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Environmental biosafety of field scale GM triticale (x*Triticosecale* Wittmack) cultivation for bioindustrial applications

by

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For my mother Stella, sister Lori-Lynn and her family, fiancé Andrew, and in loving memory of dad.

ABSTRACT

Triticale (x*Triticosecale* Wittmack) is an intergeneric hybrid of wheat (*Triticum aestivum* L. and *T. Durum* Desf.) and rye (*Secale cereale* L.) that is primarily utilized as an animal feed crop, but is being genetically modified (GM) to take advantage of its bioindustrial qualities. Prior to release of GM triticale cultivars, the potential for pollen-mediated gene flow (PMGF) and adventitious presence (AP) needs to be assessed to determine if it can coexist with weedy relatives and conventional wheat and triticale without causing unacceptable market harm.

We evaluated the potential for PMGF from triticale to wild and weedy relatives in Canada. In North America, triticale has few relatives with the exception of cereal crops wheat (spring and durum) and rye. Outside of parental genera, triticale is at highest risk for hybridization with intermediate wheatgrass (*Agropyron intermedium* (Host) Beauv.) and jointed goatgrass (*Aegilops cylindrica* Host).

Small and large plot experiments were conducted to quantify intraspecific PMGF from a triticale with a blue aleurone dominant trait to another triticale cultivar and spring and durum wheat. Combining small and large plot data, average intraspecific PMGF from 0.2-1.4 m was 0.76%. Large plot experiments recorded an average 3.4% PMGF adjacent the BA donor that, following an exponential decay model, declined to 0.09% by 50 m. Directional differences were detected with highest PMGF corresponding to prevailing wind

directions at flowering. The estimated AP of GM triticale after harvest blending within a 50 m conventional field was 0.22%.

We quantified interspecific PMGF from triticale to wheat because triticale is compatible with spring and durum wheat, exhibits synchronous flowering and may be grown in proximity. In small plot experiments, PMGF ranged from 0.0008% for spring wheat to 0.0006% for durum wheat, well below international labeling thresholds. Data indicated interspecific hybrids were rare.

Based on this research, intra- and interspecific PMGF may not prevent approved GM triticale from co-existing with weedy relatives and conventional triticale and wheat cultivars.

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List of Abbreviations

AIC – Akaike Information Criterion

ANOVA – Analysis of Variance

AP – Adventitious Presence

BA – Blue Aleurone Triticale

CFIA – Canadian Food Inspection Agency

CIMMYT – The International Maize and Wheat Improvement Center

CSH – Heterogeneous Compound Symmetry

CTBI – Canadian Triticale Biorefinery Initiative

EdRS – Edmonton Research Station

ElRS – Ellerslie Research Station

GM – Genetically Modified

HR – Herbicide Resistant

IR – Insect Resistant

LAC – Lacombe Research Centre

LLP – Low Level Presence

LRS – Lethbridge Research Station

PBO – Plant Biosafety Office

PIC – Polymorphic Information Content

PMGF – Pollen-Mediated Gene Flow

PNT – Plant with Novel Trait(s)

RFLPs – Restriction Fragment Length Polymorphisms

SMGF – Seed-Mediated Gene Flow

SSRs – Single Sequence Repeats

Chapter 1: Biosafety Assessment of Pollen-Mediated Gene Flow in Triticale (*xTriticosecale* Wittmack)

Introduction

Triticale (*xTriticosecale* Wittmack) is an intergeneric hybrid between wheat (common or durum) and rye and the first crosses were reported by Wilson in 1875 (Wilson, 1875). Incorporating desirable qualities from both parental species, triticale was introduced as an agricultural crop in the late 1960's (Ammar et al., 2004; Mergoum et al., 2004; Salmon et al., 2004). Triticale is largely an animal feed crop, but shows potential as a bio-industrial crop (CTBI, 2010; Government of Alberta Agriculture and Rural Development, 2010). Global production of triticale has increased and this trend is expected to continue as breeding programs release new cultivars with a range of improved traits (Salmon et al., 2004). Triticale biology and origins are discussed in more detail in **chapters 2 & 3**.

Triticale is amenable to genetic transformation (Stolarz and Lörz, 1986; Zimny and Lörz, 1996; Nadolska-Orczyk et al., 2005; Chugh et al., 2009); enabling breeding programs to create novel cultivars. Development of genetically modified (GM) triticales is currently underway in Canada to exploit the potential of triticale as a bioindustrial platform cereal (CTBI, 2010). Triticale is a good candidate for bio-industrial cereal feedstocks for biocomposites, biofuels, bioplastics and chemicals. It is a minor crop, has higher yields and starch compared to other cereals such as wheat and requires moderate water and nutrient

inputs (CTBI, 2010; McMenamin, 2010; Goyal et al., 2011). Before commercial release of any GM crop cultivar the Canadian Food Inspection Agency (CFIA) requires that safety assessments are conducted to ensure release does not cause unacceptable harm to food, feed and the environment (CFIA, 2011).

The Canadian Triticale Biorefinery Initiative (CTBI) is a ten year program to "…develop triticale as a dedicated bio-industrial crop supplying locally established, world-scale biorefineries producing sustainable materials, energy, and platform chemicals" (CTBI, 2010). Release of GM triticale cultivars is predicated on a thorough biosafety assessment. A substantial concern is that pollen-mediated gene flow (PMGF) from GM triticale to compatible wild or weedy relatives can lead to more invasive or persistent weedy species or disturbance of vulnerable native populations (Tiedje et al., 1989; Conner et al., 2003; Mallory-Smith and Zapiola, 2008). Alternatively, PMGF can lead to market disruptions through the adventitious presence (AP) of GM seed or material in conventional products. Markets have limits on the amount of approved GM product they will allow in a conventional shipment. The European Union (EU) has an AP limit of 0.9% (EUROPA, 2007) and conventional shipments over that threshold at the very least would be stopped at entry and at worst closure for the commodity and country of origin (EUROPA, 2007; Ramessar et al., 2009; Ramessar et al., 2010). To date, few publications have addressed triticale PMGF under traditional agronomic conditions.

Research Objectives

This thesis will expand our understanding of how triticale from a localized area can interact with neighbouring triticale, wheat, and wild relatives; and may assist in development of best management practices for breeders, seed growers and farmers. As part of the Canadian government funded CTBI, information gathered from this project will be used to create a biology document pursuant of an environmental biosafety component for transgenic release. The aim of the research described in this dissertation was to test the following objectives:

1. To determine the occurrence and distribution of wild and weedy relatives of triticale in Canada and their potential hybridization with commodity triticale to predict the risk of inter-specific transgene movement
2. To determine inter-specific pollen-mediated gene flow between triticale and parental wheat species to assess potential for co-existence of GM triticale with wheat
3. To determine intra-specific pollen mediated gene flow in triticale under natural agronomic conditions to evaluate the potential for co-existence of GM triticale with conventional triticale
4. To describe the potential benefits, risks, regulations and mitigation of transgene movement from GM triticale.

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Chapter 2: Literature Review

Triticale

Origin

Triticale (*xTriticosecale* Wittmack) is an intergeneric hybrid of wheat (*Triticum aestivum* L. or *T. durum* Desf.; female parent) and rye (*Secale cereale* L.; male parent) that was first crossed in the late 1800`s and introduced as a cereal crop in the late 1960`s (Wilson, 1875; Ammar et al., 2004). Triticale incorporates desirable traits from both parents including increased yield and starch content from wheat and abiotic stress resistance and disease resistance from rye (Lelley, 1992; Ammar et al., 2004; Mergoum et al., 2004; Government of Alberta Agriculture and Rural Development, 2006a). Triticale is generally taller than wheat and has up to 20% higher biomass and seed production (Government of Alberta Agriculture and Rural Development, 2001a; Salmon et al., 2004; Government of Alberta Agriculture and Rural Development, 2006a). Depending on which wheat is used, the resulting hybrid genome may be octoploid [2N=56; AABBDD (common wheat) x RR (rye) = AABBDDRR] or hexaploid [2N=42; AABB (durum wheat) x RR (rye) = AABBRR] (Lelley and Gimbel, 1989; Lelley, 1992; Ammar et al., 2004). Crossing octoploid triticales produces a hexaploid offspring (Jenkins, 1969). Globally hexaploid cultivars are grown almost exclusively, with the exception of China (US National Research Council, 1989; Government of Alberta Agriculture and Rural Development, 2001a). First generation primary triticales are repeatedly backcrossed to wheat to fix desirable

agronomic traits, resulting in a more prominent wheat background in triticale than rye (Briggs, 2001; Ammar et al., 2004; Mergoum et al., 2004). The biology and origins of triticale are discussed in more detail in **chapter 3**.

Reproductive Biology

Triticale and wheat are cleistogamous and are self-pollinated and rye is chasmogamous and cross-pollinated (Oelke et al., 1990; Waines and Hegde, 2004; Singh and Jauhar, 2006). Self-pollinated grass species usually do not open florets and expose stigmas or anthers to the outside environment until after pollination has occurred. Cross-pollinated grass species frequently have genetic-based constraints to self-pollination and open their flowers at maturity, promoting outcrossing and the likelihood of pollen-mediated gene flow (PMGF). Triticale and wheat share similar reproductive qualities. Stigmas are receptive for four days and usually remain within the floret (D'Souza, 1972). Anther protrusion begins after pollination (Wilson, 1968) and first occurs in the middle of the main spike and proceeds upwards and downwards (D'Souza, 1972; Cook and Veseth, 1991; Kociuba and Kramek, 2004). The period when pollination is possible (anthesis) is longer in triticale (~7-11 days) compared to wheat (~ 5 days) (Salmon, D., personal communication, June 2007; D'Souza, 1972; Cook and Veseth, 1991; Kociuba and Kramek, 2004). Tillers generally flower later than the main stem due to developmental lag and can extend the pollination window. Unsuccessful self-fertilization is followed by floret opening. Moreover, like wheat, environmental stress can induce pollen sterility causing triticale florets to open and increasing the

frequency of cross-pollination (Dorofeev, 1969; Waines and Hegde, 2004; Singh and Jauhar, 2006; Kavanagh et al., 2010).

Anther length of triticale is intermediate to wheat and rye; wheat being the shortest (Yeung and Larter, 1972; Sapra and Hughes, 1975). Sapra and Hughes (1975) examined anther extrusion in 12 triticale lines and found frequencies ranged between 31 and 72% extrusion. The single wheat and rye cultivar tested had an extrusion frequency of 34 and 45% respectively. Pollen production of triticale can be up to three times that of wheat, but half the amount rye generally produces (Sapra and Hughes, 1975). Triticale pollen production averaged 20,100 grains anther^{-1}, compared to wheat at 9,400 grains anther^{-1} and rye at 42,300 grains anther^{-1} (Yeung and Larter, 1972; Sapra and Hughes, 1975). de Vries (1971) in the Netherlands reported wheat pollen production at 1000-3800 grains anther-^{1} , however two subsequent studies by Yeung and Larter (1972) and Sapra and Hughes (1975) using North American cultivars found average pollen production to be almost three times that amount (~9,400 grains anther^{-1}). Pollen viability assessed under greenhouse conditions determined triticale pollen survived longer (110-120 min post-dehiscence) than wheat (~65-70 min post-dehiscence; Fritz and Lukaszewski, 1989). When tested under desiccating conditions pollen longevity for triticale decreased to 60-70 min compared to 35-40 min pollen longevity for wheat and 220 min pollen longevity for rye. Greater pollen longevity can be predicted for rye where outcrossing is required for reproduction.

The caryopses (seed+pericarp) of triticale and other cereals are typically referred to as 'seeds' in the agricultural literature and to maintain consistency we use their classification. Triticale seed size is intermediate to wheat and rye - longer than common wheat and plumper than durum wheat and rye and with a thousand kernel weight approximately 20% higher than wheat (Figure 2-1; Salmon, 2004). Reproduction biology and outcrossing frequency of triticale is described in more depth in **chapters 3** and **5**.

The first commercial triticale released for cultivation in Canada (cultivar Rosner) was reported to have a 5% PMGF frequency under nursery conditions (small pollen source, short distances; Yeung and Larter, 1972). However recent triticale cultivars have been extensively backcrossed to wheats and outcrossing frequencies have not been reported. Gene flow via pollen is expected to be within the range of wheat cultivars because triticale exhibits characteristics more similar to wheat than rye. This is reflected in regulations for pedigreed and non-pedigreed seed growers require an isolation distance of 3 m between pedigreed triticale and wheat plots that are grown in proximity to other pedigreed and non-pedigreed cultivars of the same species (Table 2-1; Canadian Seed Growers Association, 2011). In comparison, pedigreed rye must have 300 m isolation distances when grown in proximity to other pedigreed and non-pedigreed rye cultivars.

Western Canadian Agronomics

Winter and spring triticale are both grown in Canada and can be used for silage, however only spring triticale is recommended for seed utilization

(Government of Alberta Agriculture and Rural Development, 2006b). Winter triticale can be used for silage and in brown soil zones may be grown for seed after grazing in the spring (Government of Alberta Agriculture and Rural Development, 2001b). Spring triticale is mainly grown for silage for feedlots, triticale crops in Alberta seldom reach grain maturity and there is limited grain processing or exporting (Salmon et al., 2004). Winter triticale is seeded early in fall similar to winter wheat. Spring triticale is later maturing than wheat and must be seeded earlier (by mid-May in Western Canada) to maximize grain yield and forage quality (Government of Alberta Agriculture and Rural Development, 2006b).

Triticale can be grown in areas with high nutrient loads (i.e. highly manured fields) as it is more resistant to lodging than wheat or barley (Government of Alberta Agriculture and Rural Development, 2006a). Relative to wheat, few herbicides are registered for triticale (Government of Alberta Agriculture and Rural Development, 2011). Four wheat herbicide combinations (florasulam + MCPA ester, clodinafop-propargyl, thifensulfuron-methyl/tribenuron-methyl, and sulfosulfuron-methyl + 2,4-D ester) have been tested for use in triticale and it is tolerant to three: florasulam + MCPA ester, clodinafop-propargyl and thifensulfuron-methyl/tribenuron-methyl (Raatz et al., 2011). Time of harvest for seed production occurs when grain moisture content is below 13.5% to reduce post-harvest moulds and insect infestations. Additionally pre-harvest sprouting during warm or wet conditions can lead to decreased yield and grain quality (Trethowan et al., 1993; Singh and Jauhar, 2006).

Production

Globally triticale is a minor grain and forage crop species having 4 M ha in production during 2009 (FAOSTAT, 2010). Hectarage in Canada remains low at <15,000 ha when compared to over 13 M ha for spring wheat and 115 400 ha for rye in 2010 (FAOSTAT, 2010). In Canada 13 spring and four winter triticale cultivars are currently available (CFIA, 2011d) however most Canadian hectarage is devoted to spring triticale and Alberta is the largest producer with 12 100 ha seeded in 2010 (Government of Alberta Agriculture and Rural Development, 2001b; FAOSTAT, 2010; Government of Alberta Agriculture and Rural Development, 2010; Government of Saskatchewan, 2010). Triticale is increasing in hectarage (Figure 2-2) due to its utility as a silage crop and other agronomic advantages, i.e. reduced lodging, tolerance to acidic soils and increased drought tolerance and yield under dryland conditions when compared to barley, oats and wheat (Government of Alberta Agriculture and Rural Development, 2006a). Spring triticale will be the focus of the balance of this thesis.

Platform Potential

Triticale agronomical properties and chemical composition make it a candidate as a bioindustrial platform. Some cultivars of triticale are more suitable than wheat as a bioethanol feedstock due to its equivalent to higher starch, lower protein and lower input to biomass ratio (Pejin et al., 2009; CTBI, 2010; Goyal et al., 2011). The starch profile and fibre content of triticale seed may be conducive for use in bioplastics and biocomposites such as fibreglass for watercraft and household fixtures (CTBI, 2010); lignin from triticale straw may be beneficial for

use in adhesives and resins (CTBI, 2010); and ferulic acid present in triticale could be used in skin care, cancer prevention and food flavouring (Dervilly-Pinel et al., 2001; CTBI, 2010). The Canadian Triticale Biorefinery Initiative (CTBI) was formed to develop triticale for bioindustrial purposes, including development of GM 'designer triticale' cultivars tailored for a specified end use (Hills et al., 2007; CTBI, 2010). Triticale may be a low risk crop for GM development because it is not widely adopted as a human food grain, is a minor crop in Canada, and has a seed that is visually identifiable from wheat (Salmon, 2004; Government of Alberta Agriculture and Rural Development, 2006a; FAOSTAT, 2010; Raatz et al., 2011). Additionally, triticale is seldom grown to flowering and seed maturity (Salmon, 2004), and may be at low risk for PMGF to crop and wild relatives (Chaubey and Khanna, 1986; Gupta and Fedak, 1986; Balyan and Fedak, 1989; Neumann and Kison, 1992; Hills et al., 2007).

Genetically Modified Crops in Canada

Genetically modified crops have altered genetic makeups through the use of recombinant DNA technology that may include gene insertion, removal, silencing, or activation (Harlander, 2002; GKCCB, 2006). Genetic material used in genetic modification originates from the same species, a related or unrelated plant species, or an entirely different organism. Therefore, traits are not limited, unlike breeding using conventional or wide outcrossing or mutagenesis. All major and many minor crop species have genetically engineered cultivars commercially available or in development (Warwick et al., 2009; James, 2010).

Most cultivated GM cultivars are either herbicide resistant (HR) or insect tolerant (IT) or a combination of these two traits (James, 2010). HR crops may allow growers to use broad spectrum herbicides that are potentially more efficient and less expensive than selective herbicides (Beckie et al., 2006; Qaim, 2009). IT crops like Bt corn and Bt cotton can reduce pesticide input and subsequent fuel and labour costs (Brookes and Barfoot, 2006). Qaim (2009) assessed increases in gross margins from 1996-2008 for countries that adopted Bt cotton and corn that ranged from 12 – 470 US$/ha. Yield increases may have resulted from better weed management and reduced pest damage. Cultivation of GM crops may decrease costs and increase margins to farmers who choose to adopt them despite a premium they may pay for seed (Gealy et al., 2007; Qaim, 2009).

