2003; Vries, 1971). Wheat varieties have also shown some increase in closed flowering or incomplete floret opening as a result of prolonged dry heat (Vries, 1971) which would decrease the chances of cross pollination. Although optimal conditions for cross-pollination are not known for jointed goatgrass, it has been shown that a temperature of 21 ˚C with a minimum of 9.5 ˚C is optimal to promote cross-pollination for wheat (Porter and Gawith, 1999). Due to the lack of information surrounding jointed goatgrass cross-pollination, backcrossing rates need to be evaluated under a variety of environments in order to determine the risk of interspecific hybridization and gene introgression. Despite the 2011-12 growing season lending itself to potentially higher levels of outcrossing via hotter and drier weather conditions, it nevertheless had significantly lower observed levels of backcrossing to jointed goatgrass than the wetter cooler conditions observed during the 2010-11 growing season.

The possibility of pollen from nearby wheat fields cannot be ruled out as a source of backcrossing to the BC_1 plants. Long-distance pollen movement has been reported by several

researchers: wheat pollen grains have been detected from a source population at a distance of 20 to 24 m (Suneson and Cox, 1964; Vries, 1971), 50-60 m (Khan et al., 1973; Gaines et al., 2007), and even as far as 1,000 m (Virmani and Edwards, 1983). In the majority of these pollen movement studies, more than 90% of pollen grains remained within 3 m of their source, and the amount of pollen collected decreased rapidly with increasing distance from the source plant (Jensen, 1968; Khan et al., 1973; Virmani and Edwards, 1983). During the 2010-11 growing season the nearest wheat pollen source was from a 0.43 acre wheat plot 200 meters north of the BC_1 field plot followed by an 18 acre wheat field 480 m east of the BC_1 field plot. For the 2011-12 growing season, the nearest wheat pollen source was from a 0.43 acre wheat plot 125 m north of the BC_1 field plot followed by a 1.75 acre wheat field 500 m away. In a study of wheat-to-wheat gene flow, for the farthest sampling distance (49 to 61 m), the average cross-pollination in 32 samples that were not frost damaged was 0.017% (Gaines et al., 2007), well below our average of 0.648% for the 2010-11 growing season reported in this study. Additionally, the maximum reported wheat-to-jointed goatgrass cross-pollination distance has been recorded at 16 m (Gaines et al., 2008) and 40 m (Hanson et al., 2005). The high density of jointed goatgrass plants that surrounded the BC_1 plants should have served to swamp any incoming wheat pollen that may not have been intercepted by the tall border of triticale.

Lastly, the possibility of self-pollination of BC_1 plants cannot be entirely ruled out as a source of seed set on BC_1 plants used in backcrossing field trials. Since BC_1 plants have shown low levels of self-fertility (Schoenenberger et al., 2006; Snyder et al., 2000; Wang et al., 2001) the production of BC_2 seed in backcrossing field trials may not be the result solely of an outcrossing event. In the greenhouse trial of this experiment, the mean rate of self-fertility of BC_1 plants was 0% for individually bagged spikes and 0.004% for whole bagged plants. These

rates are well below the backcrossing rate of 0.648% observed in the field for the 2010-11 growing season. The rates of self-fertility of BC_1 plants observed in the greenhouse trials in this experiment are below the observed rates in other experiments. Mean BC_1 self-fertility has been observed at a rate of 0.06% (Wang et al., 2001). In a separate study all but two of the BC_1 plants were completely self-infertile, with two individual plants producing self-seed at 0.16% and 5.21% (Schoenenberger et al., 2006). When examining the rate of backcrossing to BC_1 plants in field studies where hand-emasculation is not done, the possibility of self-seed is an inherent part of the experimental design. We felt the best way to confirm backcrossing is to withhold a random subset of plants from the BC_1 backcrossing field plot, and exclude all other pollen sources in a greenhouse experiment to determine the rate of self-fertility of a random sample of our particular genotypes. Given the very low self-fertility of BC_1 plants in our greenhouse trials, the progeny from field BC_1 plants are most likely the result of a backcrossing event and not seed set due to self-pollination.

Although BC_1 and BC_2 plants were observed from emergence through flowering, it has been shown that plant morphology and phenotypic characteristics are unreliable in differentiating the pollen donor due to variation in the morphology of the backcross progeny. The BC_2 plants germinated from the field trials showed a wide range of phenotypes for both spike morphology and plant growth type. Spike morphology and plant growth type appeared to be highly correlated. BC_2 plants having spike morphology more similar to wheat also produced fewer, more erect tillers while BC_2 plants having spike morphology resembling jointed goatgrass spikes produced more tillers and were less erect (data not shown). It has been shown one cannot determine paternity of the first generation backcross progeny based on their coleoptile color, leaf width, number of hairs on the leaf margin, presence or absence of hairs on the leaf margin,

number of veins in the leaf, spike appearance, spike length, plant height, growth habit, or number of spikes produced on the BC_1 plant (Snyder et al., 2000). Due to the wide range of phenotypes observed in this study, the inability to determine the paternity based on plant phenotypes may carry over to the BC_2 generation. All BC_1 and BC_2 plants examined in this study showed a non-shattering spike characteristic; regardless if the spikes appeared to be similar to wheat or to jointed goatgrass.

The results presented here demonstrate that backcrossing with jointed goatgrass can occur from the BC_1 to the BC_2 generation under field conditions. With two backcrossing generations complete, it is possible that self-pollinating generations could introgress a wheat gene conferring a selective advantage into a jointed goatgrass population. Further studies investigating the rate of subsequent self-pollinating generations are necessary in order to fully assess the degree of gene flow to jointed goatgrass. It will also be necessary to understand which chromosome segments from wheat are most often being transferred into jointed goatgrass populations. The transfer of novel genes from wheat to its weedy relative has been reported with genes located on the D-genome, the shared genome between wheat and jointed goatgrass (Perez-Jones et al., 2006a; Perez-Jones et al., 2006b). It has been proposed that the risk of transferring a novel transgene from wheat to jointed goatgrass would be lower if the novel gene is inserted into one of the two genomes not shared between these two species (i.e. A-genome or B-genome). However, there is still a chance for novel transgenes in the unshared genomes to move into jointed grass's genomes via chromosome translocation events and retention of the whole wheat chromosomes (Wang et al., 2000). A comparison of the frequency of A- and B-genome chromosome movement would provide important information on transgene insertion locations that would decrease the risk of transferring a wheat gene conferring environmental stress

tolerance to its weedy relative. The Great Plains region is under extensive dryland wheat production and transgenic cultivars conferring environmental stress tolerance, such as drought stress, would benefit production in the region. Gaines et al. (2008) showed that interspecies hybridization between wheat and jointed goatgrass occurs in the Great Plains at a rate of 0.0% to 1.6%, while Econopouly et al. (2011) reported hybrids backcross to jointed goatgrass at a rate of 0.0% to 1.1%. The study reported here confirms that fertility exists in the BC_1 plants demonstrating that introgression into populations of jointed goatgrass in the Great Plains is a potential reality.

Gene introgression through hybridization and backcrossing can be minimized by effectively controlling jointed goatgrass and wheat x jointed goatgrass hybrids in wheat crops and fallow fields. Clearfield™ wheat varieties resistant to imazamox (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid) provide the only selective herbicide means for controlling jointed goatgrass during the growing season. Recommendations for the west-central Great Plains include the use of clean wheat seed, management practices that enhance crop competitiveness, appropriate crop rotations, chemical control of jointed goatgrass in fallow fields and field borders, and cultural methods including seed sanitation, spring cropping, or fallow rotations.