Potential environmental benefits from GM crop adoption vary with the trait. Growing Bt crops may reduce the amount of chemical pesticides released to the environment and potential ecological and health repercussions (Harlander, 2002; Brookes and Barfoot, 2008; Qaim, 2009). From 1996-2006 it was estimated Bt cotton cultivars reduced global pesticide ingredient use by 128 M kg (Harlander, 2002; Brookes and Barfoot, 2008; Qaim, 2009) and by 2001, 15 M fewer pesticide applications per year were reported from growing Bt cotton (Carpenter and Gianessi, 2001). In the US, average kg ha^{-1} herbicide ingredients were reduced by 25-33% from 1995-2005 through adoption of HR canola, cotton, maize and soybean (Kleter et al., 2007). Adopting HR crops enables the use of less toxic or persistent herbicides. In India and Argentina by 2005, the use of the two most toxic classes of herbicides decreased between 70-80% (Qaim and de

Janvry, 2005; Qaim, 2009). Indirectly, HR crops may be beneficial by encouraging more farms to employ no-till practices (Brookes and Barfoot, 2008; Qaim, 2009) potentially reducing soil erosion, fuel consumption and greenhouse gases associated with tillage (Beckie et al., 2006; Brookes and Barfoot, 2006).

From 1996 to 2010 there has been an 87 fold increase in the hectarage of global GM crop cultivation with 29 countries devoting 148 M ha to GM crops in 2010 (James, 2010). The majority of modified crop plants in cultivation worldwide incorporate HR trait(s) (i.e. canola, & soybean) at 89.3 M ha, IT (i.e. BT corn & cotton) at 26.3 M ha or have stacked genes (two or more novel traits) for HR & IT with 32.3 M ha in cultivation globally in 2010 (Figure 2-3; James, 2008; Warwick et al., 2009; James, 2010).

GM cultivars must be approved by the Canadian Food Inspection Agency (CFIA) prior to sale or commercial release (see following section). Canada approved its first GM crop, a glufosinate ammonium-tolerant canola, in 1995 (CFIA, 2011b). Canada is the fifth largest GM crop producer with 8.9 M ha in 2010 compared to the largest producer the USA with 66.8 M ha (Figure 2-4). Expansion of GM hectarage in Canada is slower than in other countries, and even though hectarage increased, Canada still declined in global ranking from fourth largest GM producer in 2008 to fifth largest in 2010 (Figure 2-4). Registered PNT crops in Canada include alfalfa, canola, lentil, potato, rice, soybean, sunflowers and wheat and novel traits most cultivated are HR (CFIA, 2011b). However, not all of these species have been commercialized in Canada (i.e. HR alfalfa).

GM technology is actively being applied to the introduction of agronomic traits. Future modifications to be incorporated or improved in crop species will be increased nutrient efficiencies and abiotic stress tolerance (Beckie et al., 2006; Warwick et al., 2009). These 'second generation' traits are in development and include nitrogen use efficiency which may reduce energy and greenhouse gas emissions through decreased fertilizer inputs; cold tolerance that will allow earlier seeding and lengthening of growing seasons; and drought tolerance which may lead to yield stability when rainfall and soil moisture is limited (Qaim, 2009; Warwick et al., 2009). The first of these crops, a drought tolerant corn, may be released in the USA in 2012 (Edmeades, 2008).

Regulation of Genetically Modified Crops in Canada

Confusion exists over appropriate classification of novel plants. The CFIA classifies GM organisms as those where "its genetic material has been altered through any method, including conventional breeding" (CFIA, 2010b). Genetically engineered organisms are defined as being modified "using techniques that permit the direct transfer or removal of genes in that organism". In Canada a GM or GE crop is broadly regulated as a plant with a novel trait (PNT) which is '…a plant that contains a trait which is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health' (CFIA, 2008). The Plant Biosafety Office (PBO), a division of the Canadian Food Inspection Agency (CFIA), is tasked with regulating PNT's in Canada.

The CFIA regulations are ‘science-based’ assessments that are aligned with domestic and international policies (CFIA, 2010a) and the CFIA claims that ‘no other country uses a broad regulatory scope as Canada does with its “novelty” approach’ (CFIA, 2010c). Consideration is given to the novel trait not the method by which it was derived, be it GM, mutagenesis or conventional breeding (Demeke et al., 2006; CFIA, 2010c) and PNTs must demonstrate “substantial equivalence” or have the same effect for use and plant pest potential as conventional cultivars (CFIA, 2010d). If a PNT is to be used for human consumption it must also be approved by Health Canada (CFIA, 2011a).

Prior to commercial release or sale of a PNT, risk assessments involving food, feed and environment must be conducted to ensure safety. Consideration of food and feed safety are beyond the scope of this document. The focus will be environmental biosafety (addressed below) and potential economic harm (see Economic and Environmental harm section).

The five research considerations for environmental risk assessments are:

1. potential of the plant to become a weed of agriculture or be invasive of natural habitats
2. potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive
3. potential for a plant to become a plant pest
4. potential impact of a plant or its gene products on non-target species, including humans
5. potential impact on biodiversity (CFIA, 2004).

Although the PBO requires specific data to make regulatory decisions, it does not dictate how risk assessments are conducted. However, to answer these questions, the PBO requires specific data from the petitioning party, including:

- personnel involved and status of the PNT
- description of the PNT and its modification
- description of the trait
- biology and interactions of the PNT
- agricultural-silviculture practices
- potential environmental effects resulting from introgression (CFIA, 2002)

The final three considerations are generally encompassed in a biology document (for example, see CFIA, 2005).

For data collection in field trials the CFIA first permits 'confined release' of the PNT which limits plot size and requires reproductive isolation of propagable material. Sites are monitored during and after the trials and propagable material biomass must be destroyed in accordance with regulations after data is acquired (CFIA, 2011c). Unapproved events have a 0% threshold in most international jurisdictions. Information from confined release trials combined with other risk assessment data contribute to the development of decision documents by the CFIA that indicate if the PNT is safe for unconfined release in Canada (CFIA, 2011c).

This thesis is focused on quantification of PMGF, to address in part, consideration #2 – "potential gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive" – but also the implications for economic harm (see below).

Pollen-Mediated Gene Flow

PMGF is the transfer and introgression of genes or genetic information via pollen from a genetically distinct plant to another plant of the same species (intraspecific gene flow) or a related species (interspecific gene flow). To be at risk for PMGF, a hybridization event must result in viable seed production and the gene of interest must be stably introgressed into the genome of the hybrid (Ennos, 1994; Gustafson et al., 2005; Hanson et al., 2005). Gene flow from pollen can lead to spatial distribution of the transgene within and between populations and fields. In angiosperms, PMGF is the main source of gene movement between related species and is responsible for much of their diversity (Levin and Kerster, 1974; Waines and Hegde, 2004). Gene flow may occur within the same species (intraspecific gene flow) or between two different species (interspecific gene flow).

The main requirements for PMGF are: genome compatibility, sympatry of compatible species and flowering synchrony between donor and receptor plants resulting in viable fertile hybrids (Arnold, 1997; Eastham and Sweet, 2002; Jenczewski et al., 2003; Waines and Hegde, 2003; Waines and Hegde, 2004). Interspecific PMGF requires genomes of donor and receptor species be genetically compatible with sufficient chromosome pairing to allow embryo and

endosperm development. In some species, mechanisms are present in the genome to prevent outcrossing with non-compatible species. For example, genes *pairing homoeologous*1&2 (*Ph*1 and *Ph*2) in *T. aestivum* have been shown to prohibit homoeologous pairing of chromosomes with other incompatible species (see **chapter 3**; Jauhar and Chibbar, 1999). Intra- and interspecific PMGF requires sympatry of pollen donor and receptor plants with minimal physical barriers and donor plants must be producing pollen when receptor plants exhibit stigmatic receptivity (flowering synchrony; Lamkey, 2002). Even if these requirements are met, environmental factors may reduce the likelihood of hybridization by influencing physical distribution of pollen and pollen longevity.

The degree of outcrossing is primarily influenced by the reproductive system of a crop, however environmental factors may be more important than genetic factors under certain conditions (Willenborg, 2009; Waines and Hedge, 2003) and cannot be discounted in PMGF assessments. Hot arid conditions may lead to pollen sterility and induce cleistogamous cultivars to open their flowers, increasing risk for PMGF (Waines and Hegde, 2004). Additionally, suitable environmental conditions are crucial when pollination vectors are wind or insects. Absence of winds may decrease distance pollen can travel in wind pollinated species (Angevin et al., 2008) and high velocity winds can decrease winged-pollinator visits for insect pollinated species (Totland, 1994). Pollen longevity can influence the frequency and distance of pollen movement and may be highly variable depending on environmental conditions. For example, cereals are known to have decreased pollen survival under low nutrient and desiccating conditions

(Fritz and Lukaszewski, 1989; Shivanna and Ram, 1993; Gul and Ahmad, 2006). If pollen remains viable, movement can be impeded by physical barriers such as non-compatible crop or weed species, buildings, and roads (Colbach et al., 2001). Pollen movement follows a leptokurtic dispersal pattern where the largest pollen counts are recorded closest to the source and declines with increasing distance (Raybould and Gray, 1993; Treu and Emberlin, 2000). As expected, PMGF also follows a leptokurtic pattern when studied in canola (Salisbury, 2002) flax (Jhala et al., 2011) safflower (McPherson et al., 2009) and wheat (Beckie and Hall, 2008).

In agriculture, PMGF is of particular concern when pollen transfer can lead to the movement of transgenes. However hybridization does not ensure the transgene will be retained or expressed in the hybrid. PMGF may be common when species are self-pollinated, or may be relatively rare when species are primarily cleistogamous. Corn (*Zea mays*) is cross-pollinated and produces large amounts of pollen (up to 2500 grains anther^{-1}) that may mature before stigmatic receptivity (Wallace et al., 1949; Goss, 1968) and is distributed by wind (Wallace et al., 1949; Goss, 1968; Purseglove, 1972; Ma et al., 2004). In Canada, a recent study of PMGF frequency between adjacent rows of corn ranged 14.2 and 17% (Ma et al., 2004). Soybean (*Glycine max* L.) is and most pollination occurs prior to flower opening. Pollen production in soybean (up to 760 grains anther^{-1}) (Reid et al., 1978) is lower than corn and much less PMGF (~0.16 – 0.52%) occurs between adjacent soybean rows (Yoshimura et al., 2006; Abud et al., 2007).

Lower PMGF is likely due to ovules being fertilized before stigmas are exposed to the outside environment to accept pollen from other sources.

Gene flow from modified crop cultivars has been intensely studied since their introduction in the mid 1990's. Rice (*Oryza sativa* ; Shivrain et al., 2007; Shivrain et al., 2008; Sanchez Olguin et al., 2009), canola (*B. napus*; Beckie et al., 2003; Warwick et al., 2003; Ceddia et al., 2007; FitzJohn et al., 2007; Warwick et al., 2008), corn (*Zea mays*; Ma et al., 2004; Messeguer et al., 2006; Van De Wiel et al., 2009), soybean (*Glycine max*; Ahrent and Caviness, 1994; Abud et al., 2007) and common wheat (*Triticum aestivum*; Matus-Cadiz et al., 2004; Gustafson et al., 2005; Hanson et al., 2005; Brûlé-Babel et al., 2006; Gatford et al., 2006) have been the focus of much of this research because they represent a large portion of global agricultural production of crops with modified or incorporated biotech traits (James, 2008; James, 2010).

Detection of GM Traits using Markers

The ability to detect introduced traits is required to assess risks from GM crops to the environment and economy. GM labeling thresholds imposed by several countries requires the ability to quantify GM trait presence in any given sample of seed or food product (Demeke et al., 2006). Markers are important tools used to detect intra- and interspecific PMGF events and require observing changes on the macro and micro levels. Three main types of markers used in hybrid identification and PMGF are HR marker genes, morphological and molecular markers (Andersen and Lubberstedt, 2003).

HR genes, through various mechanisms, lessen effects of herbicides that would otherwise cause mortality (Twyman et al., 2002; Miki and McHugh, 2004; Goodwin et al., 2005). Because HR markers are dominant, putative hybrids between HR and non-HR plants that survive herbicide application would confirm gene flow. HR genes have been widely used as selectable markers, inserted with other transgenes. They also have been used to detect gene movement, for example in canola (Powles, 2008), wheat (Willenborg et al., 2009; Beckie et al., 2011) and safflower (McPherson et al., 2009). Although effective and cost efficient, the same gene flow considerations that accompany GM plants – i.e. gene movement to unintended species – make the use of other markers more desirable for PMGF detection.

Morphological markers are types of phenotypic markers that are the traditional method of detecting different cultivars and hybridization in plant breeding programs. Single dominant traits that produce an easily detected morphological change such as flower or seed colour make it relatively easy to identify hybrid offspring. Some available marker traits are dominant and exhibit xenia (Acquaah, 2007). Xenia is a visible endosperm trait transferred through pollen and is expressed in hybrid offspring (Hucl and Matus-Cadiz, 2001; Acquaah, 2007). The xenia exhibited in seed texture, colour and shape have been reliably used to detect maize and wheat hybrids (Castillo-Gonzalez and Goodman, 1997; Hucl and Matus-Cadiz, 2001; Matus-Cadiz et al., 2004; Acquaah, 2007). However markers such as xenia may not be observed during all stages of plant development. In addition, when parental genomes are polyploid, or when

introduced morphological traits are variable (i.e. biomass, yield and height) it is possible altered genetic expression may be interpreted as natural genomic or phenotypic variation (Rieseberg and Ellstrand, 1993; Mohan et al., 1997).

Molecular markers can be used to detect genome hybridization and gene introgression on a DNA level and can detect 10 or fewer base pair changes within a genome (van Tienderen et al., 2002; Bernardo, 2008; Kumar et al., 2009). Although there are many types of markers, selection of marker type depends on the purpose of the test. Random DNA markers detect DNA polymorphisms not necessarily associated with phenotypic changes within the plant genome and are well suited for hybrid confirmation (Sunnucks, 2000). Random SSR markers are often selected because of their short segment length, ease of use, co-dominance and high degree of polymorphisms that make them useful in distinguishing closely related cultivars (Kuleung et al., 2006; Asif et al., 2009; Kumar et al., 2009; Mangini et al., 2010).

Molecular markers are also important tools in the detection and quantification of GM traits that may be present due to PMGF or through contamination during processing. Real-time PCR using molecular markers specific for introduced genes (transgenes) has been used internationally where GM crops may not be approved or there are limits to allowable GM content in conventional seed and materials (Berdal and Holst-Jensen, 2001; Permingeat et al., 2002; Pla et al., 2006; Alexander et al., 2007; Gulden et al., 2007; Christianson et al., 2008). When GM traits are approved by exporting countries,

these countries must provide means to screen for the transgenes and PCR analysis is the most desirable (Permingeat et al., 2002).

Two recent examples of the success and sensitivity of molecular markers and real-time PCR occurred in Germany and Brazil in 2009 when routine screening of imported flax shipments detected the presence of the nopaline synthase (T-nos) gene from a deregistered unapproved Canadian GM flax cultivar (CDC Triffid; CFIA, 2001; Flax Council of Canada, 2009; Pratt, 2009; Flax Council of Canada, 2010). Subsequent testing for other genes specific to the CDC Triffid flax including the kanamycin resistance gene nptII and the promoter P-nos confirmed the initial results (CFIA, 2001; GeneticID, 2009). Both countries have zero tolerance policies for unapproved GM events and markets were temporarily closed to Canadian flax across Europe and mandatory testing was implemented for all flax shipments entering Brazil (CFIA, 2001; Flax Council of Canada, 2009; Pratt, 2009; Flax Council of Canada, 2010).

The most robust analyses incorporate both morphological and molecular markers. The additional benefit from combining methods is the possibility of linking DNA regions to morphological expression (Varshney et al., 2005). Despite the method employed, the results must consider the probability of the observed trait being a spontaneous mutation and not an introgression or hybridization event.

Potential Environmental and Economic Harm via Gene Flow

Environmental risks may arise from interspecific gene flow that make crop weeds more difficult to control (weedy or invasive) or may genetically alter native

or weedy plant populations thus affecting population size, fitness and indirectly, biodiversity (Ellstrand and Hoffman, 1990; Conner et al., 2003; Warwick et al., 2009). If GM traits such as IR introgressed into related crop weeds through gene flow localized insect populations could be unintentionally targeted. Additionally, HR or abiotic stress introgression into related crop weeds may reduce weed control options and reduce profits. Transfer of HR has been a concern for canola (*Brassica napus* L.) in Canada where related crop weeds may be sexually compatible (Warwick et al., 2003; Simard et al., 2006). HR canola is widely grown in Canada and populations of field mustard (*B. rapa* L.) can be found in proximity to canola in some areas of Canada and may successfully hybridize with canola depending on density, spatial arrangement and distance from the pollen source (Simard et al., 2006). However hybrids were not as fertile as parental species and did not persist past four years. Traits such as HR may not convey an advantage weeds in natural habitats where herbicides are not applied, however IR and second generation GM traits that express abiotic stress tolerance (See genetically modified crops in Canada section) may be problematic and offer fitness advantages over non-introgressed populations (Gealy et al., 2007; Warwick et al., 2009; Wilkinson and Tepfer, 2009). If a fitness advantage is conveyed, populations may expand past original boundaries consuming resources previously used by other species, thereby affecting population dynamics. Conversely, if a gene conveys a fitness disadvantage, hybrid populations may exhibit new vulnerabilities to stressors putting the population at risk (Ellstrand, 2003; Warwick et al., 2009). Also, interspecific hybrids may serve as a bridge for

transgene introgression from a GM crop to a more distantly related species (Simard, 2006).

Economically, intra- and interspecific gene flow from GM crops can lead to market disruptions if they exceed AP thresholds for GM seed or material in non-GM products (Kershen and McHughen, 2005; Ramessar et al., 2010). AP from gene flow can have negative economic effects on a local scale by preventing growers from selling conventional or organic products without penalty. On an international scale, AP from approved events can lead to market and economic consequences should it surpass imposed thresholds.

AP thresholds that have been implemented in some countries represent 'low-level, technically-unavoidable and unintended presence' (CEC, 2002). The EU threshold for approved GM events is 0.9% and for Japan is 5% after which grains or grain products must be labelled as GM (Gealy et al., 2007; Ramessar et al., 2009; Ramessar et al., 2010). Because of proof of safety and substantial equivalence requirements surrounding approval of GM crops, these thresholds are based on consumer and political sentiments rather than risk to consumer health or the environment.

Thresholds were implemented in the advent of GM crops as requirements for coexistence between GM and conventional crop cultivars (Levidow and Boschert, 2008; Ramessar et al., 2010). Coexistence implies both cultivars can be grown together without causing unacceptable harm to either commodity. International market GM AP thresholds range from 0% for unapproved events or

approved events in organic systems, 0.1% for low-level presence of asynchronously approved events and 0.9% approved GM AP in the EU and 5% for approved GM AP Japan (EUROPA, 2007; Ramessar et al. 2009; Ramessar et al., 2010; EUROPA, 2011). Canada and the USA currently have no labeling standards. For the purpose of hypothesis testing, this research will use the EU 0.9% threshold for labelling of GM products.

Performing Risk Assessments

The purpose of risk assessments is to quantify the potential of PNT cultivation to cause unacceptable harm to humans, animals, environment or the economy (Raybould and Cooper, 2005; Garcia-Alonso et al., 2006; Raybould, 2006; Wolt, 2009; Beckie et al., 2010). Risk is defined as a function (f) of the ability to cause harm (hazard) combined with the probability of exposure [risk=f(hazard, exposure)] (Wilkinson et al., 2003; Raybould and Cooper, 2005). The potential for harm investigated in this thesis are: 1) potential for environmental harm by assessing potential for PMGF to wild relatives; 2) potential for economic harm by quantifying PMGF to crop relatives and comparing frequencies to international thresholds.

In Canada, the PBO of the CFIA requires specific data to make regulatory decisions, however does not provide guidelines on how risk assessments are conducted. All thorough assessments will determine risks by asking appropriate questions through the scientific method while seeking to minimize the amount of data required to accurately predict an outcome (Raybould and Cooper, 2005; Raybould, 2006; Wolt, 2009; Raybould et al., 2010). Hypothesis testing during

assessment cannot prove absence of risk or that GM crops or other PNTs are safe (Wolt, 2009; Raybould et al., 2010). Rather, testing can determine if risks are higher than pre-set thresholds. Regulatory decisions are made after examining outlined risks in association with environmental and government policies thresholds that will determine if they are "acceptable" or "unacceptable" (Raybould and Cooper, 2005).

Risk assessments should be iterative and hypothesis testing repeated on a progressively more complex scale only when initial worst case scenario results exceed a predetermined threshold (Poppy, 2000; Wilkinson et al., 2003; Wolt, 2009). The 'tiered system' utilizes this approach starting with conservative "worst case scenario" testing (Tier I) and progressing to qualitative experiments under progressively more natural conditions if set thresholds are exceeded (Tiers II & III; Wilkinson et al., 2003; Poppy and Wilkinson, 2005; Raybould and Cooper, 2005; Andow and Zwahlen, 2006; Garcia-Alonso et al., 2006). This method is routinely used to quantify risks of GM crop cultivation, test toxicity of pesticides and assess food safety and is designed to be time and resource efficient.

Tier I experiments are generally conducted under controlled laboratory or greenhouse conditions and are considered "worst case scenario" tests because they provide ideal conditions for hazards to occur (Poppy, 2000; Wilkinson et al., 2003; Raybould and Cooper, 2005). In PMGF assessments, tier I includes emasculation of pollen receptor plants and embryo rescue of hybrids (Wilkinson et al., 2003). If hazards are not observed, testing may be terminated and risk can be deemed low. If hazards are observed, hypotheses may be refined and Tier II

testing can proceed. Because not considered realistic, tier I tests may identify low risks but cannot confirm high risk under natural conditions (Wilkinson et al., 2003; Raybould and Cooper, 2005).

Tier II testing may include more laboratory or greenhouse experiments if additional hazards were identified in tier I or may progress to "semi-field" conditions such as controlled small plot experiments (Poppy, 2000; Wilkinson et al., 2003; Raybould and Cooper, 2005; Garcia-Alonso et al., 2006). If sufficient data is gathered during the experiment to decide risk and make regulatory decisions testing may be end, however if further information is required an additional tier may be explored.

Tier III tests are usually field studies and are intended to assess risks under more natural conditions (Poppy, 2000; Wilkinson et al., 2003; Raybould and Cooper, 2005; Garcia-Alonso et al., 2006). Specific questions answered during this tier have been refined throughout experimentation. For example, for PMGF:

Tier I: Are these plants sexually compatible?

Tier II: How likely is hybridization under natural conditions?

Tier III: Will PMGF in large field conditions exceed the selected 0.9% threshold?

If more information is required this tier may be refined further and retesting may proceed (Poppy, 2000; Wilkinson et al., 2003; Garcia-Alonso et al.,

2006). If risks are negligible or acceptable with "reasonable certainty", testing may stop (Garcia-Alonso et al., 2006).

Risk Assessments to Quantify PMGF in Triticale

To determine the economic and environmental risks of PMGF from GM triticale we followed the tiered risk assessment system. **Chapter 3**, a literature review examining the potential for triticale to hybridize with wild and weedy relatives, may be considered 'tier 0' as it identifies probability of hazard and exposure and assists in formulation of a targeted hypothesis to be tested in Tier I (Garcia-Alonso et al., 2006).

Tier I greenhouse experiments were completed prior to the initiation of the PhD and tested the sexual compatibility between wheat (common and durum), rye and triticale (**Appendix 1**; Hills et al., 2007). Approximately 2000 florets were emasculated per cultivar and pollinated with triticale pollen to determine crossing ability.

Tier II testing is described in **chapter 4** where small plot trials were conducted to test the likelihood of wheat x triticale hybridization under normal agronomic conditions. 1.9 M seeds were screened from two common wheat and one durum wheat pollen receptors in three locations over two years (four site years) to establish base-line interspecific PMGF rates.

Tier III experiments are described in **chapter 5**. Large plot trials were performed under normal agricultural conditions to quantify outcrossing and potential transgene movement across various distances up to 50 m and compare to

EU thresholds. Trials were conducted at two locations over two years (four site years) using a blue aleurone triticale (pollen donor) and conventional triticale (pollen receptor). Over 17 M seeds were screened to establish base-line intra-specific PMGF rates.

This thesis will contribute to a biology document intended for the CFIA to evaluate GM triticale coexistence potential with conventional triticale and wheat. Moreover, techniques employed in its completion will provide additional tools for genomic testing and cultivar and hybrid determinations (**Appendix 2**).

Table 2-1. Isolation distances for pedigreed and non-pedigreed triticale, wheat and rye seed producers. (*Adapted from Canadian Seed Growers Association, 2011*)

Crop	Proximity Crop	Isolation Distance
Triticale	• Pedigreed triticale – same cultivars	1 m
	• Pedigreed triticale – different cultivar • Non-pedigreed triticale • Barley, buckwheat, common & durum wheat, oat and rye	3 m
Wheat	• Pedigreed wheat – same cultivar	1 m
	• Pedigreed wheat – different cultivar • Non-pedigreed wheat • Barley, buckwheat, durum wheat, oat, rye and triticale	3 m
Rye	• Pedigreed rye – same cultivars	1 m
	• Pedigreed rye – different cultivar • Non-pedigreed rye	300 m
	• Barley, buckwheat, common & durum wheat, oat and triticale	3 m

Figure 2-1. Seeds of common wheat (A), durum wheat (B), rye (C) and triticale (D).

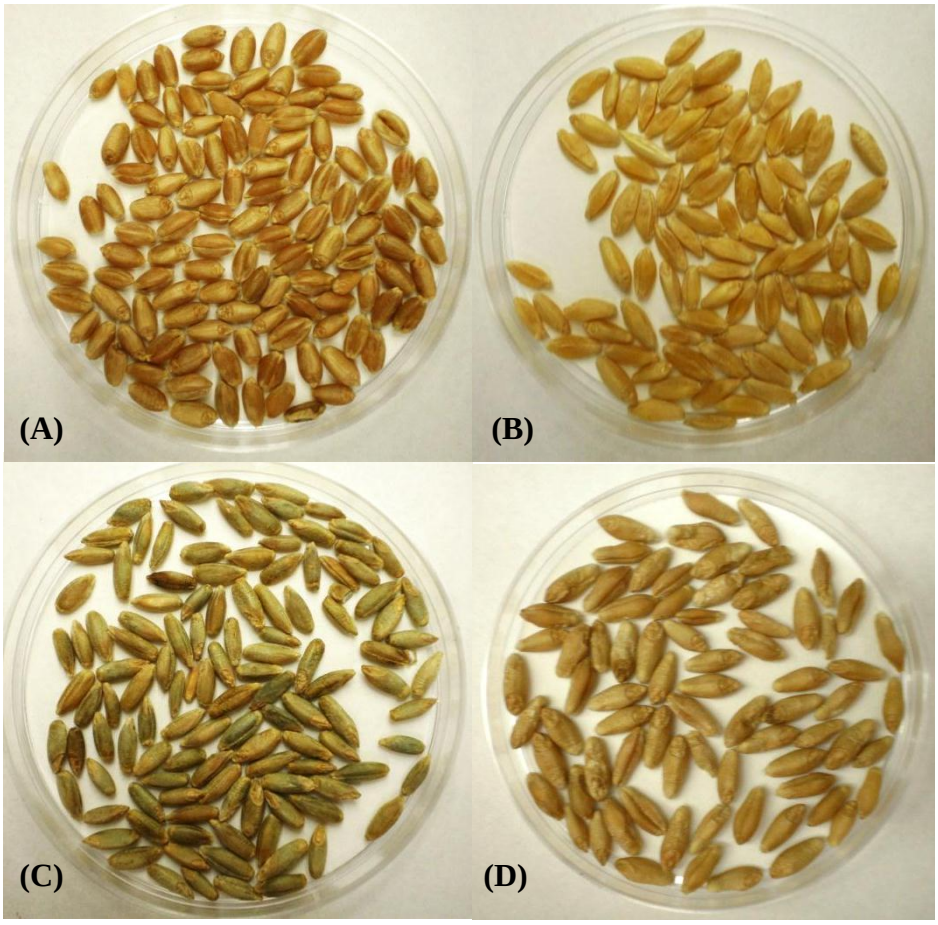


Figure 2-2. Hectares of triticale harvested globally since 1975 (FAOSTAT, 2011).

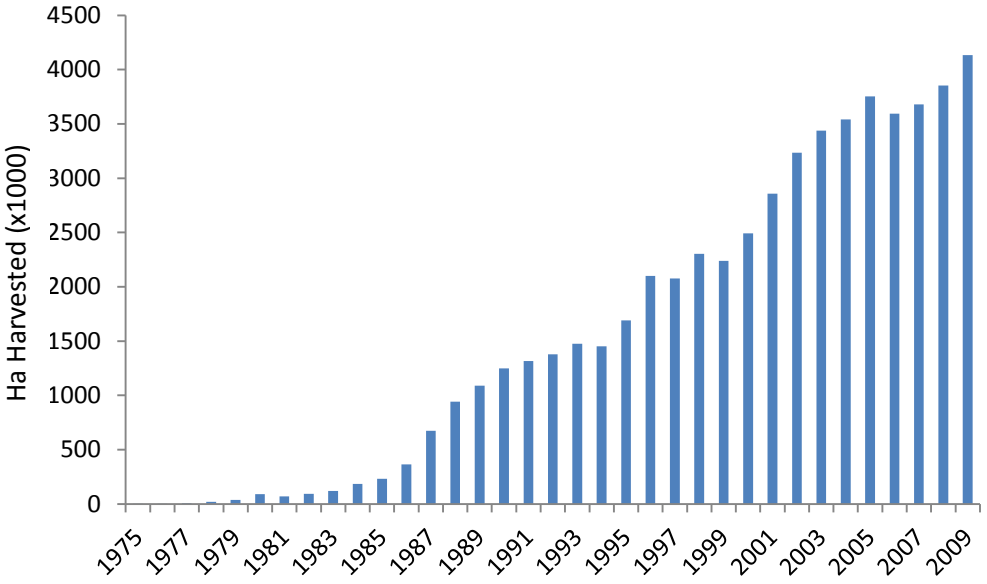


Figure 2-3. Proportion (%) of approved GM traits (insect resistance, herbicide resistance, and a combination or 'stack' of more than one trait) from the 148 M ha in global cultivation in 2010 (James, 2010).

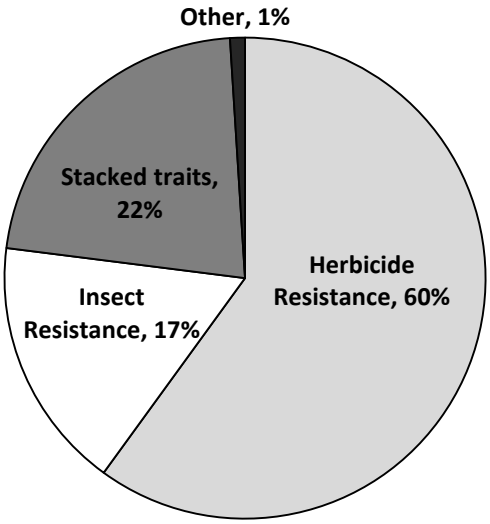
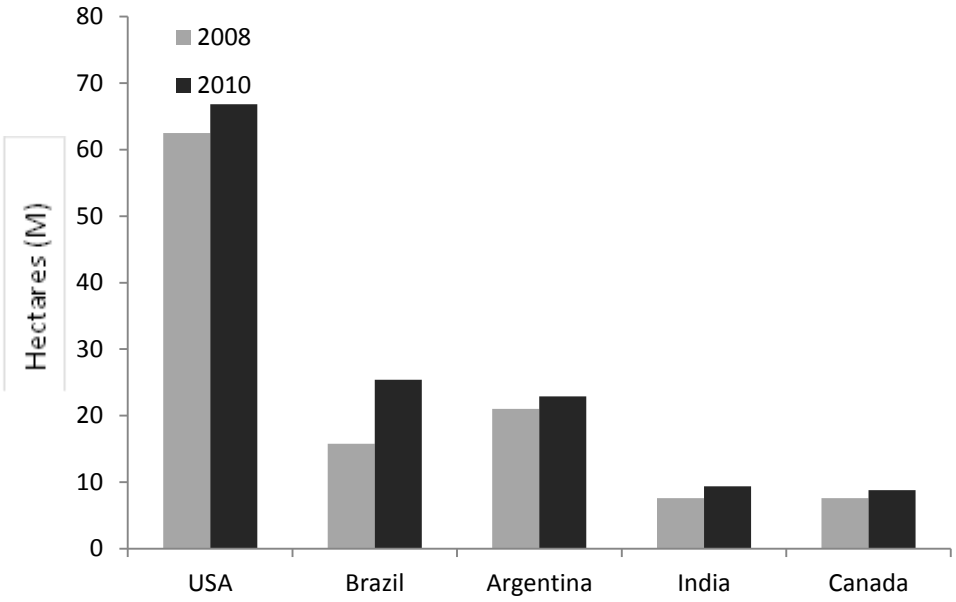


Figure 2-4. Area (ha) occupied by GM crops the top five producing countries in 2008 and 2010 (James 2008 & 2010).



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Chapter 3: Potential Hybridization of Genetically Modified Triticale with Wild and Weedy Relatives in Canada

Introduction

The movement of genes between plant species is a regularly occurring process and gene flow has been documented between many crops and wild species (Ellstrand et al., 1999). Recent attention has focused on this process because of the introduction of genetically modified (GM) plants with novel traits. Although GM of crop plants has the potential to increase profitability and sustainability of agriculture, there are concerns regarding potential environmental risks (Ellstrand et al., 1999; Snow et al., 2005; Tiedje et al., 1989; Wolfenbarger, 2000). One concern is the movement of transgenes from crops to wild or weedy species and effects on their genetic diversity and population dynamics. Depending on the trait introduced, hybrids may become invasive or persistent and impact community diversity (Conner et al., 2003; Mallory-Smith and Zapiola, 2008; Tiedje et al., 1989; Wolfenbarger, 2000). Gene flow from herbicide-resistant crop species to wild or feral relatives was reported in common wheat (*Tritium aestivum* L.), rice (*Oryza sativa* L.), and oilseed rape (*Brassica napus* L.) (Massinga et al., 2003; Seefeldt et al., 1998; Warwick et al., 2003; Sanchez Olguin et al., 2009; Shivrain et al., 2007). Herbicide-resistance genes moved from common wheat to jointed goatgrass (*Aegilops cylindrica* Host), giving rise to herbicide-resistant hybrids (Perez-Jones et al., 2006; Seefeldt et al., 1998). Shivrain et al. (2007). Sanchez Olguin et al. (2009) demonstrated imidazolinone and glufosinate resistance movement from herbicide resistant cultivated rice to feral rice cultivars.

In addition, herbicide-resistant oilseed rape hybridizes with wild relative field mustard (*Brassica rapa* L.) creating a weedy hybrid that has the potential to become a problem in cropping systems and surrounding areas (Massinga et al., 2003; Warwick et al., 2003, 2008). These examples underscore the need for detailed environmental risk assessments before release of new genetically modified crops.

Genetically modified triticale (tribe Triticeae, = Poaceae) is being examined for a range of uses, including bioproducts (CTBI, 2008; Hills et al., 2007; Zimny et al., 1995). Relatively new to agriculture, triticale is an intergeneric hybrid between wheat and cultivated rye (*Secale cereale* L.). Triticeae includes 32 genera and ~300 species (Watson and Dallwitz, 1992) and is of economic importance due to the germplasm potential of wild members for cereal breeding programs and the inclusion of key forage or reclamation species (Kellogg et al., 1996).