Econopouly et al. (2013) assessed the phenotypic diversity of jointed goatgrass as a factor for determining the risk of gene introgression from wheat to jointed goatgrass. The main objective of the study was to evaluate the response of jointed goatgrass populations to drought stress in order to assess the risk of transgenic drought-tolerant wheat by evaluating the selective advantage that jointed goatgrass might gain by acquiring a drought tolerance transgene via gene flow. Results indicated there was a significant ($p<0.005$) effect of the interaction of accession

with watering treatment for plant height, number of tillers produced per plant, number of spikelets per spike, and number of spikelets per plant (Econopouly et al., 2013). Variation in these traits suggests that the competitiveness, fitness, and ability of jointed goatgrass to hybridize with wheat would also vary across accessions acquiring a drought tolerance gene from wheat (Econopouly et al., 2013). The variations in sensitivity to water treatment observed among 30 different accessions of jointed goatgrass evaluated in Colorado suggest that the risk of introgression would not be uniform among jointed goatgrass populations (Econopouly et al., 2013). Since traits such as tolerance to environmental stress could already exist in jointed goatgrass populations, the selective advantage of gaining wheat genes conferring similar traits will vary among jointed goatgrass populations (Econopouly et al., 2013).

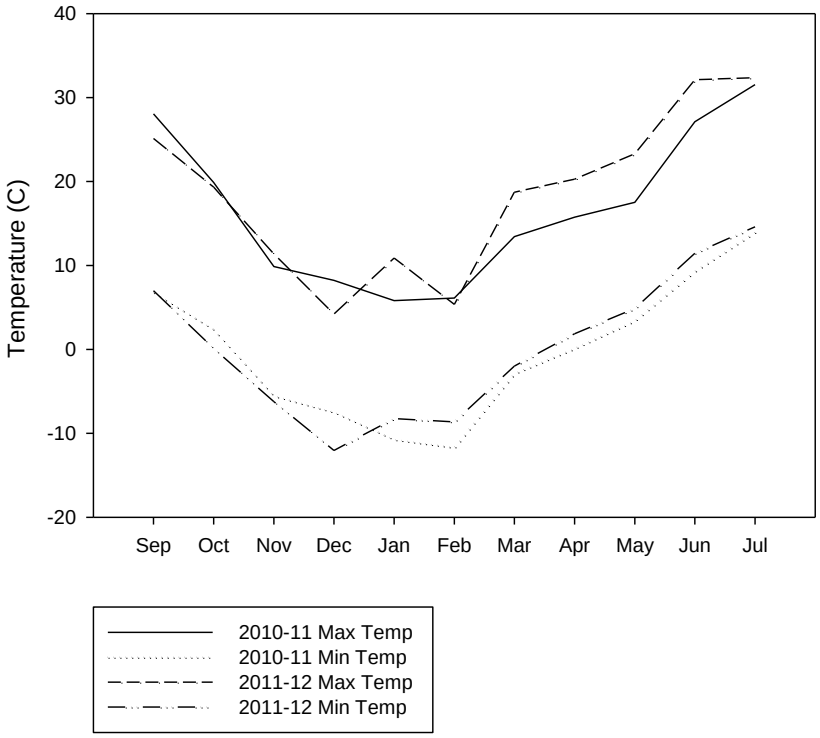
It is timely to address the issue of gene movement from wheat into a weedy relative as a large amount of research is still needed to fully assess the risks associated with potential gene flow from wheat to jointed goatgrass before commercial release of GE wheat varieties. Here we have considered the rate at which wheat x jointed goatgrass BC_1 plants backcross to jointed goatgrass for a second generation in the field. Despite many of the same risks being possible with traditionally bred wheat cultivars, the environmental risks associated with gene flow of GE crops must be thoroughly addressed to prevent undesirable consequences for both agricultural and natural ecosystems. The primary objective of the field trials was to estimate the backcrossing rate of BC_1 plants as a result of pollination by jointed goatgrass under natural field conditions. Results from these field trials will be informative in understanding the risk of introgression of wheat genes to its weedy relative, as a second generation of backcrossing of wheat x jointed goatgrass hybrids to jointed goatgrass under field conditions had never been performed. Since gene flow from wheat to jointed goatgrass can occur via hybridization and

recurrent backcrossing, an effective stewardship program should accompany the release of wheat cultivars containing novel traits to avoid the development of new jointed goatgrass phenotypes and to ensure the longevity of the new technology. The conclusions drawn from the field and greenhouse experiments are of critical importance as new genetic and breeding techniques become available for wheat improvement. New technologies must take into account the risk of transferring novel traits from *T. aestivum* into nearby populations of *Ae. cylindrica*.

Transgenic technology may become heavily reliant on herbicide management practices that are effective in limiting weed populations in the field. The differences in backcrossing rates for the two growing seasons in this study showed that less dense populations of jointed goatgrass and a greater distance between BC_1 plants and jointed goatgrass can reduce the rate of backcrossing in the field. The development of wheat varieties simultaneously resistant to several chemical classes of herbicides would allow more herbicide rotation options for growers to keep jointed goatgrass populations at lower numbers, decreasing levels of jointed goatgrass pollen and thus reducing the potential for backcrossing in the field. Breeding for herbicide resistance in wheat may need to become more prevalent as widespread adoption of a limited number of herbicide-resistant crops could aggravate problems with resistance, especially if the same herbicides are used repeatedly in crop rotations.

Figures

A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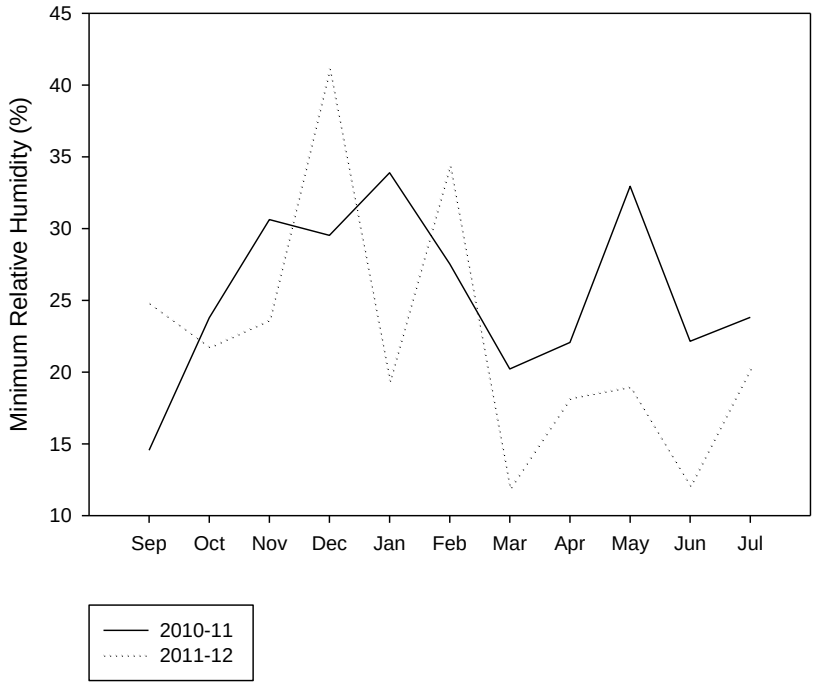
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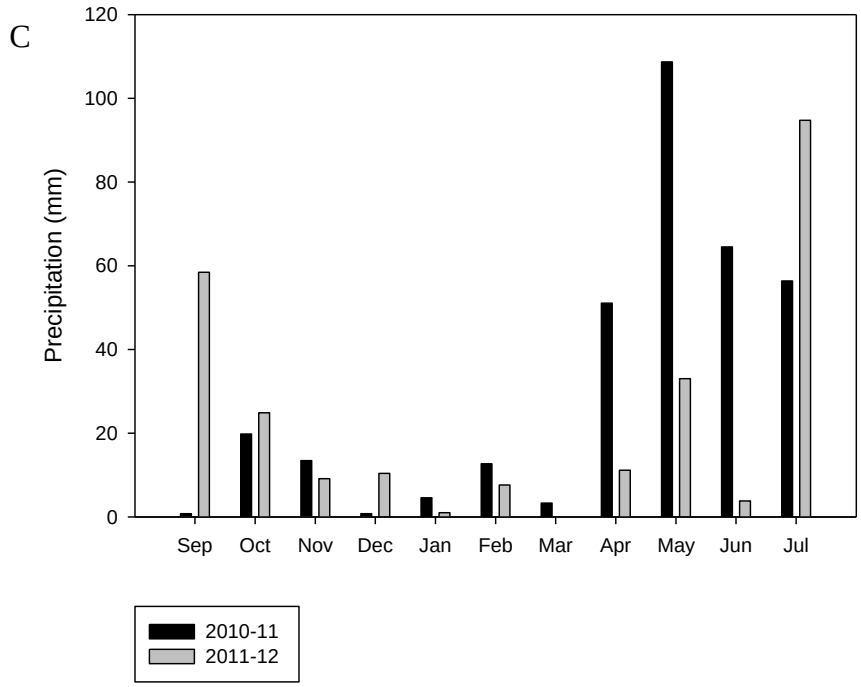

Figure 1.1. Yearly variation between two growing seasons at Fort Collins, CO, as observed in (A) mean maximum and minimum temperature (˚C), (B) mean minimum relative humidity (%), and (C) total monthly precipitation (mm) by month.

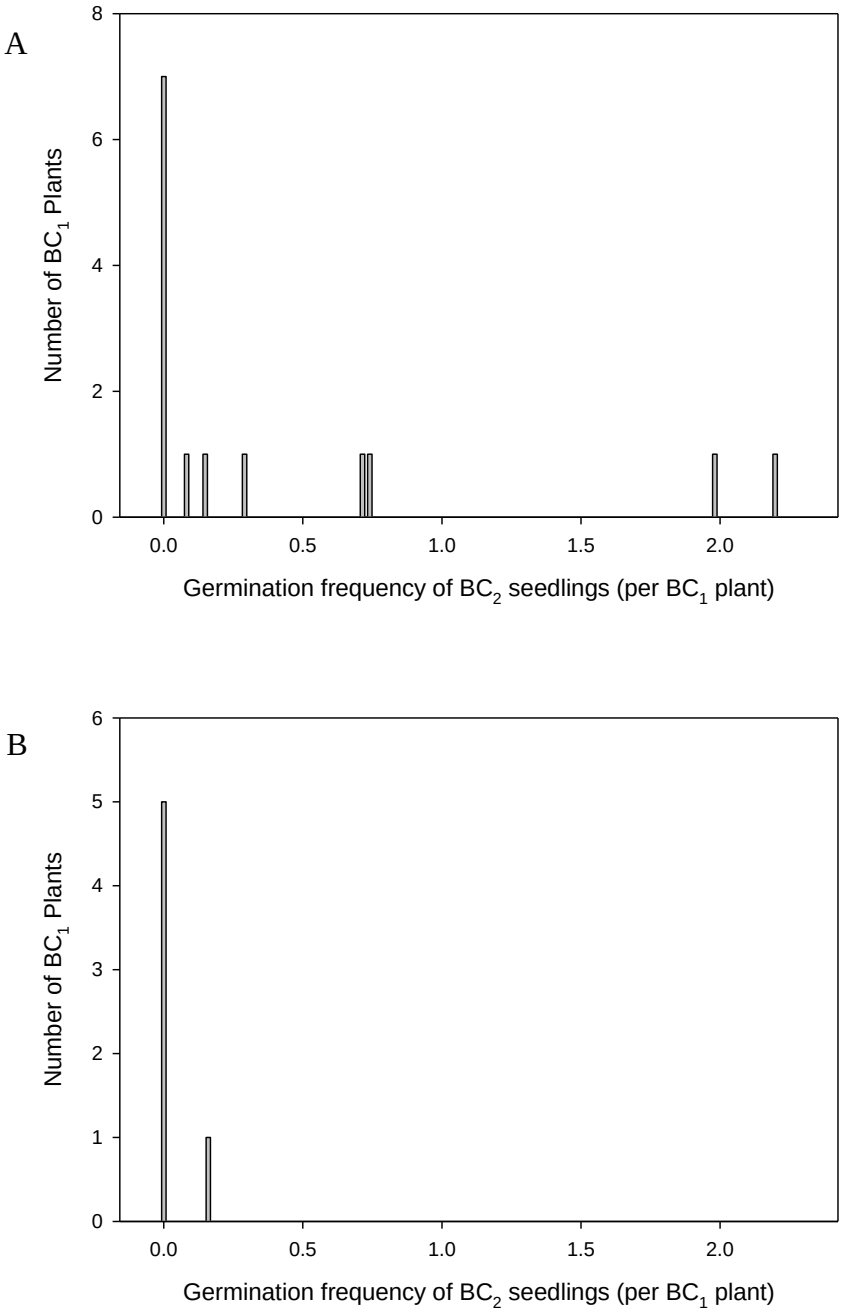

Figure 1.2. Frequency distribution of BC_2 seedlings that germinated from the seeds on spikes collected from BC_1 plants from Fort Collins during (A) the 2010-11 growing season and (B) the 2011-12 growing season.

Table 1.1. The fertility of *Triticum aestivum* x *Aegilops cylindrica* first generation backcross progeny.

Cross Type	# of Plants	# of Spikes	# of Spikelets	# of Florets	# of Progeny	Mean Emergence %	Range %
BC_1 x JGG – Field 2010-11	14	418	3,315	6,630	43	0.648	0.0 - 2.19
BC_1 x JGG – Field 2011-12	6	249	2,122	4,244	1	0.024	0.0 – 0.16
BC_1 x JGG- Greenhouse	66	215	1,632	3,264	1	0.03	0.0 – 1.19
BC_1- Bagged Spikes	17	47	352	704	0	0	0.0
BC_1- Bagged Plants	37	1,130	11,697	23,394	1	0.004	0.0 – 0.11

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CHAPTER 2

Visualizing Chromosome Composition of Wheat (*Triticum aestivum* L.) x Jointed Goatgrass (*Aegilops cylindrica* Host.) Backcross Progeny Using Multi-Color Genomic *In Situ* Hybridization

INTRODUCTION

Jointed goatgrass (*Aegilops cylindrica* Host.) is an agricultural weed that has been negatively impacting winter wheat production in the Great Plains and Western United States since it was introduced to the U.S as a contaminant of winter wheat seed in the late 19th century (Mayfield, 1927). These two species are similar in plant size and height, flowering timing, spikelet arrangement, vernalization response, and germination timing (Donald and Ogg, 1991). Their genetic relatedness and similar development phenology allows them to hybridize, providing a bridge for gene flow from wheat to the weedy relative (Gaines et al., 2008; Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Morrison et al., 2002; Perez-Jones et al., 2010; Seefeldt et al., 1998; Wang et al., 2001; Zemetra et al., 1998).