Triticale was developed to combine the yield potential of common or durum wheat (*T. aestivum* or *T. durum* Desf.) with the adaptability to less optimal growing conditions of rye (Ammar et al., 2004; Merker, 1985). Production of triticale is growing worldwide, in part due to an increase in its use as a feed stock, specialty food bioproduct and biofuel grain (FAOSTAT, 2007; Salmon et al., 2004; U.S. National Research Council, 1989; Varughese et al., 1996a,1996b). Triticale has similar protein and energy to other cereals and surpasses other grains in starch digestibility for ruminant animals (Bird et al., 1999; Hill, 1991). More recently, triticale has been used as a food-grade, bioproduct, and biofuel grain

(CTBI, 2008; Wang et al., 1997). Triticale is also a less expensive biofuel alternative to common wheat for bioethanol production (Wang et al., 1997). Briggs (2001) identified several different types of triticale cultivars suitable for the production of bioplastics, glues, and building materials.

An understanding of species relationships within *Triticum* L. and *Secale* L. as well as other members of the genera in Triticeae is essential to identifying species at risk for hybridization with triticale. *Triticum* and *Secale* both have complicated taxonomic and phylogenetic histories (e.g., Dvořàk and Zhang, 1992; Frederiksen and Petersen, 1998). Phylogenetic information and hybridization data used to determine these relationships have the potential to provide insight into which relatives may hybridize with triticale. In addition, evaluation of natural and artificial crosses between triticale, parental species, and their wild relatives is essential. Hybridization is also dependent on sympatry of species, synchronous pollen production for donor and stigmatic receptivity species, viability of hybrid seeds, and genetic compatibility of species. This paper examines potential hybridization risks between triticale and its wild and weedy relatives, an essential first step before experimental testing of gene flow from triticale to related species. We reviewed (i) the origin of triticale, (ii) the taxonomic and phylogenetic history of both parental genera in the tribe Triticeae, (iii) crossability of triticale and parental species in light of potential barriers to outcrossing, and (iv) biology and genomic constitutions of relatives of triticale.

Hybrid Origin of Triticale

To generate triticale, wheat (common or durum) functions as the pollen receptor (female parent) and rye acts as the pollen donor (male parent). Alexander Wilson reported this cross in 1875 but the resulting hybrids were sterile (Wilson, 1875). Later crosses by Elbert S. Carman (1884) and Wilhelm Rimpau (1891) produced hybrid progeny that were partially fertile. The successful intergeneric cross was named x*Triticosecale* Wittmack by combining the generic names of its parents (see Stace 1987 for review of triticale nomenclature). Meister (1921) reported the occurrence of natural hybrids between common wheat and rye, although fertile hybrids were not observed until 1928 (Meister and Tjumjakoff, 1928). These natural hybrids were termed primary triticales because they were the first generation of wheat x rye crosses (Kiss, 1966).

Primary triticales of Carmen and Rimpau were octoploids (2N=56), the result of crossing hexaploid wheat (2N=42) with diploid rye (2N=14; Carman, 1884; Oettler, 2005; Rimpau, 1891). Octoploid triticale exhibited unpredictable fertility despite numerous attempts using different crossing techniques and varied parental lines (Ammar et al., 2004; Lelley, 1992; Oettler, 2005; U.S. National Research Council, 1989). In the late 1930s colchicine was used to induce chromosome doubling and produce fertile hybrids (Blakeslee and Avery, 1937).

Because the large genome of octoploid triticales was implicated in poor crop performance, research began to develop triticale to lower ploidy levels. Jesenko (1913) crossed both tetraploid wheat (2N = 28; usually *T. dicoccoides* (Körn.) Körn. ex Schweinf. and *T. durum*] and hexaploid common wheat with rye.

Tetraploid crosses resulted in more seedlings than those with hexaploid common wheat, presumably due to their smaller genomes (Lelley and Taira, 1979; Oehler 1931; Oettler, 1982). Crossing two octoploid primary triticales produced hexaploid progeny and breeding programs in Canada, Hungary, and Russia focused on crossing octoploid triticales with the newly created hexaploids (Ammar et al., 2004; Jenkins, 1969; Oettler, 2005; Pissarev, 1966). Hexaploid progeny were more consistent in their productivity than their octoploid counterparts (Kiss and Videki, 1971; Muntzing, 1979; Oettler, 2005) and research efforts shifted to hexaploid triticales.

Secondary triticales are those produced through several different approaches: (i) crossing two primary triticales, (ii) any triticales generated after the primary triticale, or (iii) backcrossing triticale with wheat (common or durum) or rye (Kiss, 1966). These crosses expanded the range of traits within triticale. Two cultivars (Triticale no. 57 and Triticale no. 64) were released in 1968 by Kiss for commercial production in Hungary (Ammar et al., 2004; Mergoum et al., 2004) shortly followed in 1969 by the release of cultivar Rosner in Canada and Cachurulu in Spain (Larter et al., 1970; Sánchez-Monge, 1973). These cultivars were limited in their success but were instrumental in introducing farmers to the potential of this novel crop (Ammar et al., 2004; Oettler, 2005). The International Maize and Wheat Improvement Center (CIMMYT) began working with triticale in the 1960s and crop improvement became more of a global collaborative effort (Ammar et al., 2004). Through an intensive breeding program, yields of these new lines of triticale were comparable or exceeded that of common wheat with

increased tolerance to marginal growing conditions (Gregory, 1974; Lapinski and Apolinarska, 1985; Mackowiak, and Lapiński, 1985). Currently, breeding programs are still in place with the mandate of crop improvement (Bernard et al., 1996; Salmon et al., 2004).

Classification and Phylogeny of *Triticum*

Specific relationships in the genus *Triticum* have been analyzed using chromosomal interchange identification (Riley et al., 1967), sequence variation in chloroplast DNA (cpDNA; Mori et al., 1995; Yamane and Kawahara, 2005) and the internal transcribed spacer DNA (Hsiao et al., 1995), sequencing data (Golovnina et al., 2007), and comparative genetic analysis (Goncharov, 2005). Classifications have been inconsistent for *Triticum*, a phenomenon highlighted by three recent studies. Gill and Friebe (2002) recognized six species with 13 subspecies, a modified system of van Slageren (1994), presumably based on morphology. In contrast, Goncharov (2005) divided 29 species of *Triticum* into five sections based on comparative genetic analysis. In the most recent analysis of cpDNA sequences, Golovnina et al. (2007) concluded there were 30 species and four subspecies. This chapter follows the most recent classification (Golovnina et al., 2007).

There is no consensus on phylogenetic relationships within *Triticum* (e.g., Gill and Friebe, 2002; Golovnina et al., 2007; Petersen et al., 2006; Yamane and Kawahara, 2005). Many members are known polyploids which results in a reticulate rather than a strictly branching phylogenetic pattern (Kellogg et al., 1996) and there is little genetic variation among species making it difficult to find

useful molecular markers (Hsiao et al., 1995; Sallares and Brown, 2004). *Triticum* and *Aegilops* L., a closely related genus, are intermixed together on phylogenetic trees derived from the chloroplast markers *mat*K and *trn*L (Golovnina et al., 2007); analysis of base pair substitutions, insertion/deletion events, and microsatellites of chloroplast noncoding sequences (Yamane and Kawahara, 2005); and β-amylase sequence analysis (Mason-Gamer, 2005).

Classification and Phylogeny of *Secale*

Species composition of *Secale* has also undergone considerable taxonomic revision. Roshevitz (1947) reported 14 species in the genus. However, many of these species were subsequently designated as subspecies in later assessments. Khush (1962) and Khush and Stebbins (1961) reduced the number of species to five and then Hammer et al. (1987) proposed four species in *Secale*. Most recently, *Secale* comprises three species: *S. cereale*, *S. strictum* (C. Presl.) C. Presl. (= *S. montanum* Guss.), and *S. sylvestre* Host (Frederiksen and Petersen, 1997, 1998). This chapter follows the latter treatment.

In all analyses *S. sylvestre* was phylogenetically distinct from the other *Secale* species examined. This separation of *S. sylvestre* was noted in morphology-based studies (Khush and Stebbins, 1961; Roshevitz 1947) and supported by molecular studies based on restriction fragment length polymorphisms (RFLPs) and ribosomal DNA spacer length (Petersen and Doebley, 1993; Reddy et al., 1990); thin layer chromatography (Dedio et al., 1969); isozymatic analysis (Vences et al., 1987); and restriction endonuclease analysis of cpDNA (Murai et al., 1989).

Also consistent with earlier, morphological studies (Roshevitz 1947; Khush and Stebbins, 1961), molecular analyses indicate the species boundaries between *S. strictum* and *S. cereale* are not well defined. Petersen and Doebley (1993) concluded both species were not monophyletic based on RFLPs. Comparing 14 isozymatic loci, Vences et al. (1987) reported very high genetic identity between the two species (0.964) and were unable to separate them using statistical analysis. Reddy et al. (1990) also could only clearly separate *S. sylvestre* when examining DNA spacer length differences. These findings agree with a more recent analysis using variation in *Adh*1 sequences of *Secale* by Petersen et al. (2004). Despite the molecular similarities they are still considered separate species in all classifications to date.

Phylogeny of Tribe Triticeae

Relationships within the tribe are poorly understood due, in part, to a wide range of chromosome numbers and inconsistencies between placements in phylogenetic trees of different members (Bouchenak-Khelladi et al., 2008; Kellogg et al., 1996; Petersen and Seberg, 1997). For example, the position of *Triticum* and *Secale* in the tribe is unclear. In analysis of multiple plastid DNA loci *rbc*L, *mat*K, and *trn*L-F, Bouchenak-Khelladi et al. (2008) found *Triticum* grouped with *Aegilops*, x*Triticosecale*, and *Taeniatherum* Nevski. Based on sequence variation in β-amylase genes Mason-Gamer (2005) also grouped *Crithopsis* Jaub. & Spach with *Triticum*, but Bouchenak-Khelladi et al. (2008) found *Crithopsis* formed a group with *Secale* more distantly related to *Triticum*. Mason-Gamer (2005) also found *Secale* with *Australopyrum* (Tzvelev) Á.Löve

and *Dasypyrum* (Coss. & Durieu) T. Durand. Because of the contradictions in relationships, it is challenging to use phylogenies alone to assess triticale hybridization potential.

Triticale Hybridization Potential with Parents and Wild Relatives

Hybridization potential of triticale and relatives can be influenced by many factors including genome constitution and compatibility, genetic barriers, floral structure, distance from pollen source to compatible pollen receptor, size of compatible populations, physical barriers, temporal barriers, and environmental conditions (Conner et al., 2003; Tiedje et al., 1989). These factors can be complicated due to interactions or environmental stochasticity at time of anthesis (i.e., wind speed, humidity, temperature). The ability of triticale to outcross with (i) wheat and rye parental species and (ii) other potentially compatible wild relatives needs to be examined.

Allopolyploidization results in sequence changes and deletion in many species including common wheat (Ozkan et al., 2002) and canola (Song et al., 2005). Xue-Feng et al. (2004) compared parental and triticale genomes and determined that 2.7 and 62% of wheat and rye expressed sequences had changed, respectively, either through sequence loss or modification. The rate of loss for wheat sequences varied depending on the ploidy level of the wheat used to create the primary triticale. Crosses with hexaploid common wheat result in an approximate 9% DNA loss compared to 28 to 30% loss with tetraploid durum wheat (Boyko et al., 1984). Due to a more than 60% decrease in rye genome

expression (Boyko et al., 1984), triticale may have more outcrossing barriers to rye than to wheat.

Moreover, triticale inherited wheat genes that reduce crossability to rye and other related species (Guedes-Pinto et al., 2001; Lelley et al., 1995; Oettler, 2005). Wheat genes *Ph*1 and *Ph*2 have been inherited by triticale and inhibit outcrossing by preventing homoeologous pairing of chromosomes (Jauhar and Chibbar, 1999; Weissmann et al., 2008; Zaharieva and Monneveux, 2006). These genes have been problematic for different wheat and triticale breeding programs when introgression from other related species is needed for introduction of new traits (Canadian Food Inspection Agency, 1999; Feuillet et al., 2008; Zaharieva and Monneveux, 2006). Conversely, the function of *Ph* genes makes them desirable for use in GM common and durum wheat development as a chaperone to prevent the movement of transgenes (Weissmann et al., 2008). Crossability genes *Kr*1, *Kr*2, *Kr*3, and *Kr*4 from wheat are also present in triticale and strongly suppress outcrossing to rye and other related species (Guedes-Pinto et al., 2001; Lelley, 1992; Oettler, 2005). In fact, Manickavelu et al. (2009) proposed using this gene to eliminate outcrossing risks with rye.

The florets of cross-pollinated grass species, such as rye, are open at anthesis, allowing pollen distribution and outcrossing via wind and/or animals (Oelke et al., 1990; Waines and Hegde, 2004). Florets usually open after anthesis for self-pollinated species like common and durum wheat (Oelke et al., 1990; Singh and Jauhar, 2006; Waines and Hegde, 2004). However, under environmental stress, florets of wheat may open, increasing the opportunity for

cross-pollination (Dorofeev, 1969; Waines and Hegde, 2004). Triticale exhibits a cleistogamous floral structure although there is some propensity for cross-pollination (Singh and Jauhar, 2006; Yeung and Larter, 1972). In addition, it has a short period for anthesis, approximately 7 to 11 d (D. Salmon, personal communication, 2007) and for hybridization to occur compatible species must have stigmatic receptivity during this time (Lamkey, 2002). Because of its closed florets and short flowering times, triticale is much more likely to self than outcross.

Greenhouse studies using manual crosses (Chaubey and Khanna, 1986; Hills et al., 2007; Lelley, 1992) confirm that triticale can hybridize with both wheat and rye parents (Table 3-1). Chaubey and Khanna (1986) found low seed set between triticale and common wheat or rye. Three cultivars of triticale (UPT7681, UPT78268, UPT75233) and two cultivars of hexaploid common wheat (UP2003, UP262) were crossed. Two combinations, UPT75233 x UP2003 and UPT7681 x UP2003, resulted in viable seeds with three and two seeds germinating from 140 and 120 florets pollinated, respectively. In crosses between triticale cultivar UP7681 and Russian rye, 20 seeds were set out of 110 florets pollinated. Of the seeds set, seven germinated. Fertility and/or viability of the resulting F1 seedlings were not discussed. Lelley (1992) found higher success rates crossing triticale with common wheat than rye producing 21 and ninr new hybrid lines, respectively. Total numbers of florets pollinated and set seed was not reported. Both Lelley (1992) and Hills et al. (2007) concluded crosses between triticale and rye exhibited lowest crossability. Furthermore, Hills et al. (2007)

observed F1 hybrids created from crosses between triticale and all three parental species were usually sterile. Crosses with wheat species were most viable when triticale was the female parent (up to 97% emergence of seed set) vs. male parent (up to 1% emergence; Hills et al., 2007). These differences in pollen donor and/or receptor success will be relevant to future hybridization assessments.

Natural crossing of triticale back to cultivated common wheat was reported in Mexico between an unknown dwarf common wheat and triticale cultivar x308. In an experimental plot, the hybrid exhibited higher grain yield and fertility (Ammar et al., 2004; Zillinsky and Borlaug, 1971). By 1970, this accidental hybrid, named Armadillo, was incorporated into the majority of cultivated triticale lines (Ammar et al., 2004; Zillinsky, 1974).

Outcrossing data of triticale with nonparental species are limited. Hybridization between triticale and *Agropyron trichophorum* (Link) K. Richt (= *A. intermedium* ssp. *trichophorum* (Link) Halac.; Gupta and Fedak, 1986a); *Hordeum parodii* Covas (Gupta and Fedak, 1986b); cultivated barley, *Hordeum vulgare* L. (Balyan and Fedak, 1989); and *T. monococcum* L. (Neumann and Kison, 1992) were successful but required embryo rescue and resulted in low numbers of hybrid plants: 0.05% plants obtained from the total florets pollinated in x*Triticosecale* x *T. monococcum*, 0.58% in *Hordeum parodii* x x*Triticosecale*, 2.0% in x*Triticosecale* x *A. trichophorum* (Link) K. Richt, 0.75% in *H. vulgare* x x*Triticosecale* (Table 3-2). No reports were found of triticale crossing naturally with wild species.

In the absence of outcrossing data on triticale and wild relatives, genome designations may shed light on hybridization potential. Designation is determined by the amount of complete meiotic pairing in F1 hybrids. If the hybrids have <50% complete pairing, they are assigned a different genome letter (Wang et al., 1995). Octoploid triticale has genome designation AABBDDRR and hexaploid has AABBRR. The AABBDD and AABB are derived from either wheat parent and the RR is from the rye parent. Zaharieva and Monneveux (2006) determined that similar designations predict successful hybridization events in *Triticum*. Because the AABB from both wheat parents are very similar (Gill and Friebe, 2002), triticale may be able to hybridize with compatible relatives of both *T. aestivum* and *T. durum*. *Triticum dicoccoides* is the parent of both cultivated wheats and shares the same genome designation as durum wheat (AABB; Dvorak et al., 1998; Gill and Friebe, 2002; Golovnina et al., 2007). While confirmed reports of hybrids between *T. dicoccoides* and *T. aestivum* are difficult to find, Liu and Tsunewaki (1991) proposed that this ancestral cross led to *T. spelta* L. Crosses between *T. dicoccoides* and *T. durum* have been recorded in the South Caucasus region, occasionally resulting in fertile offspring (Dorofeev, 1968, 1969). No natural hybridization events have been recorded between either *T. durum* or *T. aestivum* and *T. urartu* and *Ae. speltoides*, the parental species of *T. dicoccoides*, which may be due to genetic differentiation in cultivated common and durum wheat.

Related species to cultivated common and durum wheats are in the genera *Triticum* and *Aegilops* (Bouchenak-Khelladi et al., 2008; Kellogg et al., 1996).