Interest in the development of herbicide-resistant and environmental stress-tolerant genetically engineered (GE) wheat cultivars has focused attention on the possibility of introgression of wheat genes into jointed goatgrass populations. Pollen-mediated gene flow leading to crop-weed hybridization and subsequent generations of recurrent backcrossing to the weedy species could lead to the introgression of advantageous genes from a crop species into a weed population (Ellstrand and Hoffman, 1990). Despite these same risks being present with traditionally bred crops, transgenic technology makes it possible for single genes or blocks of

genes to be inserted into a genome of interest as a compact unit. Movement of a single wheat chromosomal segment, containing a novel transgene, into a jointed goatgrass genome could lead to the expression of the novel transgenic trait. Within the next decade, we will begin to see an increase in the number of crops that possess many novel traits that were identified in distantly related (tertiary) germplasm and thus incorporated using transgenic technologies. These traits will be novel due to the absence or rarity of these genes in wild plant populations. Introgression of genes from a crop species to a weedy species facilitating improved tolerance to biotic and abiotic stresses such as herbivory, pathogens, drought or salinity could increase the competitive ability of the weedy species and lead to new management challenges (Darmency, 1994; Warwick et al., 1999). Applied transgenic technology has been used to improve drought tolerance in maize and it is likely this same technology could be used to improve drought tolerance in wheat cultivars (Castiglioni et al., 2008). Semi-arid areas, like the western U.S., have become an area of focus as introgression of a drought tolerance transgene from improved wheat cultivars into jointed goatgrass could result in a more adapted and competitive species, allowing it to expand its range, invade new habitats, and increase its competitiveness with domesticated and native species (Arnold, 1992; Snow and Palma, 1997; Stewart et al., 2003).

Bread wheat (2n=6x=42, genomes ABD) and jointed goatgrass (2n=4x=28, genomes CD) share the common ancestor *Ae. tauschii* Coss., the donor of the D-genome (Kimber and Sears, 1987). Despite wheat and jointed goatgrass both being highly self-pollinating species, when these two species grow in close proximity and their flowering times overlap, cross-pollination leading to hybridization of these two species can occur. The difference in ploidy between wheat and jointed goatgrass (wheat is a hexaploid and jointed goatgrass is a tetraploid) does not serve as an adequate genetic barrier to prevent the production of F_1 hybrid plants. Wheat x jointed

goatgrass F_1 hybrids are pentaploid and contain 35 chromosomes (2n=5x=35, genomes ABCDD). During meiosis, the D-genome chromosomes of wheat form bivalents with the D-genome chromosomes of jointed goatgrass while the A- and B-genome chromosomes from wheat and the C-genome chromosomes from jointed goatgrass remain univalent.

Although there are several documented cases of gene flow from crops to wild relatives (Ellstrand et al., 1999), little information is available about the long-term persistence of crop genes in wild populations or the impact of fitness-related genes on weedy species' population dynamics. Ecological consequences of GE wheat are difficult to predict and assess, yet cytogenetic experiments examining chromosome retention and translocation events in wheat x jointed goatgrass backcross progeny could provide new insights into strategies to minimize the occurrence of gene escape from GE wheat. The movement of genetic information from wheat to jointed goatgrass can occur via several mechanisms: gene recombination through homologous chromosome pairing, translocation, and chromosome addition. The D-genome chromosomes of wheat can pair with the D-genome chromosomes of jointed goatgrass, forming bivalents and allowing for crossing over to occur. Novel genes on the D genome of wheat have moved to wheat x jointed goatgrass backcross progenies by means of homologous chromosome pairing and gene recombination (Perez-Jones et al., 2006a, 2006b). With each successive backcross to jointed goatgrass the backcross progeny's genetic composition should more closely resemble that of jointed goatgrass. With each backcross to jointed goatgrass there is a greater number of C-genome bivalent chromosome pairings during meiosis resulting in the restoration of the C-genome chromosomes to complement the already stable D genome. Since the retention of chromosomes requires that they pair with each other during meiosis, the crop chromosomes in the hybrid (A- and B-chromosomes) will likely be lost during generations of recurrent

backcrossing of the hybrid to jointed goatgrass. This has made chromosomes of the A and/or B genomes of wheat ideal candidates for the location of transgene insertion. Despite this potential genetic barrier, the possibility still exists for novel genes of the A or B genomes of wheat to move into jointed goatgrass. A translocation event where a portion of an A- or B- wheat chromosome broke off and became attached to a jointed goatgrass chromosome, was reported in a BC_2S_2 progeny (second selfed progeny from a hybrid backcrossed to jointed goatgrass twice) (Wang et al., 2000). The third method of gene transfer from wheat to jointed goatgrass is chromosome addition. Wheat x jointed goatgrass backcross individuals with more than 28 chromosomes are retaining extra A- and/or B-genome chromosomes from wheat (Wang et al., 2000). Identification of chromosome addition or translocated chromosome segments of wheat DNA by genome may provide insight into the occurrence of gene escape from GE wheat. Jointed goatgrass's lack of the A and B genomes present in wheat has led scientists to speculate that novel transgenes inserted into the A or B genomes of wheat would minimize the escape and introgression of a transgene from GE wheat. A genetic barrier to introgression could lower the occurrence of transgene escape from crops to wild relatives, whereas the lack of such a barrier could lead to a high occurrence of transgene movement and persistence in a wild population.

In order to determine the potential risk of wheat gene transfer to jointed goatgrass it is important to determine the genomic composition of backcross progeny throughout the introgression process. Genomic *in situ* hybridization (GISH) has become a widely applicable cytogenetic technique for examining genome structure in polyploid species. The GISH technique uses labeled genomic DNA as a probe to hybridize to complementary chromosomes which are then visually analyzed and karyotyped by their fluorescent emission. Advances in *in situ* hybridization methods have made it possible to identify different genomes within polyploid

plant species simultaneously using multiple fluorescently labeled genomic probes (Han et al., 2003; Mukai et al., 1993; Sanchez-Moran et al., 1999; Shishido et al., 1998). The GISH technique has been particularly useful in the tribe Triticeae, in which it has been used to identify alien chromatin introduced into wheat via hybrid crosses (Cai et al., 2001; Le et al., 1989; Malysheva et al., 2003; Molnár-Láng et al., 2000; Schwarzacher et al., 1989). Translocation events have also been visually identified in backcross progenies of wheat hybrids using GISH (Molnár-Láng, 2000; Wang et al., 2000).