Triticum aestivum is compatible with all other hexaploid wheat species and subspecies with the same genome designations (AABBDD; Korber-Grohne, 1988). Zaharieva and Monneveux (2006) examined 13 European species, one *Triticum* and 12 *Aegilops* that are wild relatives of *T. aestivum*. Of these, eight species had confirmed reports of spontaneous hybridization with *T. aestivum*: *Ae. biuncialis* Vis., *Ae. columnaris* Zhuk., *Ae. cylindrica* Host, *Ae. geniculata* Roth., *Ae. neglecta* Req. ex Bertol., *Ae. speltoides*, *Ae. triuncialis* L., and *Ae. ventricosa* Tausch. but the majority of offspring were sterile. Zaharieva and Monneveux (2006) reported the following species hybridizing with *T. aestivum* under assisted laboratory conditions: *Ae. biuncialis* Vis., *Ae. columnaris* Zhuk., *Ae. cylindrica* Host, *Ae. geniculata* Roth., *Ae. neglecta* Req. ex Bertol., *Ae. speltoides*, *Ae. triuncialis* L., and *Ae. ventricosa* Tausch. However, laboratory and assisted hybridization experiments alone do not predict successful crossing in natural environments and can involve highly technical procedures such as artificial chromosome doubling, embryo rescue, and/or climatic and edaphic controls. Boguslavski (1978, in Zaharieva and Monneveux, 2006) assessed potential for natural hybridization between 22 *Aegilops* species and *T. aestivum*. *Aegilops* x *Triticum* hybrids were confirmed using *Ae. biuncialis*, *Ae. cylindrica*, *Ae. neglecta*, *Ae. speltoides*, and *Ae. triuncialis* with fertility rate ranging from 0.2 to 6%.

Although no wild species of *Triticum* are distributed in North America, the Canadian Food Inspection Agency (1999) identified four *Aegilops* species that have potential to outcross with *T. aestivum*: *Ae. crassa* Boiss.; *Ae. cylindrica*; *Ae.*

geniculata; and *Ae. truncialis*. *Aegilops cylindrica* has been recently found in two small monitored sites in Ontario, however no other *Aegilops* species are present in Canada (CFIA, 2005; Haber, 2006). Natural hybrids between *T. aestivum* and *Ae. cylindrica* have been reported in the United States (Zemetra et al., 1998). The success of this cross is likely due to homology of the D genome in both species (Kimber and Zhao, 1983; Zaharieva and Monneveux, 2006). Cultivated triticale in North America is mostly hexaploid and, as a result, does not have the D genome present in *T. aestivum* and *Ae. cylindrica*. *Aegilops cylindrica* is a weed in wheat cropping systems just south of the Canadian border hybridizes naturally with common wheat and therefore this species should be examined for crossing with triticale.

Hybrids between *T. durum* and wild relatives are less frequently observed than *T. aestivum* (Ceoloni and Jauhar, 2006). *Triticum boeticum*, *T. monoccocum*, and *T. urartu* share portions of the *T. durum* genome (AA). However, artificial or hand crosses resulted in sterile hybrids, which were stabilized only after subsequent backcrossing to *T. durum* (Ceoloni and Juahar, 2006). Using embryo cultures, wild *Triticum* species *T. boeticum* and *T. dicoccoides* are currently being used in durum wheat improvement breeding programs to introduce desirable traits (Singh et al., 1998; Zitelli, 1973).

There is less information on rye outcrossing with relatives than wheat. Khush (1962) assessed the crossability between wild rye species *Secale sylvestre*, *S. strictum*, and cultivated *S. cereale*. Crossability was determined as a function of percent seed set of parents, germination of hybrid seed, and percentage of F1

hybrids that set seed. Artificial crosses between *S. cereale* and *S. sylvestre* exhibited lowest crossability at 0.02 to 0.08% followed by *S. strictum* x *S. sylvestre* at 6.9% and *S. cereale* x *S. strictum* at 26.3% (Khush and Stebbins, 1961). Stutz (1957) reported successful crosses between *S. cereale* and *S. strictum* but reduced crossability. There have been no reports of crossing between *Secale* and putative close relative *Crithopsis*. The low capacity for cultivated rye to cross with wild relatives suggests lower risk for hybridization between triticale and *S. strictum* or *S. sylvestre*. This risk is even lower given that wheat *Kr* genes within triticale have been may inhibit crossing from triticale to rye (Guedes-Pinto et al., 2001).

Wild Canadian Species at Potential Risk for Crossing with Triticale

With little information on triticale outcrossing available, compatibility of parental wheat and rye species were investigated as risk indicators. No wild *Triticum* or *Secale* species are distributed in Canada and, as such, hybridization with triticale cannot occur here (Morrison, 2007). However, other relatives of *Triticum* and *Secale* are present and may pose hybridization risks. Species that exhibit confirmed compatibility with both wheats and rye are at highest potential risk for hybridization with triticale. Six species hybridize with common wheat, durum wheat, and rye in artificial settings: *Aegiliops cylindrica*, *Agropyron intermedium*, *Elymus repens, A. trichophorum*, *Hordeum vulgare*, and *Leymus arenarius* (Table 3-3; Gandhi et al., 2006; Knobloch, 1969 and referenced therein; and Morrison et al., 2002). It is important to note *Agropryon intermedium* and *Agropyron trichophorum* have been reclassified as the same species: *Thinopyrum*

intermedium (Host) Barkworth & D.R. Dewey (Flora of North America Editorial Committee, 2007). However we discuss them as distinct entities because hybridization literature regard them as different species and report variable crossing successes.

Resistance to fungal and viral disease, drought and heat tolerance and its perennial nature has made *Agropyron intermedium* an attractive genetic donor species for common wheat. *Triticum-Agropyron* substitution lines have been developed and used but successful introgressions are rare because of undesirable gene linkages and instability of larger chromosome fragments (Friebe et al., 1996; Garg et al., 2008; Khan, 2000; Sibikeev et al., 1995). Mujeeb-Kazi et al. (1989) hand pollinated several cultivars of common wheat (Chinese Spring, Glennson 81, Nacozari-76, and Pavon-76) and *A. intermedium* in the field. Out of 1260 total crosses, 619 seeds were set and 92 germinated and grew into healthy plants. The majority successful hybrid plants were the result of crosses with Chinese Spring, a known *kr* recessive wheat cultivar. A more recent study detailing the results of crosses between three cultivars of durum wheat (Cocorit 71, Yavaros 79, and Cappelli) and *A. intermedium* obtained 107 hybrid plants out of 236 total crosses (Mujeeb-Kazi et al., 2007). For both studies, hand pollinations, ovary treatments, embryo rescue and plantlet culturing were employed (Mujeeb-Kazi et al., 1989; 2007). Smith (1943) also performed crosses between durum wheat and *A. intermedium* with 7.7 to 24.2% of florets pollinated producing seeds. In this earlier research, crossing methodology was not reported and hybrid confirmation was based on morphological examinations alone. All three experiments described

above used *A. intermedium* as the male parent in the crosses and did not examine behavior of *A. intermedium* as the female pollen receptor. Hybrid crossing efficiencies are rarely reciprocal. For example, triticale exhibits marked hybridization differences when used as a pollen donor vs. pollen receptor (Hills et al., 2007). Introgression of genes from *A. intermedium* to common and durum wheat is of more interest for wheat breeding, but information on the reciprocal movement of transgenes to wild and weedy relatives will be required before release of GM triticale cultivars.

Agropyron intermedium is an open-pollinated species found in Alberta, British Columbia, Saskatchewan, and Yukon where it is both naturalized and cultivated (Hannaway and Larson, 2004). In British Columbia, *A. intermedium* is included in some hydro-seed blends to control erosion in forested areas (Homosky, 1996). The species is not considered invasive; however, it has been planted along roadsides and waste areas and can become a monoculture in favourable conditions (Hannaway and Larson, 2004; Homosky, 1996). Despite the widespread distribution of *A. intermedium* and its proximity to wheat fields, no natural hybrids have been reported. However, relatively high rates of recovery of hybrid seed following hand pollination of common and durum wheat with *A. intermedium* as a pollen donor, and its distribution warrants further testing to better quantify potential outcrossing with triticale.

Elymus repens and *A. trichophorum* both exhibit traits that increase hybridization risk with triticale. *Elymus repens* is an open-pollinated weedy species found in all Canadian provinces and adapted for many habitats including

waste areas, cultivated fields and grasslands (Crompton et al., 1988; Darbyshire, 2003; Frankton and Mulligan, 1993). Hybridization potential with cultivated triticale is low as *E. repens* has never successfully crossed with cultivated wheat in a natural setting (Canadian Food Inspection Agency, 1999, 2006; Knobloch, 1969). Tsitsin (1940) reported successful hybridization between *T. aestivum* and *E. repens* in laboratory settings, however the results were not repeatable and have since been dismissed (Smith, 1943; Canadian Food Inspection Agency, 1999, 2006). More recently, Mujeeb-Kazi et al. (1989) attempted this cross in laboratory settings and were only able to retrieve one hybrid seed out of 414 pollinations. The seed was produced using a *kr* recessive cultivar of wheat that can increase the likelihood of natural crosses. Triticale and most cultivated wheat cultivars used in Canada are dominant wild *Kr* types and are less likely to cross (H. Randhawa, personal communication, 2009).

Agropyron trichophorum is classified as a naturalized species in British Columbia, Saskatchewan, and the Yukon Territory and is often planted with *A. intermedium* (Hannaway and Larson, 2004). Like *A. intermedium* and *E. repens*, it grows in a range of conditions that, coupled with its open florets, presents a higher risk for hybridization with triticale than other less adaptive and/or closed pollinated species. In crossing experiments with common wheat and *A. trichophorum*, *kr* recessive wheat plants were more successful in obtaining viable hybrids (Mujeeb-Kazi et al., 1989). Crossing of triticale with *A. trichophorum* in artificial settings resulted in low seed set (1.6–2.6%) and required hormone

treatments and embryo rescue from the mother plant (Gupta and Fedak, 1986a) suggesting minimal outcrossing risk with triticale in crop settings.

The remaining two species that artificially hybridize with triticale's parents, *Hordeum vulgare* and *Leymus arenarius*, are considered to be at minimal risk of crossing with triticale. *Hordeum vulgare* is widely cultivated in Canada and is often grown in close proximity to common and durum wheat and rye fields. Despite this, no known cases of natural hybridization between *H. vulgare* and wheat (bread or durum), or rye have been recorded, implying minimal outcrossing risk with triticale (Morrison, 1955; Petersen, 1991; Smith, 1951). *Leymus arenarius* is found in Ontario, Quebec, and the Northwest Territories and is well established in sandy locations as a dune stabilizing grass (USDA, 2009). Occasionally, *L. arenarius* has been cultivated but is undesirable for this purpose because of its tendency to be invasive (Barkworth, 2007). *Leymus arenarius* poses limited risk for outcrossing with triticale because it generally does not grow where triticale is cultivated.

Confirmed compatibility with wheat (bread and/or durum) or rye species is also an important indicator of risk. Ten species in *Agropyron*, *Elymus*, and *Leymus* occur in Canada and have reportedly hybridized with either common or durum wheat in laboratory settings: *Agropyron campestre* Godr. & Gren., *A. cristatum* (L.) Gaertn., *Thinopyrum ponticum* Barkworth and D.R. Dewey, *Elymus albicans* (Scribn. & J.G. Sm.) A. Löve, *E. alaskanus* (Scribn. & Merr.) Á.Löve, *E. dahuricus* Turcz. ex Grisb., *E. lanceolatus* (Scribn. & J.G.Sm.) Gould, *E. trachycaulus* (A.Nelson) Á.Löve, *Leymus mollis* (Trin.) Pilg. and *L. triticoides*

(Buckley) Pilg. (Table 3-3; Mujeeb-Kazi et al., 1987; Osborne and Elliott, 1955; Smith, 1943; Tsitsin, 1940). Of species found in Canada, only *Hordeum* has confirmed hybridizations with rye: *H. brachyantherum* Nevski, *H. depressum* Rydb., *H. jubatum* L., *H. marinum* Huds., and *H. murinum* L. (Table 3-3; Petersen, 1991). All of these crosses involving wheats and rye were difficult to produce artificially and resulted in limited seed set and low seed viability.

Conclusions

Triticale presents unique challenges when assessing outcrossing risks with wild and/or related species. As a new addition to agriculture and a minor crop, triticale is grown on lower acreage than other major crops (i.e., common wheat, corn, or canola; FAOSTAT, 2007). Crop breeders have less experience with triticale than established crops and thus the potential gene flow from GM triticale may be more difficult to predict.

Examination of phylogenetic relationships was used to identify which closely related species represent a risk for outcrossing. However, understanding these relationships for the parents of triticale (*Triticum* and *Secale*) and the entire Triticeae tribe remains elusive and challenging. It is noteworthy and unexpected that tribal affinities are more informative for predicting outcrossing potential of triticale than species found within parental genera. That is, species that artificially cross with cultivated wheat and rye are spread throughout the tribe and are not restricted to *Triticum* or *Secale*, as might be predicted. In fact, aside from parental species only one member of either parental genus, *Triticum monococcum*, has been shown to cross with triticale in artificial crossing experiments (Neumann and

Kison, 1992). All other potential at risk species identified in this paper are from other genera in the tribe. This finding is perhaps unsurprising because the entire tribe behaves more like a species complex than distinct related genera (Kellogg et al., 1996). Thus, when identifying wild relatives potentially at risk of crossing with triticale, the entire tribe must be considered.

When assessing outcrossing and gene flow potential compatibility with parental species, geographic distribution and floral structure must be considered. Related species found in conjunction with agricultural regions and with open florets have a higher potential to receive pollen from the triticale crop. Hybridization is only the first step to examine gene flow and introgression of the transgene(s). Successful introgression will depend on the location of the transgene on the genome with some portions less likely to be retained than others. For example, the R genome from triticale is unlikely to be preserved in hybrids of species without a corresponding R genome.

In Canada, the most likely candidates for outcrossing with transgenic triticale are cultivated and feral parental wheats (*T. aestivum* and *T. durum*) and rye (*S. cereale*). While outcrossing rates have been established through hand pollination of emasculated flowers (e.g., see Hills et al., 2007), the rates of outcrossing under field conditions are not known and should be examined. Triticale is grown in proximity to the crop species *Hordeum vulgare*, however hybridization between triticale and barley required embryo rescue and is therefore considered to be unlikely to occur under field conditions. Gene flow to crop

species is unlikely to present an environmental risk but the presence of transgenes in other crops does present concerns for international marketing of cereals.

The barriers to gene flow from triticale to wild and weedy species are high. As an interspecific hybrid, triticale contains portions of several genomes, which reduces genomic compatibility, hybridization, and introgression potential. It also contains the *Ph* and *Kr* gene families, known to reduce homeologous pairing of chromosomes. Extrapolation from parental species, common and durum wheat and rye, suggests only a few species naturalized to Canada need to be considered. *Elymus repens*, *A. trichophorum*, and *L. arenarius*, have limited success when crossed with cultivated wheat species and rye in artificial settings. No natural crosses between wheat and *A. trichophorum* and *Leymus arenarius* have been reported and are considered unlikely. *Agropyron intermedium* is open pollinated and located proximal to agricultural areas in Canada. It crosses with common and durum wheats, rye and triticale in laboratory settings, but only crosses with wheat were shown to be fertile and only after subsequent backcrossing (Mujeeb-Kazi et al., 1987; Smith, 1943; Stebbins and Pun, 1953). Another weedy species that should be examined is *Aegilops cylindrica*. Although shown to be in two small isolated populations in Ontario, it is a major crop weed of wheat in the northeastern United States and is predicted to move north sometime in the future. *Aegilops cylindrica* has been found to hybridize with wheat under natural field conditions and gene flow has been demonstrated. Further research to define the level of outcrossing between triticale and *A. intermedium* and *Ae. cylindrica* should be considered.

Table 3-1. Success of greenhouse crosses between Triticale and parental species common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*) and rye (*Secale cereale*) showing the number of florets pollinated, hybrid seed set (expressed as a percentage of pollinations) and seeds germinated (expressed as a percentage of the seed set) as reported by Chaubey and Khanna (1986) and Hills et al. (2007).

Species Crossed	Florets pollinated	Hybrid Seed %	Germinated Seeds %
Chaubey and Khanna, 1986			
x*Triticosecale* UPT75233 x *Triticum aestivum* UP2003	140	3.6	60
x*Triticosecale* UPT7681 x *Triticum aestivum* UP2003	120	3.3	50
x*Triticosecale* 7681 x *Secale cereal* (Russian)	110	16.7	35
Hills et al., 2007			
x*Triticosecale* AC Alta x *Triticum aestivum* AC Barrie	~2000	25	94
x*Triticosecale* AC Alta x *Triticum durum* Kyle	~2000	5	0
x*Triticosecale* AC Alta x *Secale cereale* Rogo	~2000	10	50
x*Triticosecale* 89TT108 x *Triticum aestivum* AC Barrie	~2000	21	97
x*Triticosecale* 89TT108 x *Triticum durum* Kyle	~2000	3	41
x*Triticosecale* 89TT108 x *Secale cereale* Rogo	~2000	15	0
Triticum aestivum AC Barrie x x*Triticosecale* AC Alta	~2000	75	<1
Triticum durum Kyle x x*Triticosecale* AC Alta	~2000	70	0
Secale cereale Rogo x x*Triticosecale* AC Alta	~2000	22	38
Triticum aestivum AC Barrie x x*Triticosecale* 89TT108	~2000	85	0
Triticum durum Kyle x x*Triticosecale* 89TT108	~2000	90	0
Secale cereale Rogo x x*Triticosecale* 89TT108	~2000	15	0

Table 3-2. Success of interspecific hybrids between triticale and *Agropyron trichophorum, Triticum monococcum, Hordeum parodii and Hordeum vaulgare* indicating the number of flowers pollinated, hybrid seeds generated, (expressed as a percentage of florets pollinated) and plants obtained (expressed as a percentage of the florets pollinated).References are included in footnotes.