In this study, the diploid progenitors of wheat's genome will be used as genomic probes to detect chromosome movement and retention of wheat DNA sequences by individual genome. GISH probes that hybridize specifically to their complementary chromosomes will need to be optimized to discriminate the closely related genomes in wheat x jointed goatgrass backcross progeny. Few techniques are known for simultaneous identification of multiple genomes in wheat x jointed goatgrass backcross progeny. In general, it is still a challenge to distinguish the A, B, and D genomes of wheat by GISH. The objective of this research is to develop and utilize a GISH procedure to identify the A- and B-genome chromosomes separately from the C- and D-genome chromosomes in backcross progenies of wheat x jointed goatgrass. This technique could be used to estimate the frequency of A- or B-genome chromosome retention occurring in the genetic background of backcross progeny in order to assess the biological risk of releasing wheat varieties with novel genes.

MATERIALS AND METHODS

Progeny/Experimental Material Development

Hybrid seed was produced in the greenhouse in 2007 using the hard red winter wheat cultivar 'Above' (Haley et al., 2003) and jointed goatgrass seed collected in Platner, CO. Florets of the wheat plants were hand-emasculated and manually pollinated with jointed goatgrass pollen to produce hybrid seed following the method of Zemetra et al. (1998). Seeds collected from the wheat spikes were planted in 5 cm square pots containing potting soil (Fafard #2 SV, Conrad Fafard, Inc., Agawam, MA 01001) and germinated in the greenhouse. The resulting hybrid plants were transplanted into a field plot at the Colorado State University Agricultural Research, Development, and Education Center (ARDEC) in Fort Collins, CO, during the 2007-08 growing season. Jointed goatgrass seed collected from Paoli, CO, was seeded over the entire plot, both within and between rows, to force close proximity of the hybrid plants with jointed goatgrass pollen. Since hybrid plants are self-infertile due to male sterility (Guadagnuolo et al., 2001; Mallory-Smith et al., 1996; Wang et al., 2001; Zemetra et al., 1998), viable seed on these hybrid plants could only have been produced through outcrossing. Spikes were collected from over 100 hybrid plants, and a random subset of spikes from the hybrids were planted into 27 x 53-cm flats containing potting soil in the summer of 2010. Seedlings that germinated from the hybrid spikes were the result of a backcross to jointed goatgrass and were determined to be first generation backcross (BC_1) plants. These BC_1 plants were transplanted into a research plot in a grid pattern with 0.6 m spacing between plants in all directions in a field study at ARDEC in the fall of 2010. Jointed goatgrass from Paoli, CO, was planted within and between rows of BC_1 plants over the entire plot to serve as the dominant pollen source. Spikes collected from the BC_1 plants in the field plot were disarticulated and planted in potting soil. The progeny that emerged

were considered second generation backcross (BC_2) seedlings and served as the experimental material for this study.

Somatic Metaphase Chromosome Preparation

Root-tips were collected from newly formed plant roots growing in 5-cm square pots with potting soil in the greenhouse. Plant roots 3 to 4-cm long that showed new growth were cut and placed in 1.5 mL Eppendorf tubes and treated with ice cold water for 24 h to accumulate cells in metaphase. Roots were then fixed in ethanol and glacial acetic acid (3:1) and stored at 4 °C until selected for use. The meristematic portions of the roots were cut from the root tip and washed thoroughly with water. The roots were then digested in a mixture of 10 mM citrate buffer, cellulase (*Trichoderma viride*, EMD Millipore), and pectinase (*Aspergillus niger*, Sigma) for 2-3 h at 37 ˚C. Roots were washed with water then moved into a mixture of 1% acetocarmine and 45% acetic acid (1:3) for 5 min. A section of meristematic root cells was then transferred via pipette onto a glass slide pre-treated with 3-(Trimethoxysiyl)propyl methacrylate (Sigma). A glass coverslip (23 x 23 mm) pre-treated with a siliconizing agent (SIGMACOTE, Sigma Aldrich) was placed over the digested root tip, tapped to spread out the cells, warmed over an alcohol flame, and then pressed firmly into place. Chromosomes were observed with a light microscope with phase contrast (Photomicroscope II Zeiss, Germany). Slides with metaphase chromosome spreads were stored at -80 ˚C until selected for use.

DNA Extraction

Total genomic DNA was isolated from fresh freeze-dried leaf tissue of *Triticum urartu*, *Ae. speltoides*, and *Ae. cylindrica.* The SDS extraction buffer and chloroform (Mallinckrodt

Chemicals): isoamyl alcohol (Fisher Biotech) (24:1) were prepared fresh for all DNA extractions following the procedure of Riede and Anderson (1996). Plant tissue was placed in 2.0 mL Eppendorf tubes with two metal ball bearings and placed in a pelletizing shaker for 30-60 seconds. Seven hundred µL of hot extraction buffer was added to the ground plant tissue. Samples were incubated at 65 ˚C for 30-60 min and mixed by inversion every 10 min. Seven hundred microliters of chloroform: isoamyl alcohol (24:1) was added to each DNA extraction and gently mixed to form an emulsion. Samples were centrifuged at 14,000 rpm for 10-15 min and the aqueous phase was transferred to a 1.5 mL Eppendorf tube. Two volumes of 100% EtOH were added to each sample and mixed by inversion then placed in a -20 ˚C freezer overnight. Samples were centrifuged at 12,000 rpm for 5 min. The supernatant was removed and the DNA pellet was washed with 70% EtOH then air dried. The dried DNA was dissolved in 400 µL TE. Four µL of RNase A (Thermo Scientific) was added and mixed in each sample and incubated at 37 ˚C for 30-60 min. Five hundred µL of phenol (OmniPur) was added to each sample, mixed by inversion, and centrifuged for 5 min. The supernatant was transferred to a 1.5 mL Eppendorf tube and 500 µL of phenol/chloroform (1:1) was added, mixed by inversion, and centrifuged for 5 min. The supernatant was again transferred to a 1.5 mL Eppendorf tube, 500 µL of chloroform was added, mixed by inversion, and centrifuged for 5 min. A 1/10 volume of 3 M sodium acetate (pH 5.2) followed by 2 volumes of 100% EtOH were added to precipitate the DNA. The samples were mixed by inversion and placed in a -20 ˚C freezer overnight for maximum yield. Samples were centrifuged at 12,000 rpm for 5 min and the supernatant was decanted. The DNA pellet was washed with 75% EtOH and air dried. DNA was dissolved in TLE (Tris-‘low’ EDTA), quantitated, and diluted as needed.

Genomic DNA Probe Labeling

Genomic DNA of *T. urartu* and *Ae. speltoides* were labeled with an amine-reactive dye by nick-translation and incorporation of aminoallyl-dUTPs (ARES DNA Labeling Kit, Invitrogen) following directions as described in the product manual with minor modifications. The volume of water was adjusted as needed to achieve a final volume of 100 µL with 2 µg of the DNA template.

1. 43 µL nuclease-free water
2. 10 µL 10X nick-translation buffer
3. 10 µL 0.1 M DTT
4. 8 µL d(GAC)TP mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP) (BioLine)
5. 2 µL 0.5 mM dTTP
6. 12 µL 0.5 mM aminoallyl-dUTP
7. 2 µL DNA template, 1µg/µL
8. 10 µL DNase I
9. 3 µL DNA polymerase I (Promega)

The reaction was incubated at 15 ˚C for 2 hours. The labeled DNA was then purified using a PCR Purification Kit (PureLink, Invitrogen). The amine-modified DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. Two µL of glycogen was added to help precipitate the DNA as this has been shown to result in greater DNA recovery.

Labeling with a Reactive Fluorescent Dye

The amine-modified DNA samples were labeled with amine-reactive dyes as described in the product manual (ARES DNA Labeling Kit, Invitrogen). The labeling buffer was prepared by adding 1 mL of nuclease-free water to a vial of sodium bicarbonate. The amine-modified DNA was dissolved in 5 µL of nuclease-free water. Prior to labeling, the amine-modified DNA was denatured by heating to 95 °C for 5 min and then placed on ice. Three µL of labeling buffer was added to the amine-modified DNA. One vial of the reactive dye was dissolved in 2 µL of DMSO solvent. The amine-modified DNA was added to the dissolved reactive dye and well-mixed. The reaction was incubated in the dark at room temperature for 1 h. The labeled DNA was then purified using a PCR Purification Kit. The amine-modified DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. Two µL of glycogen was added before the DNA was incubated at -80 ˚C over-night. The DNA pellet was washed with 75% EtOH and then allowed to air dry. Fluorophore labeled DNA samples were suspended in 20 µL of TE buffer.

Hybridization of the Probe to Chromosomes

GISH was conducted as previously described for a fluorescent *in situ* hybridization procedure (Jiang et al., 1996). Slides were removed from the -80 ˚C freezer and placed on dry ice. Coverslips were removed with a razor blade and the slides were dehydrated in an ethanol series (70%, 95%, and 100%, 5 min each) at room temperature. The hybridization mixture was prepared with:

1. 25 µL deionized formamide
2. 5 µL 20X SSC

3. 2 µL sheared salmon sperm DNA
4. DNA probes (~40-50 ng/probe)
5. 10 µL 50% dextran sulfate
6. Blocking DNA

The hybridization mixture was denatured at 80 °C for 5 min and then immediately chilled. One hundred fifty µL of 70% formamide in 2X SSC solution was pipetted on the dried slides and placed on an slide warmer at 80 °C for 1.5 min. The cover slips were removed and the slides were immediately dehydrated in a cold ethanol series (70%, 95%, and 100%, for 5 min) at -20 °C. The slides were then air dried. The hybridization solution was applied to each slide and placed in a wet chamber at 37 °C for overnight hybridization to mitotic chromosome spreads.

Detection of GISH signals

Following hybridization, slides were washed in 2X SSC for 5 min at room temperature, 2X SSC for 10 min at 42 °C, 2X SSC for 5 min at room temperature, and a final wash of 1X PBS for 5 min at room temperature. DAPI (4',6-diamidino-2-phenylindole) with slow fade was added followed by a glass coverslip before *in situ* hybridization results were examined using a fluorescence microscope. A Zeiss Axioskop 4FL filter series was used to detect fluorescence signals.

Fluorophores with different excitation and emission maxima allowed simultaneous detection of two genomic probes. The different fluorophores had sharp emission peaks which prevented spectral overlap between the two probes and DAPI, eliminating background problems and bleed-through. DAPI was effective in locating chromosomes spreads and to confirm chromosome counts on the slide. An Axioskope microscope (Zeiss) was used for all trials. A

ProgRes C5 camera (Jenoptic) was used to take pictures while the ProgRes CapturePro 2.8.2 (Jenoptic) software program was used to visualize and edit pictures.

RESULTS

Chromosome numbers for six individual BC_2 plants were confirmed using at least two separate root squashes with three to four chromosome spreads per slide. A range of chromosome numbers from 28 to 47 were observed for the population of BC_2 plants developed for this study. The origin of extra chromosomes in individuals with more than 28 chromosomes is of interest from the standpoint of potential gene transfer from wheat to jointed goatgrass via chromosome retention after initial hybridization.

In this study, the ability to distinguish the chromosomes in wheat, jointed goatgrass, or wheat x jointed goatgrass BC_2 progeny by genome was poor. Whole genomic probes showed extensive cross hybridization across all genomes when hybridized with individual genomic probes or hybridized with multiple genomic probes. This resulted in fluorescent hybridization signals that were nearly homogeneously distributed among all chromosomes leading to an inability to karyotype chromosomes by genome. Hybridization attempts with single probes gave stronger fluorescent signals than when multiple probes were used. This was particularly true for the green fluorescent probe which would often become undetectable when high concentrations of blocking DNA or multiple fluorescent probes were added to the hybridization mixture.

Whole genomic probes of *T. urartu* and *Ae. speltoides* showed extensive cross hybridization when probed to wheat chromosomes (Figures 2.1 and 2.2). DNA from jointed goatgrass, durum wheat, and barley were examined as potential blocking DNAs with single and multiple genomic probes at varying concentrations in an attempt to prevent the cross

hybridization seen on chromosome spreads. The total genomic DNA from these three species when used as blocking DNA only served to dull the hybridization signals across all chromosomes rather than limiting the cross hybridization required to provide a clear fluorescent signal for the chromosomes unique to the specific genomic probes. The use of low concentrations of *Ae. tauschii* as blocking DNA appeared to provide minor differences in differentiating the D-genome chromosomes from the A- and B-genomes chromosomes in wheat chromosome spreads. However, the cross hybridization between the A- and B-genomes prevented these two genomes from being able to be differentiated from each other.

DISCUSSION

Interspecies hybridization leading to gene flow has long been proposed as a mechanism for evolution in natural populations (Anderson, 1949). Plant breeders have used interspecies hybridization to exploit genetic diversity and to identify novel genes for crop improvement. However, sexual compatibility and gene flow between a domesticated species and a wild relative when not controlled is cause for concern. The introgression of crop genes into other plant species has received considerable attention as more crops species are being modified through the use of transgenic technologies. The movement of novel wheat genes and their expression in wheat x jointed goatgrass backcross progeny has previously been reported (Lin, 2001; Perez-Jones et al., 2006a; Perez-Jones et al., 2006b). The higher rates of gene movement observed from genes located in the D genome of wheat compared to the A or B genomes of wheat have made the A and B genomes of wheat prime locations for novel gene insertion. However, genes belonging to the A and B genomes of wheat can still be retained and expressed in wheat x jointed goatgrass backcross progeny. Wang et al. (2000) were able to show that BC_2S_2 plants with 29

chromosomes had one chromosome that fluoresced when hybridized with a *T. durum* probe, BC_2S_2 plants with 30 chromosomes had two chromosomes that fluoresced when hybridized with a *T. durum* probe, and BC_2S_2 plants with 31 chromosomes had three chromosomes that fluoresced when hybridized with a *T. durum* probe. This confirmed that extra chromosomes from the A or B genomes of wheat were being retained in wheat x jointed goatgrass backcross individuals with more than 28 chromosomes. In general, the number of D-genome chromosomes present through the introgression process tends to remain 14 in every generation. This is because the D-genome chromosomes pair as seven bivalents during meiosis and when backcrossed to jointed goatgrass a complete set of seven new D-genome chromosomes are introduced for pairing. Wang et al. (2000) used *Ae. markgrafii* DNA (2n=2x=14, genome C) as a labeled probe to visualize C-genome chromosomes of BC_2S_2 individuals. They showed that all BC_2S_2 individuals had 14 C-genome chromosomes including individuals with 29 and 30 chromosomes. In this study, BC_2 individuals with more than 28 chromosomes were thought to carry extra A- and/or B-genome chromosomes.