Species Crossed	Florets Pollinated	% Hybrid Seed Set	% Plants Obtained
x*Triticosecale* x *Agropyron trichophorum*[1]	204	3.9	2.0
x*Triticosecale* x *Triticum monococcum*[2,3]	*NR	0.48	0.05
Hordeum parodii x x*Triticosecale*[4]	995	4.7	0.58
Hordeum vulgare x x*Triticosecale*[5]	816	20	0.75

*NR = Not Reported

[1]Gupta and Fedak, 1986a
[2]Neumann and Kison, 1992
[3]Kison and Neumann, 1992
[4]Gupta and Fedak, 1986b
[5]Balyan and Fedak, 1989

Table 3-3. Reported successful hybridizations between common wheat (*Triticum aestivum*), durum wheat (*T. durum*) and rye (*Secale cereale*) with wild and weedy relatives that occur in Canada. Success was determined by germination of hybrid seed and/or hybrid plants obtained from embryo rescue. Type of cross (artificial = A or natural = N) is indicated. References are included in footnotes.

Species crossed	Type
Triticum aestivum L. *x Agropyron cristatum* (L.) Gaertn[1,2].	A
Triticum aestivum L. *x Agropyron cylindrica*[7,17]	A/N
Triticum aestivum L. *x Thinopyrum ponticum* Barkworth and D.R. Dewey [2,3]	A
Triticum aestivum L. *x Agropyron intermedium* (Host) P. Beauv.[2,4,9]	A
Triticum aestivum L. x *Elymus repens* (L.) Gould [6]	A
Triticum aestivum L. *x Agropyron trichophorum* (Link) K. Richt.[2,4,]	A
Triticum aestivum L. *x Elymus dahuricus* Turcz. ex Grisb.[6]	A
Triticum aestivum L. *x Hordeum vulgare* L.[8]	A
Triticum aestivum L. *x Leymus arenarius* Hochst.[6]	A
Triticum aestivum L. *x Leymus mollis* (Trin.) Pilg.[6]	A
Agropyron campestre Godr. & Gren. *x Triticum aestivum* L.[5]	A
Thinopyrum ponticum Barkworth and D.R. Dewey *x Triticum aestivum* L. [10]	A
Agropyron trichophorum (Link) K. Richt. *x Triticum aestivum* L. [5]	A
Elymus lanceolatus (Scribn. & J.G.Sm.) Gould *x Triticum aestivum* L. [5]	A
Elymus albicans (Scribn. & J.G. Sm.) A. Löve *x Triticum aestivum* L.[5]	A
Agropyron cristatum (L.) Gaertn. *x Triticum durum* Desf.[5]	A
Agropyron intermedium (Host) P. Beauv. *x Triticum durum* Desf.[5,11]	A
Elymus repens (L.) Gould *x Triticum durum* Desf.[12]	A
Agropyron trichophorum (Link) K. Richt. *x Triticum durum* Desf.[5]	A
Elymus alaskanus (Scribn. & Merr.) Á.Löve *x Triticum durum* Desf.[5]	A
Elymus trachycaulus (A.Nelson) Á.Löve *x Triticum durum* Desf.[5]	A
Leymus triticoides (Buckley) Pilg. *x Triticum durum* Desf.[5]	A
Elymus lanceolatus (Scribn. & J.G.Sm.) Gould *x Triticum durum* Desf.[5]	A
Elymus albicans (Scribn. & J.G. Sm.) A. Löve *x Triticum durum* Desf.[5]	A
Secale cereale L. *x Elymus repens* (L.) Gould [2]	A
Secale cereale L. *x Agropyron intermedium* (Host) P. Beauv.[13,14]	A
Agropyron intermedium (Host) P. Beauv. *x Secale cereale* L.[6]	A
Agropyron trichophorum (Link) K. Richt. *x Secale cereale* L.[2]	A
Hordeum brachyantherum Nevski *x Secale cereale* L.[15]	A
Hordeum depressum Rydb. *x Secale cereale* L.[15,16]	A
Hordeum jubatum L. *x Secale cereale* L.[15,18]	A
Hordeum marinum Huds. *x Secale cereale* L.[15]	A
Hordeum murinum L. *x Secale cereale* L.[15,19]	A
Hordeum vulgare L. *x Secale cereale* L.[15]	A
Leymus arenarius Hochst. *x Secale cereale* L.[20]	A

[1] Mujeeb-Kazi et al., 1987 [2] Smith, 1942 [3] Sharma and Ohm, 1990 [4] Mujeeb-Kazi et al., 1989 [5] Smith, 1943
[6] in Knobloch, 1969 [7] Gandhi et al., 2006 [8] Morrison, 1955 [9] Veruschkine and Shekhurdin, 1933
[10] Osborne and Elliott, 1955 [11] Peto and Young, 1942 [12] Tsitsin, 1940 [13] Stebbins and Pun, 1953
[14] Zennyozi, 1963 [15] Petersen, 1991 [16] Morrison and Rajhathy, 1959 [17] Morrison et al., 2002 [18] Wagenaar, 1959
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Chapter 4: Conventional Wheat and GM Triticale: Meeting the Coexistence Requirement

Introduction

Genetically modified (GM) major crops have been widely adopted, with over 148 M ha in cultivation in 2010 (James, 2010), but most minor crops have yet to undergo trait development. The major barriers to adoption of GM technologies have been the lack of harmonization of regulatory requirements between countries (Farre et al., 2010), the associated regulatory complexity and cost (Kalaitzandonakes et al., 2007), the risk of litigation due to the presence of unapproved events (for example: Bayer Rice Litigation, 2007), asynchronous approval of traits between countries and regions (FDA, 2006; Flax Council of Canada, 2009a; Flax Council of Canada, 2009b) and coexistence regimes imposed subsequent to approval for cultivation (Devos et al., 2009). Thresholds for GM traits vary, i.e. zero for unapproved events, 0.1% for low-level presence and 0.9% for approved events within the EU (EUROPA, 2007 & 2011) and 5% for approved events in Japan (Ramessar et al., 2009). In contrast, in many countries where GM crops are widely grown (i.e. Canada and the USA), there is no requirement to segregate crops following regulatory approval and release. Commercial requirements, in comparison to those of governments, can be more variable. For example, the imposition of a ban on major GM ingredients by European food retailers and manufacturers exceeds and preempts regulatory requirements (Kalaitzandonakes and Bijman, 2003).

Triticale is a candidate for development through GM. Spring wheat (*Triticum aestivum* L.), durum wheat (*T. durum* Desf.), and triticale (x*Triticosecale* Wittmack) are closely related species with spring or durum wheat providing the initial maternal and rye the paternal components of the triticale genome (Oettler et al., 1991; Lelley, 1992; Ammar et al., 2004). In addition to the need to quantify the potential for AP resulting from PMGF and SMGF to conventional triticale crops, inter-specific hybridization with spring and durum wheat must be quantified to address concerns of inadvertent movement of GM traits to wheat crops. While there is interest in GM triticale for use as a novel cereal bio-industrial platform (Canadian Triticale Biorefinery Initiative, 2010; Goyal et al., 2011), if GM triticale can hybridize with wheat, it may pose too great a commercial risk for conventional wheat markets.

Spring wheat is a major agricultural crop grown on 227 M ha globally with over 20.7 M metric tonnes produced on 7.2 M ha in Canada in 2011 (Canadian Wheat Board, 2011). Globally durum wheat is the second most important *Triticum* species, grown on 18 M ha. Canada is the second largest producer of durum wheat in the world, producing 4.6 M metric tonnes on 1.6 M ha annually. Triticale is grown on ~4.3 M ha globally, and 25,000 metric tonnes are produced in Canada on 12,100 ha annually (most recent data unavailable; FAOSTAT, 2011). Glyphosate-resistant wheat, the first GM wheat to reach field trials, was voluntarily withdrawn prior to release by the developer in 2004 (Stokstad, 2004) because of grower and consumer concerns.

The potential for PMGF from triticale to its related species appears to be low, but has not been quantified in large field trials. Intraspecific PMGF in crops varies widely, primarily based on pollination biology, from allogamy (i.e. rye and corn; Hallauer and Darrah, 1985; Oelke et al., 2011) to primarily autogamy (i.e. triticale and wheat). Autogamy in triticale and wheat reduces the opportunity for interspecific hybridization, however environmental stress can cause pollen sterility and promote floret opening, increasing PMGF (Dorofeev, 1969; Waines and Hegde, 2003; Singh and Jauhar, 2006; Kavanagh et al., 2010). In addition to autogamy, triticale and wheat possess *Ph*1 and *Ph*2 genes that suppress homeologous pairing and recombination of alien chromosomes reducing F1 vaiability (Jauhar and Chibbar, 1999; Guedes-Pinto et al., 2001; Zaharieva and Monneveux, 2006; Weissmann et al., 2008) and crossability genes *Kr*1-4 that inhibit interspecific outcrossing to rye (Guedes-Pinto et al., 2001; Oettler et al., 2005). However, emasculation and manual crossing of triticale and spring and durum wheat may produce seeds, some of which were viable (Table 4-1; Chaubey and Khanna, 1986; Lelley, 1992; Hills et al., 2007). Hybrid seed viability and seed morphology were influenced by direction of the cross and triticale cultivar. With triticale acting as the pollen receptor, few seeds were formed. Triticale x common wheat produced plump seeds with high viability. Triticale x durum wheat produced shriveled seeds with viability depending on the triticale cultivar selected as the pollen receptor. When triticale was the pollen donor, more seeds were produced, but all were shriveled and non-viable (Hills et al., 2007). Only a single confirmed wheat x triticale hybridization event in the field has been

reported which occurred between triticale and wheat planted in adjacent plots in the 1970's (Zillinsky and Borlaug, 1971; Zillinsky, 1974; Ammar et al., 2004).

Identification of hybrids between triticale and spring and durum wheat is problematic. Hybrids are expected to exhibit an intermediate morphology, but may be difficult to reliably differentiate from parental types. Hybridization and inter-specific gene flow has been quantified using a variety of markers including herbicide resistance and a visual blue aleurone trait which expresses a strong xenia effect (Rieseberg and Ellstrand, 1993; Warwick et al., 2003; Hucl et al., 2004; Matus-Cadiz et al., 2004; Matus-Cadiz et al., 2007; Beckie et al., 2011). However, it is difficult to predict the retention of a single trait and effective trait screening may be problematic, particularly in assays where a single missed hybridization event may significantly impact results. SSR (simple sequence repeat) markers are well characterized, readily available, and are time and cost efficient. These co-dominant markers are particularly useful in distinguishing closely related species and cultivars because of their high degree of polymorphism (Kuleung et al., 2006; Asif et al., 2009; Mangini et al., 2010). Wheat SSRs have been used to determine genetic diversity between genomes of triticale cultivars (Kuleung et al., 2006; Vyhnanek et al., 2009) and spring wheat (Salem et al., 2008) and to differentiate durum wheat cultivars (Mangini et al., 2010). Wheat SSR markers polymorphic for wheat and triticale may be useful tools in detecting wheat x triticale interspecific hybrids and quantifying AP (**Appendix 2**). The combined use of morphological markers, morphology and SSR markers may result in more rigorous hybrid identification.

An initial (Tier 1; *sensu* Raybould and Cooper, 2005) interspecific hybridization experiment conducted in a greenhouse under "worst case scenario" testing with emasculated manual crosses demonstrated that hybridization between triticale and spring and durum wheat can occur, but hybridization to rye was unlikely (Hills et al., 2007). Here we describe a Tier 2, interspecific hybridization field trial, conducted in two years at four locations to quantify PMGF from a single triticale cultivar containing a blue aleurone (BA) trait to two cultivars of spring wheat and one cultivar of durum wheat. Seeds from spring and durum wheat grown in close proximity to BA triticale were visually screened for the presence of a blue aleurone, or a shriveled appearance consistent with known hybrid seed morphology. Hybridity of putative hybrid plants was confirmed morphologically and molecularly by screening a panel of 55 SSR markers. F1 plants containing at least one triticale-sized fragment detected by a SSR marker(s), or exhibiting intermediate morphology, or a blue aleurone colored seed, were identified as hybrids. This approach provided a conservative estimate of PMGF between triticale and spring and durum wheat.

Materials and Methods

Plant Material

Spring wheat cultivars AC Barrie (DePauw et al., 1997) and AC Crystal (Fernandez et al., 1998) and durum wheat cultivar AC Avonlea (Clarke et al., 1999) were used as pollen receptors. The pollen donor was a blue aleurone line (BC_4F_4), from a cross between AC Alta/Purendo-38 with 4 subsequent backcrosses to AC Alta (BA; provided by Agriculture and Agri-Food Canada in Lethbridge, Alberta). The blue aleurone is a single-gene dominant trait (Keppenne

and Baenziger, 1990; Acquaah, 2007). When the dominant allele is present in the male or female gamete, the aleurone layer of the seed will be blue. This trait has previously been utilized as a morphological marker in spring wheat to quantify outcrossing and gene flow (Matus-Cadiz et al., 2004; Hanson et al., 2005; Matus-Cadiz et al., 2007) and establish isolation distances between cultivars (Hucl and Matus-Cadiz, 2001).

Greenhouse Experiments

To generate hybrid seed, manual crosses between the three above spring and durum wheat pollen receptors and the BA pollen donor were conducted in greenhouse experiments. Seeds were planted 1 seed per 1 L pot using soil-less media (Sunshine mix #4, Sun Gro Horticulture, Vancouver, BC, Canada) under a lighting schedule of 16 hours light at 18^{o}C and 8 hours dark at 12^{o}C. Wheat cultivars were emasculated prior to flowering and pollinated with BA triticale. Ten seeds were tested for viability and the remainder retained to assist in putative hybrid seed identification and morphological hybrid analyses.

Field Experiments

To maximize the probability of hybridization, BA triticale was planted in a central 50*1.4 m strip, with adjacent strips of either spring wheat, cultivars AC Barrie or AC Crystal, or durum wheat, cultivar AC Avonlea. Each wheat cultivar was planted on two dates (Table 4-2) to maximize the probability of flowering synchrony with the BA pollen donor. ‘Sundry’ barley was seeded in the 10 m between plots to reduce PMGF between treatments. To determine if there were environmental effects influencing PMGF, trials were established in two years at Lacombe, Alberta, (LAC, latitude 52.28, longitude 113.44) and Ellerslie Research

Station (ElRS, latitude 53.4232, longitude 113.581) in 2007 and ElRS and Edmonton Research Station (EdRS, latitude 53.4886, longitude 113.569) in 2009. Experiments were designed as a randomized complete block with the three wheat cultivars, planted at two dates, with three replicates.

Flowering synchrony was assessed as the number of days the main head of the receptor species flowered together with the donor species divided by the total number of flowering days, multiplied by 100. Triticale was removed from plots after flowering had ceased to reduce the probability of seed contamination. Wheat strips were harvested mechanically using a two-row Suzue harvest-binder and threshed using a custom built cereal thresher (Bill's Welding, Pullman, Washington U.S.A.), and seeds retained for screening.

Seed Sampling

Minimum sampling size was established *a priori* by conducting a power analysis using binomial probabilities as described by Zar (2010) and reported in Jhala et al. (2011) which estimates the minimum sample size for different theoretical frequencies. The theoretical frequencies at the different α values served as the null hypotheses levels used to declare significance of gene flow.

The observed frequencies from the study were then compared with the theoretical frequencies and if the observed frequencies were above the theoretical frequency, there was significant gene flow. For this analysis a theoretical frequency of 0.01% and a 99% confidence level (α=0.05%) was considered acceptable. Therefore a sample size of 636,000 seeds/ cultivar was screened.

$$\beta = P\left(Z < \frac{p_0 - p}{\sqrt{\frac{pq}{n}}} - Z_\alpha \sqrt{\frac{p_0 q_0}{pq}} \right) \quad \text{(Eq. 1)}$$

$$n = \frac{pq\left(\Phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0 q_0}{pq}}\right)^2}{(p_0 - p)^2} = \frac{(p_0 - \Delta)(1 - p_0 + \Delta)\left[\Phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0(1-p_0)}{(p_0-\Delta)(1-p_0+\Delta)}}\right]}{\Delta^2} \quad \text{(Eq. 2)}$$

Where n is the minimum number of seeds required; 1-β is the power of the statistical test; Z is the random variable that follows the standardized normal distribution, i.e., Z ~N (0, 1); p is the theoretical frequency of PMGF with q = 1-p; p_0 is the true value in null hypothesis- hypothesized parameter; Δ is the effect size ($p_0 - p$); Z_a is the critical value for significant level a; and Φ^{-1} (β) is the inverse function of the normal density curve for the probability β.

Statistical Analysis

PMGF was calculated as the number of confirmed hybrids divided by the number of seeds screened multiplied by 100. The number of hybrid seeds identified was subject to an analysis of variance (ANOVA) using ProcMix in SAS with locations nested within years to determine if there were significant differences between sites, years and cultivars.

Putative Hybrid Screening

Seed samples were collected from seeding dates that exhibited highest flowering synchrony with the pollen donor for each replicate per site/year.

Samples were visually screened and putative hybrid seeds identified, either as having a blue colouration or shriveled appearance as observed in known hybrid seeds from greenhouse crosses (Figure 4-1; Hills et al., 2007). Putative hybrid seeds were planted in individual cells using a soil-less Cornell mix (Boodley and Sheldrak, 1977) and grown in a growth chamber using a lighting schedule of 16 hours light at 15^{o}C and 8 hours dark at 12^{o}C. Seeds that did not germinate were discarded. At three weeks post-emergence, leaf tissue was harvested from each seedling, DNA extracted, and plants were retained in a greenhouse under the same temperature and lighting regime. Plants were grown to maturity and morphological characteristics, including plant height and colour, inflorescence and awn length, maturation date, presence/absence of seeds and seed colour, were recorded and compared to parental species. F2 seeds, if present, were harvested and retained.