The detection of alien chromosomes using GISH has become a routine technique for species and genomes that are distantly related. The limitation of this technique occurs when attempting to differentiate genomes of species that are closely related. This is due to similarities in genomic DNA sequences between groups and genera within the same tribe (Flavell et al., 1981). When using GISH to identify closely related genomes, the common or repetitive DNA sequences shared among genomes within the same genus can lead to fragments of the probe binding to non-targeted regions. This non-specific hybridization of a labeled probe leads to cross-hybridization and decreases the specificity of the probe. Non-specific hybridization is said to be greater for probes made from large DNA samples and also when probing to polyploid

species due to their likelihood of containing repetitive elements. The bread wheat genome is composed of two *Aegilops* genomes and one *Triticum* genome. Despite the close genetic relationship between *Triticum* and *Aegilops*, GISH has been successful in differentiating the three genomes in wheat but has only been reported twice (Mukai et al., 1993; Sanchez-Moran et al., 1999). The success of GISH is often dependent on the use of a hybridization mixture that prevents cross-hybridization of common or repetitive DNA sequences shared between closely related genomes while still allowing for hybridization and detection of single or low-copy DNA sequences. Probes with entirely unique or low-copy sequences require little or no blocking DNA while probes containing highly repetitive elements require high levels of blocking DNA in order to obtain distinct GISH signals (Hanson et al., 1995; Zwick et al., 1997). Due to the genetic relatedness of the *Aegilops* and *Triticum* genomes, a range of plant DNAs were evaluated in hybridization mixture experiments in an attempt to block the fluorescently labeled dispersed repetitive sequences and homoeologous sequences responsible for non-specific hybridization. I was unable to block the dispersed repetitive sequences and homoeologous sequences needed to prevent cross-hybridization while still allowing the low copy genome specific sequences needed to provide a strong signal on the targeted sites of their respective chromosomes or chromosome regions. This study showed that DNA sequences shared between *Triticum* and *Aegilops* will bind non-specifically labeled DNA sequences to non-specific chromosome regions and lead to challenges in karyotyping chromosomes by genome. Even when the A and B genomes could be distinguished from the C and D genomes in wheat x jointed goatgrass backcross progeny, I was unable to differentiate the A and B genomes from each other. This may be due to greater similarity in DNA sequences shared between the A and B genomes than between the A or B genomes and the C and D genomes. The inability to differentiate the A and B genomes

prevented us from being able to make any statements about the frequency or risk of A-genome vs. B-genome chromosome retention or translocation.

Wang et al. (2000) were able to detect the presence of extra A- or B-genome chromosomes in BC_2S_2 progeny with more than 28 chromosomes as well as a translocation event involving an A- or B-chromosome segment. The use of *T. durum* (2n=4x=28, genomes AB) as a probe prevented Wang et al. (2000) from determining if A- or B-genome chromosomes were preferentially being retained or involved in translocation events. The use of *T. durum* as a probe also prevented Wang et al. (2000) from determining if the extra chromosomes were being retained as a homologous pair in BC_2S_2 progeny with 2 or more extra chromosomes. Since the stable transfer of genes from the A- or B-genome chromosomes of wheat to jointed goatgrass requires that homologous chromosomes pair with each other during meiosis, retained crop chromosomes will likely be lost during backcrossing events to jointed goatgrass. If extra chromosomes retained in wheat x jointed goatgrass backcross progeny are homologous pairs, these chromosomes can become stably introgressed in a wheat x jointed goatgrass backcross progeny's genome over generations, despite being from the A or B genomes of wheat. A multi-color GISH procedure effective in simultaneously identifying the A and B genomes separately holds the potential to provide critical information that cannot be obtained with the single probe approach used by Wang et al (2000). Although GISH has proven itself as a powerful and versatile tool, it cannot be used to distinguish one chromosome from another within a particular genome. Fluorescent *in situ* hybridization (FISH) probes that provide individualized fluorescent chromosome banding patterns may allow chromosomes to not only be identified by genomes but also by individual chromosome number or even individual chromosome segments. GISH analysis coupled with FISH analysis could provide a more reliable technique for wheat

chromosome identification in jointed goatgrass backcross progeny. This may be particularly valuable in identifying frequencies of translocation events of a particular chromosome or chromosome segment.

Direct fluorescent labeling of our genomic probes was chosen as it is one of the fastest and cheapest ways to label genomic DNA since it does not require any further detection steps after hybridization (Moter and Göbel, 2000). Using the direct labeling method, a fluorescent dye molecule is directly bound to a receptive oligonucleotide that has been enzymatically incorporated into the DNA sample via nick-translation. Despite the ease of preparing samples, the direct labeling method is not widely used because of its relatively low signal intensity (Chevalier et al., 1997). It has been shown that the sensitivity of *in situ* hybridization assays can be increased by linking the probes to a reporter molecule like digoxigenin or biotin that is then detected by a primary or secondary antibody (Chevalier et al., 1997). Fluorescence using this indirect labeling method was reported to be eight times more intensive than that of oligonucleotides carrying the single label using the direct labeling method (Moter and Göbel, 2000). To determine if our direct labeling method was limiting our ability to discriminate chromosomes by genome we also performed a trial using digoxigenin and biotin labeled genomic DNA detected with antibodies. Our results showed that the indirect labeling method did not improve the signal contrast between the targeted genomic DNA sequences and non-targeted sequences among the chromosomes. We did observe a significant increase in the background signal under the indirect labeling method compared to the direct labeling method. Consequently, the indirect labeling method with its high background signal decreased the likelihood of accurately detecting small translocation segments. It was formerly thought the direct labeling method was not providing a fluorescent signal with enough intensity to allow for

minor changes in signal intensity to be seen. We felt this lack of a fluorescent signal intensity gradient was preventing us from being able to detect minor changes in fluorescence required to be able to identify the difference between a chromosome that fluoresced due to its specificity of the probe or a result of crossing over. After comparing the results of both the direct labeling method and the indirect labeling method (Figures 2.1 and 2.2), we have concluded that our inability to differentiate the chromosomes by genomes was not a result of an inferior labeling technique or poor fluorescent signal intensity.

Although the method used in this study did not allow us to determine whether there is a difference in the ability of A- or B-genome chromosomes to be retained, once a reliable method is developed the progeny of these tested individuals can be used to track gene introgression by genome in progeny that have been self pollinated for several generations. Translocations are a major concern for novel gene transfer from wheat to this weedy relative. Even if extra A- or B-chromosomes were eliminated during meiosis, a translocated segment from either of these two genomes would likely be maintained in backcrossed individuals, especially if there was a fitness advantage conferred by the genes on the translocated segment within the new genetic background. An important area of research will be to determine if BC_2S_x generations of wheat x jointed goatgrass plants which show high levels of translocations from the A and B genome, affect plant growth or fitness. If there is a dramatic fitness cost associated with retaining extra chromosome segments from the A or B genomes it may offset any advantage a novel gene would provide if located on one of the translocated segments. Without more information pertaining to the genetics of gene flow, no decisions can be made in favor of the A- or B-genome chromosomes as a safer location for novel gene location.

Figures

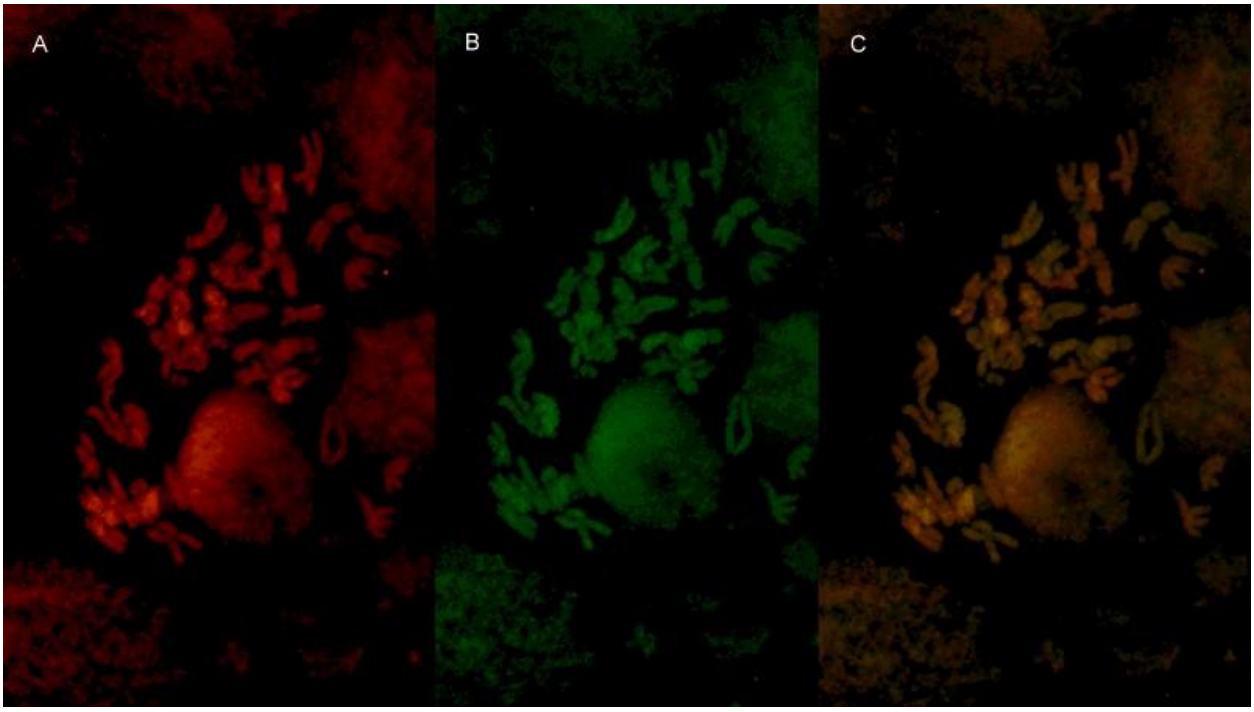

Figure 2.1. Fluorescent genomic *in situ* hybridization images of a wheat mitotic chromosome spread using the direct DNA labeling method. A) *T. urartu* DNA labeled with Alexa Fluor® 546 showing some specificity in binding to A-genome chromosomes but also showing extensive cross-hybridization with B- and D-genome chromosomes. B) *Ae. speltoides* DNA labeled with Alexa Fluor® 488 showing less specificity in binding to B-genome chromosomes while showing cross-hybridization with A- and D-genome chromosomes. C) A merged image of photo A and B showing some differences in fluorescence intensity for each genome.

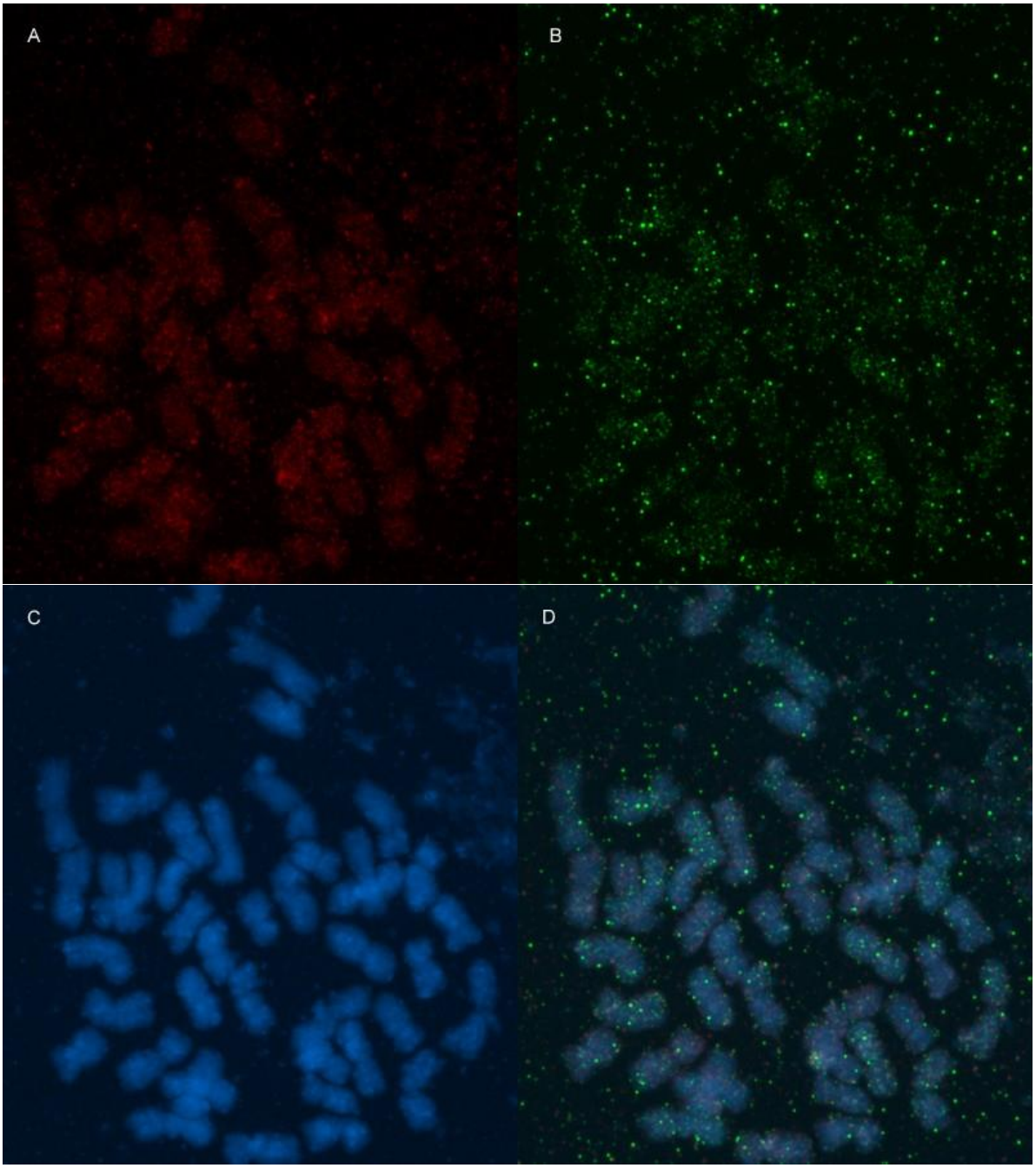

Figure 2.2. Fluorescent genomic *in situ* hybridization images of a wheat mitotic chromosome spread using an indirect DNA labeling method. A) *T. urartu* DNA labeled with digoxigenin showing extensive cross-hybridization with all genome chromosomes. B) *Ae. speltoides* DNA labeled with biotin showing cross hybridization and a strong signal backgound. C) Chromosomes stained with DAPI D) A merged image of photo A, B, and C showing minimal differences in fluorescence intensity for each genome.

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