DNA extraction was performed using a modified CTAB method (Randhawa et al., 2009) and DNA dissolved in 500 µl TE buffer and stored at −20°C. DNA samples from five plants were pooled and screened with 55 SSR markers (**Appendix 2**). Forward primers incorporated a 5' M13 tail (CACGACGTTGTAAAACGAC) as did three fluorescently labeled (6-FAM, VIC and PET) universal primers (Invitrogen, Burlington ON, Canada) for detection with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCR was performed in a PX2 Thermal cycler (Thermo Fisher Scientific Inc. Waltham, MA, USA). The PCR reactions contained 25–100 ng of genomic DNA, 1· PCR buffer, 2.5 mM dNTPs, 50 mM $MgCl_2$, 1 pmol of dye M-

13 forward tail primer, 1 pmol each of forward and reverse primers and 0.1 U Taq DNA polymerase (New England Biolabs, Beverly, MA, USA) in a 10 μL volume. The PCR program was run with an initial denaturation temperature of 94°C for 3 min, followed by 35-40 cycles of denaturation at 94°C for 1 min, annealing for 1 min at either 50, 55 or 60°C depending on the individual primers, 2 min extension at 72°C and a final extension for 7 min at 72°C. Amplification was confirmed by electrophoresis on a 2% (w/v) agarose gel in 1.0×TBE buffer. Dye-labeled PCR products from the pooled DNA were triplexed and analyzed on an automatic capillary array based ABI 3100-Avant Genetic Analyzer. The product was diluted with TE buffer at 1/10 before loading and mixed with 9.0 μl of the loading dye Hi-Di formamide and 0.05 μl of Liz500 size standard (Applied Biosystems), denatured at 94°C for 5 min and chilled on ice for 5 min. GeneScan® and Genotyper® Software (Applied Biosystems) used to extract the data. Fragment sizes from samples were compared to profiles from triticale and wheat controls. When triticale-sized fragments were detected, DNA from plants in the pooled cell were tested individually. A conservative method of hybrid identification was used where the presence of a minimum of 1 triticale-sized SSR product resulted in a positive molecular identification of hybridity.

Results

Greenhouse Experiments

Manual crossing produced few hybrid seeds between AC Barrie x BA and AC Crystal x BA, and no seeds between AC Avonlea x BA. Most seeds produced were sunken and shriveled (Figure 4-1) with some appearing as empty flakes and all were non-viable. It has been previously reported that F1 hybrids with triticale

as a pollen source suffer from endosperm abortion due to excessive or fast nuclear division of hybrid endosperm (Gill and Waines, 1978; Gill et al., 1981). This made identification of the blue aleurone trait in shriveled seeds difficult.

Field Experiments and Seed Screening

Crop emergence and plant stands for all species and cultivars were excellent for all site years. Pollen receptor strips exhibited various levels of flowering synchrony with the pollen donor depending on the cultivar and seeding date: AC Barrie seeding date 1 all locations and years; AC Crystal seeding date 1 with the exception of ElRS 2009; and AC Avonlea 2007 & 2009 ElRS seeding date 1, Lacombe 2007 & EdRS 2009 seeding date 2 (Table 4-2). A total of 1.9 M seeds (636,000/ cultivar) were screened and 2031 seeds were identified as putative hybrids: 731 from AC Barrie plots, 665 from AC Crystal plots and 635 from AC Avonlea plots. No seeds intermediate in appearance between triticale and spring and durum wheat or with a blue aleurone were identified.

Putative Hybrid Screening

Only 448 of the 2031 putative hybrid seeds germinated: 185 seeds (25%) from AC Barrie spring wheat plots, 195 seeds from AC Crystal spring wheat plots and 68 seeds from AC Avonlea durum wheat plots (Table 4-3). The ANOVA was not informative due to the large number of zero values for hybrids. It was not possible to distinguish differences between environments. Subsequent discussion of hybrids is distinguished only by species and cultivar.

Morphological assessments of viable putative hybrids identified five plants with intermediate morphological characteristics: two spring wheat (two AC

Barrie x BA; Table 4-4) and three durum wheat (AC Avonlea x BA). No hybrids were detected morphologically amongst the spring wheat AC Crystal x BA putative hybrids. With the exception of one AC Barrie hybrid, plants exhibited characteristics more similar to triticale than wheat: glaucous leaves, ~20% longer flowering heads, later maturation and up to 50% increased height than control plants (avg. 82 cm vs. 121 cm). The unique AC Barrie x BA hybrid closely resembled its wheat parent, but with intermediate-length awns, later maturation and ~40% increased height (avg. 82 cm vs. hybrid 113 cm). Hybrids were fertile and produced small shriveled conventional coloured F2 seeds in AC Barrie x BA crosses, larger shriveled conventional colour F2 seeds in AC Crystal x BA crosses and shriveled blue aleurone F2 seed in AC Avonlea x BA crosses (Figure 4-2).

Initial molecular screening of the 448 putative hybrids using 55 informative SSR molecular markers identified eight pooled samples that contained unique triticale DNA fragments identified previously in BA and AC Alta. Seven markers amplified triticale-sized fragments: BARC51, BARC54, BARC151, BARC170, BARC199, BARC240 and WMC254 (Table 4-5). Individual hybrid DNA from each of the pooled samples, retested with the same informative SSR markers, confirmed 11 seedlings as hybrids. A maximum of five and minimum of one SSR marker confirmed a single hybrid. The maximum number of hybrids confirmed by a single marker was seven and the minimum number of hybrids confirmed by a single marker was three (Tables 4-4 & 4-5). Markers were located on chromosomes 3, 4 & 6 of the A genome and chromosome 1 & 4 of the B genome. No hybrid seed was identified with

informative SSR markers from the D genome which may be from the loss of the D genome in triticale.

Combining morphological and marker data, 14 hybrids were identified: 10 spring wheat x triticale, seven AC Barrie x BA and three AC Crystal X BA, and four durum wheat x triticale, AC Avonlea x BA. PMGF from triticale to the spring wheat cultivar AC Barrie was 0.001% and to AC Crystal, 0.0005%. PMGF from triticale to the durum wheat cultivar Avonlea is 0.0006%.

Discussion

Viability of putative hybrid seeds was low (22%). Lack of viability may have been due to several causes including hybrid origin, diseased kernels, seed immaturity or abiotic stress (Henry and Kettlewell, 1996) but non-viable hybrids could not contribute to volunteer populations and are therefore not considered an important source of gene flow.

Hybrids between triticale and common wheat (avg. 0.0008) and durum wheat (0.0006%) were rare under field conditions despite inter-relatedness of triticale and spring and durum wheat. Autogamy of spring and durum wheat reduces the probability of hybridization with triticale pollen. As expected, hybridization between these species under field conditions was lower than reported in greenhouse experiments where removal of pollen competition through emasculation enhanced hybridization potential (Chaubey and Khanna, 1986; Hills et al., 2007). Inter-specific PMGF between spring wheat x triticale (0.0008%, combined) and durum wheat x triticale (0.0003%) and was much lower than intraspecific triticale PMGF (0.76%; Kavanagh et al. *in press*) under the same

field conditions. *Ph*1, *Ph*2 and *Kr*1-4 genes present in spring and durum wheat and triticale are presumed to reduce hybridization (Jauhar and Chibbar, 1999; Guedes-Pinto et al., 2001; Oettler et al., 2005; Zaharieva and Monneveux, 2006; Weissmann et al., 2008).

Hybridization and inter-specific gene flow has been measured using a variety of tools, including herbicide resistant and visual markers (Rieseberg and Ellstrand, 1993; Warwick et al., 2003; Hucl et al., 2004; Beckie et al., 2011). To be conservative, in this study hybrids were confirmed by the presence of a single triticale-sized fragment or morphological trait because of the potential for phenotypic plasticity or genomic instability to influence the results. Fewer hybrids were confirmed with the morphological analysis than with the molecular screening. Of the 55 polymorphic SSR markers between triticale and spring and durum wheat, only 13% of markers detected triticale-sized fragments in the putative hybrids. During initial hybridization, genetic instability, including the loss of entire chromosomes, is known to occur (Ma and Gustafson, 2008; Dou et al., 2006) suggesting transgene retention and position in hybrids may be unpredictable. Single polymorphisms may have also been attributable to random mutation, i.e. point mutations or structural changes to chromosomes (den Dunnen and Antonarakis, 2001; Casella, 2011) or possible taq error. These results underscore the need for a large marker pool with broad genome coverage when performing cereal hybrid analyses.

Only two of the five hybrids (40%) identified in morphological examinations were confirmed by molecular analyses. It is possible that too few

markers were selected for the molecular component and they did not encompass the genome portion containing triticale fragments in the other three plants. Conversely, only two of the 11 hybrids (18%) confirmed in molecular analysis were detected in morphological comparisons. The potential that sequence variation in the wheat cultivars or triticale resulted in false positive or negative results cannot be ruled out. Thus consideration of both morphological and molecular results provides a conservative estimate of PMGF.

The viability of the F2 seed and the fitness of the F1 plants or their potential progeny in the field have not been determined. Hybrid seed, due to its small and shriveled morphology (Figure 4-1) is unlikely to be collected or retained during combine harvesting and seed cleaning, reducing further the potential impact on the export market. Shriveled hybrid seeds seem less likely to successfully produce a viable volunteer that may contribute to gene flow. However fertile F1 hybrid plants could be a source of SMGF in the future. The fertility of identified hybrids in this experiment differs with previous studies that report wheat x triticale F1 sterility (Hills et al., 2007). Because of the conservative approach of this study, it is possible identified hybrids were false positives, especially when confirmed by few molecular markers. Hybridization between triticale and spring and durum wheat may occur but rarely under field conditions when grown in proximity.

Conclusions

The frequency of hybrids between the triticale and spring and durum wheat tested in this experiment was below the EU 0.9% AP threshold. Additional

triticale cultivars should be tested to assess PMGF variability across genotypes. Based on this research, PMGF from GM triticale to common and durum wheat may not prevent coexistence.

Table 4-1. Success of manual crosses between triticale (x*Triticosecale*) and common wheat (*Triticum aestivum*) and durum wheat (*T. durum*) showing the number of florets pollinated, hybrid seed set (expressed as a percentage of pollinations) and seeds germinated (expressed as a percentage of the seed set) as reported by Chaubey and Khanna (1986) and Hills et al. (2007).

Species Crossed	Florets Pollinated	Hybrid Seed %	Germinated Seeds %
Chaubey and Khanna, 1986			
x*Triticosecale* UPT75233 x *Triticum aestivum* UP2003	140	3.6	60
x*Triticosecale* UPT7681 x *Triticum aestivum* UP2003	120	3.3	50
Hills et al., 2007			
x*Triticosecale* AC Alta x *Triticum aestivum* AC Barrie	~2000	25	94
x*Triticosecale* AC Alta x *Triticum durum* Kyle	~2000	5	0
x*Triticosecale* 89TT108 x *Triticum aestivum* AC Barrie	~2000	21	97
x*Triticosecale* 89TT108 x *Triticum durum* Kyle	~2000	3	41
Triticum aestivum AC Barrie x x*Triticosecale* AC Alta	~2000	75	<1
Triticum durum Kyle x x*Triticosecale* AC Alta	~2000	70	0
Triticum aestivum AC Barrie x x*Triticosecale* 89TT108	~2000	85	0
Triticum durum Kyle x x*Triticosecale* 89TT108	~2000	90	0

Table 4-2. Seeding, flower initiation, synchrony and harvest dates for wheat cultivars AC Avonlea, AC Barrie and AC Crystal for all sites in 2007 and 2009. Flowering synchrony was assessed as the number of days the main head of the receptor cultivar flowered together with the donor cultivar divided by the total number of flowering days, multiplied by 100.

Site, year	Cultivar	Seeding date #	Seeding date	Synchrony %	Harvest date
Ellerslie, 2007	AC Avonlea	1	May 14	90	Sept 13
	AC Avonlea	2	May 17	85	Sept 13
	AC Barrie	1	May 14	90	Oct 4
	AC Barrie	2	May 25	40	Oct 4
	AC Crystal	1	May 14	90	Sept 26
	AC Crystal	2	May 17	65	Sept 26
Lacombe, 2007	AC Avonlea	1	May 8	82	Sept 20
	AC Avonlea	2	May 12	95	Sept 20
	AC Barrie	1	May 8	88	Oct 4
	AC Barrie	2	May 22	65	Oct 4
	AC Crystal	1	May 8	93	Sept 20
	AC Crystal	2	May 12	75	Sept 20
Ellerslie, 2009	AC Avonlea	1	May 11	85	Sept 10
	AC Avonlea	2	May 15	37	Sept 10
	AC Barrie	1	May 11	85	Sept 10
	AC Barrie	2	May 21	12	Sept 10
	AC Crystal	1	May 11	0	Sept 10
	AC Crystal	2	May 15	33	Sept 10
Edmonton, 2009	AC Avonlea	1	May 11	39	Sept 9
	AC Avonlea	2	May 15	57	Sept 9
	AC Barrie	1	May 11	86	Sept 9
	AC Barrie	2	May 21	30	Sept 9
	AC Crystal	1	May 11	87	Sept 9
	AC Crystal	2	May 15	49	Sept 9

Table 4-3. Total seeds screened, putative hybrid seeds identified, putative hybrid seeds germinated, confirmed hybrids and % pollen-mediated gene flow (PMGF) from triticale to each pollen receptor cultivar (AC Avonlea, AC Barrie and AC Crystal).

Cultivar	Seeds screened	Putative hybrids	Seeds germinated	Confirmed hybrids	PMGF (%)
AC Avonlea	636000	635	68	4	0.0006
AC Barrie	636000	731	185	7	0.001
AC Crystal	636000	665	195	3	0.0005

Table 4-4. Morphological and molecular screening of putative hybrid plants and the number of SSR markers that confirmed hybridity (if applicable).

Parental wheat	Morphological confirmation	Molecular confirmation	Number of markers
AC Avonlea	Y	Y	4
AC Avonlea	Y	N	-
AC Avonlea	N	Y	2
AC Avonlea	Y	N	-
AC Barrie	Y	N	-
AC Barrie	N	Y	2
AC Barrie	N	Y	1
AC Barrie	N	Y	1
AC Barrie	Y	Y	4
AC Barrie	N	Y	2
AC Barrie	N	Y	5
AC Crystal	N	Y	2
AC Crystal	N	Y	2
AC Crystal	N	Y	4

Table 4-5. Molecular markers that detected hybrid DNA. Chromosome designation and number of hybrids confirmed are given for each marker

Marker	Chromosome	Hybrids detected
BARC51	3A	5
BARC 54	3A	5
BARC151	5A	7
BARC170	4A	3
BARC199	4B	4
BARC240	1B	3
WMC254	6A	3

Figure 4-1. Seed of hybrid wheat x triticale crosses obtained in manual crosses between: (A) AC Barrie x BA, (B) AC Crystal x BA. BA seed is added for comparison.

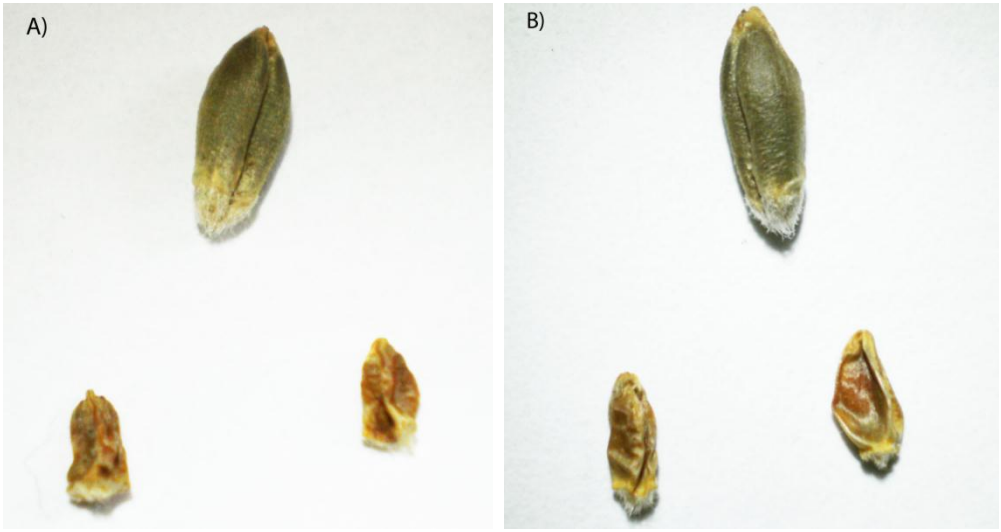

Figure 4-2. Seeds from confirmed AC Barrie x BA crosses (A), AC Crystal x BA (B) and AC Avonlea x BA. A BA seed is added for comparison.

A

B

C

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Chapter 5. Pollen-Mediated Gene Flow in Triticale (xTriticosecale Wittmack)

Introduction

Triticale is an intergeneric hybrid of wheat and rye with a high yield potential and higher stress tolerance than other grains (Mergoum et al., 2004; Mergoum et al., 2004; Government of Alberta Agriculture and Rural Development, 2006; Government of Alberta Agriculture and Rural Development, 2006). Since its introduction in the late 1960's triticale production has grown globally with over 4 M ha in cultivation in 2009 (FAOSTAT, 2010). In Western Canada it is a minor crop with 12 100 ha in cultivation compared to over 13 M ha of spring wheat (FAOSTAT, 2010; Government of Alberta Agriculture and Rural Development, 2010; Government of Saskatchewan, 2010). Although commonly used as an animal feed crop (Dodge, 1989), triticale has been used as a food-grade, bio-product, and bio-fuel grain (Mergoum et al., 2004; Pena, 2004; Government of Alberta Agriculture and Rural Development, 2009). It is being developed as a crop for novel bio-based products using genetic engineering (Canadian Triticale Biorefinery Initiative, 2010).

Prior to the commercial use of GM triticale cultivars, risk assessments must be conducted. GM triticale may become an agricultural weed, become invasive, or may outcross to wild or weedy species resulting in more weedy or invasive hybrids (Conner et al., 2003; Ellstrand and Schierenbeck, 2006; Warwick et al., 2009; Kavanagh et al., 2010). Economic risks include the potential for GM

triticale seeds to become mixed with other crops, either by inadvertent mixing during grain handling, through the harvest of persistent triticale volunteers, or from PMGF from GM triticale to nearby related conventional crop species (i.e. triticale, common wheat, durum wheat and rye) (Demeke et al., 2006). The potential for AP of GM traits would require food, feed and environmental approval of the GM trait in most markets. In some markets, products containing GM traits over a specified threshold would require product labelling (European Union, 2003; Ramessar et al., 2010).

PMGF may be the primary source of AP in outcrossing crops, including corn (Messeguer et al., 2006) and sugar beet (Darmency et al., 2007); however, PMGF frequency is reduced in predominantly self-pollinating crops such as canola (Beckie et al., 2003; Hall et al., 2003; Knispel et al., 2008) and flax (Jhala et al., 2011). In these crops seed-mediated gene flow becomes a more important source of AP (Beckie and Hall, 2008). Given the economic consequences of GM traits in conventional grains, AP of the approved trait must be below international thresholds for GM labelling and trace amounts of unapproved traits can be detrimental (EUROPA, 2007; Ramessar et al., 2009). Determining the frequency of PMGF as the distance from the pollen source increases may assist in the development of regulations and mitigation measures to reduce AP.

Triticale's progenitors differ in their outcrossing; wheat generally exhibits cleistogamy and rye chasmogamy. As a hybrid, triticale has a reduced compliment of wheat and rye genes, and the rye genome exhibits the greatest reduction in expression (Boyko et al., 1984; Xue-Feng et al., 2004). Triticale is considered a

predominantly cleistogamous species, but like rye is reported to have higher levels of ergot (Lorenz and Hoseney, 1979), which is associated with floret opening. For this reason we hypothesize PMGF will be higher in triticale than wheat.

Spring wheat PMGF has been reported previously in Western Canada, in part to anticipate or determine the consequences of the introduction of herbicide resistant (HR) wheat cultivars (Matus-Cadiz et al., 2004; Brûlé-Babel et al., 2006; Willenborg and Van Acker, 2008; Willenborg et al., 2009a; Willenborg et al., 2009c; Beckie et al., 2011). Similar to other crops, empirical modeling shows that PMGF frequency declines rapidly with distance from the pollen source. For larger fields, estimates of the AP due to PMGF in seed following harvest-blending (the mixture of seeds from near and farther distances from the pollen source) are predicted to contain less than 0.1% hybrids (Gustafson et al., 2005). PMGF in spring wheat differs widely by cultivar (Hucl, 1996). Cultivar differences were associated with differences in pollen viability and spikelet opening at anthesis. Growing conditions can also affect the frequency of outcrossing. Environmental factors such as drought, copper deficiency and abiotic stress may decrease pollen fertility and pollen number, and increase floret opening and outcrossing frequency (Dorofeev, 1969; Owuoche et al., 1994; Waines and Hegde, 2004). Outcrossing frequency can also be affected by agronomic conditions such as seeding dates and crop stand density (Willenborg et al., 2009a; Willenborg et al., 2009b). The size of the pollen donor and the width of the recipient fields also affect PMGF in

wheat (Beckie et al., 2011) and other crops, including canola (Beckie et al., 2003; Husken and Dietz-Pfeilstetter, 2007).

Triticale outcrossing rates have not been previously reported under field conditions. The objectives of this research were: 1) to quantify PMGF between triticale cultivars growing in small plots. Small plot data was used to design appropriate parameters for larger scale testing; 2) to quantify PMGF at medium distances under typical agronomic conditions in Western Canada; 3) to compare the PMGF in triticale to spring wheat outcrossing experiments conducted at a similar scale. A triticale containing a blue aleurone (BA) trait was used as a pollen source and triticale cultivar AC Alta (McLeod et al., 1996) was used as the pollen receptor. Outcrossing from the blue pollen source was identified by the expression of a light blue pigment in the triploid aleurone layer of F1 seed. An empirical model of triticale outcrossing was developed up to a distance of 50 m.

Materials and Methods

Plant Material

For both experiments the triticale cultivars were the same. The pollen receptor was spring triticale cultivar AC Alta (McLeod, 1996) which is the most widely grown spring triticale in Western Canada. The BA triticale pollen donor was an unregistered blue aleurone line (BC_4F_4), from a cross between AC Alta/Purendo-38 with 4 subsequent backcrosses to AC Alta obtained from Agriculture and Agri-Food Canada.

Small Scale Experiments

Trials were conducted in 2007 in Lacombe Alberta, (LAC, latitude 52.28, longitude 113.44) and Ellerslie Research Station (ElRS, latitude 53.4232, longitude 113.581) and in 2009 ElRS and Edmonton Research Station (EdRS, latitude 53.4886, longitude 113.569). Each plot was 50 m long and arranged with a central 1.4 m 8-row plot of BA between two 8-row plots of AC Alta, direct seeded on the same date (seeding date 1) and 7 to 10 days later (seeding date 2) with a Fabro 8-row air seeder equipped with atom-jet openers at a rate of 300 seeds m^{-2}. Seeding dates were extended to optimize flowering synchrony. There were three replicates at each site. The minimum distance between pollen source and pollen receptor was 0.2 m. To reduce potential pollen flow between plots, a minimum of 10 m of 'Sundry' barley was seeded between plots and replicates. Phosphorus fertilizer (44 Kg ha^{-1}) was applied with the seed and nitrogen (80 Kg ha^{-1}) was side banded at seeding. Roundup WeatherMAX® herbicide was applied prior to seeding and Buctril® M + liquid Achieve® herbicides were applied in-crop at recommended label rates to control weeds prior to the flag leaf stage. Flowering synchrony was assessed as the number of days the main head of the receptor species flowered together with the donor species divided by the total number of flowering days, multiplied by 100. To reduce the possibility of admixture, the centre BA rows were mowed and removed after completion of flowering. AC Alta plots were harvested on Oct 4 at ElRS and Oct 5 at LAC in 2007 and Sept 10 at ElRS and Sept 9 at EdRS in 2009 using a Wintersteiger plot combine and seeds stored at room temperature prior to analysis. Flowering stages were determined weekly (data not shown). For each site year seeds from the

receptor strips that exhibited the highest flowering synchrony with the pollen source were analyzed.

Large Scale Experiments

Field trials were conducted in 2008 and 2009 at ElRS and Lethbridge research station (LRS, latitude 49.6838, longitude 112.628) under conventional agronomic conditions. The soil texture at ElRS was clay loam with 28% sand, 41% silt, 31% clay, 11% organic matter and pH 6.5. At LRS the texture was clay loam with 35% sand, 36% silt, 29% clay, 6% organic matter and pH 7.8. A 20 x 20 m plot of BA triticale was direct seeded as the pollen source and surrounded in all directions by 50 m of AC Alta triticale as a pollen receptor. The trial was direct seeded using a Fabro 8-row air seeder with atom-jet openers and the total trial size was 120 x 120 m. Phosphorus fertilizer (35 Kg ha^{-1}) was applied with the seeds and nitrogen was side banded (100 Kg ha^{-1}). In 2008, both pollen source (BA triticale) and receptor (AC Alta) were seeded on May 6 at LRS and May 13 at ElRS (Table 5-1). In 2009, the pollen receptor was seeded 4-5 days earlier than the pollen source to better capture flowering synchrony. Seeding of the pollen receptor was May 18 and the pollen donor May 23 at ElRS; at LRS the pollen receptor was seeded on May 18 and pollen donor on May 22 (Table 5-1). The seeding rate was 310 seeds m^{-2} at a depth of 3.2 cm. Roundup WeatherMAX® herbicide was applied before seeding for all sites and Frontline™ A&B (2008) and Buctril® M + liquid Achieve® (2009) herbicides were applied in-crop at recommended label rates to control weeds prior to the flag leaf stage. Flowering synchrony was assessed as the number of days the receptor cultivar flowered

together with the donor cultivar divided by the total number of flowering days, multiplied by 100. Following flowering, the BA triticale was removed to minimize the risk of BA seed admixture into the pollen receptor. When ripe, the surrounding AC Alta was subdivided into 20 m strips oriented in the four cardinal directions and wedges in the four ordinal directions and the crop between the strips removed. Samples were harvested distal to the pollen source and progressed inward to reduce cross contamination. The pollen receptor was harvested every 5 m from 50 m to 10m, every 1.5 m up to 3 m, every 0.4 m up to 1 m, followed by every 0.2 m up to 0.2 m adjacent to the pollen source for each of the 8 directions. A Wintersteiger 2001 Elite research combine was used to harvest the pollen receptor at distances >3 m and a two-row Suzue harvest-binder was used closer to the pollen source. Harvesting was completed on Sept 25 and Oct 3 in 2008; and Sept 29 and Sept 14 in 2009 at LRS and ElRS, respectively (Table 5-1). Plants were threshed and seeds cleaned and stored at room temperature prior to analysis.

Weather Data

The seasonal and 30 year temperature and precipitation averages were obtained from the National Climate Data and Information Archives website maintained by Environment Canada (http://www.climate.weatheroffice.gc.ca). For ElRS data from the Edmonton INT'L A weather station was used and for LRS, the Lethbridge AWOS A weather station.

Seed Screening

The blue-aleurone xenia trait in the pollen donor was used as a marker as previously described (Hucl and Matus-Cadiz, 2001; Hucl et al., 2004).

Outcrossing events were identified as a light blue color in the aleurone of the F1 seed.

Sample sizes for small and large plot seed screening were established with power analyses using binomial probabilities as described by Zar (2010) and reported in Jhala et al. (2011). This method determines the sample size required to detect one transgenic seed for different theoretical frequencies based on degrees of confidence using the formulae below (Eq. 1 and 2). Three different confidence intervals (α) and power values (1-β) were used to determine sampling size. When the actual PMGF frequencies were greater than the theoretical frequencies (H_0) PMGF was significant. A 95% confidence level was selected to ensure highest accuracy.

$$\beta = P\left(Z < \frac{p_0 - p}{\sqrt{\frac{pq}{n}}} - Z_\alpha \sqrt{\frac{p_0 q_0}{pq}} \right) \qquad \text{(Eq. 1)}$$

$$n = \frac{pq\left(\Phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0 q_0}{pq}}\right)^2}{(p_0 - p)^2} = \frac{(p_0 - \Delta)(1 - p_0 + \Delta)\left[\Phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0(1-p_0)}{(p_0 - \Delta)(1 - p_0 + \Delta)}}\right]}{\Delta^2} \qquad \text{(Eq. 2)}$$

Where □ is the minimum number of seeds required; 1-β is the power of the statistical test; *Z*, is the random variable that follows the standardized normal distribution, i.e., Z ~N (0, 1); p is the theoretical frequency of PMGF with q = 1-p; p_0 is the true value in null hypothesis- hypothesized parameter; Δ is the effect size ($p_0 - p$); Z_a is the critical value for significant level a; and $\Phi^{-1}(\beta)$ is the inverse function of the normal density curve for the probability β.

Statistical Analysis

Small Plot Experiments

PMGF was calculated as the number of blue seeds detected divided by the total number of seeds screened multiplied by 100. An ANOVA was performed to test for differences between sites and years. There were no significant differences between sites or years and data over different environments were subsequently combined.

Large Plot Experiments

PMGF was calculated as described in the above section. Exponential decay curves were generated for each replicate (direction) at all sites and years using nonlinear regression with PROC NLMIXED (SAS Institute Inc., 2007; McPherson et al., 2008; Jhala et al., 2011). Estimation of the slopes was completed using a binomial distribution and fitting the data to the exponential decay function reported by Hanson et al. (2005):

$$p = ae^{-bd}$$

(Eq. 3)

where, □ is the predicted frequency of PMGF; *a* is the intercept; *b* is the curve parameter; and *d* is the mean distance from the source (m). Standard errors and 95% confidence intervals were calculated for each parameter estimate. Distances where PMGF decreased by 50% (O_{50}) and 99% (O_{99}) were estimated using the exponential decay function and the equations reported by Macpherson et al. (2008) and Jhala et al. (2011):

$$O_{50} = \frac{\ln(0.5 \times a) - \ln a}{-b}$$

(Eq. 4)

$$O_{99} = \frac{\ln(0.01 \times a) - \ln a}{-b}$$

(Eq. 5)

where, □ is the intercept and *b* is the slope.

Differences in PMGF between sites and years were evaluated by using the rate of PMGF decline or slope (*b*) generated from the exponential decay curves as a new variable. An ANOVA was performed on the slopes to determine whether there were site and year differences given that replicates were considered to be random. To assess whether there were directional effects between replicates, the mixed-model analysis based on SAS PROC MIXED (SAS Institute Inc., 2007) was used. More than 20 covariance structures were tested to account for variation between directions (data not shown). Of all the covariance structures examined, the heterogeneous compound symmetry (CSH) allowed for the best model fit using the Akaike Information Criterion (AIC). We performed pre-planned non-orthogonal estimate statements to determine whether there were significant directional differences in PMGF by direction (SAS Institute Inc., 2007).

To determine the impact of harvesting blending on PMGF, a simple numerical integration of the empirical model of PMGF as a function of the width of the field was performed as described by Gustafson et al. (2006).

Results and Discussion

Small Plot Experiments

In all sites and years, BA triticale initiated flowering earlier than AC Alta (data not shown) and multiple seeding times optimized flower synchrony. As previously reported for wheat, emergence timing is related to flowering initiation and flowering synchrony (Willenborg et al., 2009b). Only seeds from AC Alta strips that demonstrated the longest flowering synchrony with the BA pollen donor (avg. 87% synchrony) were analyzed. All plots had excellent emergence and similar plant densities (data not shown). A total of 144 000 seeds were screened in small plot experiments. There were no significant differences in PMGF between sites or years and therefore data was pooled.

The mean PMGF between BA and AC Alta averaged over a distance of 1.6 m from the pollen source was 0.76% (SE 0.006). Using a similar blue aleurone marker in spring wheat, Hucl (1996 and 2010) reported a wide range of PMGF (0.35 to 6.7%) between 12 cultivars at 0.2 m distance and 3.6 m, respectively (Table 5-2). We anticipate that triticale cultivars may also vary in PMGF. Triticale cultivars are heterogeneous, in part because of their variable genetic background and the amount of retention of the rye genome (Merker, 1971; Jouve et al., 1989; Kuleung et al., 2006). Quantification of PMGF between small plots has implications for the isolation distances used during cultivar development. As Hucl (2010) maintained for wheat, the maximum impurity tolerance of 0.01% in foundation seed would be exceeded using the current isolation distance of 1 m recommended for triticale (Canadian Seed Growers Association, 2011).

Large Plot Experiments

During all site years flowering synchrony exceeded 75% (Table 5-1). Plant stands were of good quality all years with the exception of ElRS in 2008. Because of poor emergence in the AC Alta pollen receptor in three directions (S, SE & SW) at ElRS in 2008 this data was excluded from directional analyses. There were no significant interactions or differences between sites or years, despite the differences in precipitation between years. Data from all sites and years were combined for further analysis.

Temperatures during 2008 and 2009 were generally cooler than the 30 year normal in the early part of the growing season in all sites and years (Figure 5-1). The temperature range during flowering for ElRS was 13.9 – 21.3 °C and LRS was 14.2 – 21.8 °C (Table 5-3). Precipitation was close to normal at ElRS in 2008, but less than normal in 2009 and recorded as a drought year. We hypothesised that PMGF would increase at this site year because dry conditions are known to correlate with increased outcrossing in other self-pollinated grass species such as wheat (Briggs et al., 1999) and rice (Weerakoon et al., 2009). At ElRS during the flowering period in 2008 total precipitation was 7.8 mm and in 2009 was 4.2 mm. In LRS precipitation was less than normal in the early growing season in 2008 and above normal in 2009. During flowering a total of 37.0 and 4.0 mm precipitation was recorded in 2008 and 2009, respectively. Humidity during the flowering period at ElRS averaged 81% and 74% in 2008 and 2009, and in LRS was lower, averaging 63% and 74%, in 2008 and 2009 respectively. Prevailing wind direction during the flowering period varied by year and location

(Table 5-3): W at ElRS in 2008; S and SW at ElRS in 2009; S and SW at LRS in 2008; and S, SW and W at LRS in 2009.

Prevailing wind direction during the flowering periods was expected to be negatively correlated with higher PMGF rates and there were significant differences ($P < 0.0001$) in outcrossing for each directional comparison. Winds were prevalent from the west, south west and south. The highest average PMGF occurred in the easterly direction at 0.2 m (5.07%) and at 50 m (0.14%; Table 5-4). The lowest PMGF frequency at 0.2 m (1.92%) was recorded in the west and the lowest at 50 m (0.04%) was in the north. Directional effects were most evident closer to the pollen source (graphically represented in Figure 5-2) and as expected, directional effects become less pronounced with distance from the pollen source. Prevailing wind direction has been previously shown to influence PMGF in wheat (Hucl and Matus-Cadiz, 2001; Hucl, 2010).

Over 17 M seeds were screened using the blue aleurone as a visible marker (Table 5-4). Sample size (minimum 1 487; maximum 2 740 800) was generally increased as PMGF events decreased to exceed the statistical confidence ($\alpha = 0.05\%$). Increasing the intensity of sampling increased the accuracy of the estimations and also increased the likelihood that rare outcrossing events were identified.

Triticale PMGF over distance best fit an exponential decay curve where the intercept was 1.57E-6 and the slope 0.2647 (Figure 5-3). Average PMGF of 3.4% was recorded at 0.2 m from the pollen source; 162 352 seeds were screened

and 6 121 blue seeds detected (Table 5-4). Outcrossing declined exponentially with distance. Average PMGF declined by 50% 2.62 m from the pollen source and by 99% 17.4 m from the pollen source. At a distance of 50 m from the pollen source, PMGF was 0.09%; 2 740 200 seeds were screened at this distanced and 2 315 blue seeds were detected. PMGF events were present in harvested samples at all distances from the pollen source, indicating that, on this scale, triticale PMGF exceeds 50 m.

PMGF is not uniform and most occurs closest to the pollen donor (Figure 5-3). Blending of seed from throughout the field occurs at harvest. The amount of AP decreases as the width of the receptor field increases (Damgaard and Kjellsson, 2005; Gustafson et al., 2005). Assuming a uniform blending of seed, the amount of AP corresponds to the integration of PMGF over distance (Eq. 3). Had the 50 m field surrounding the pollen source been harvest blended, it is estimated the total level of AP would have been 0.22%. The harvest blended AP for spring wheat was estimated at 0.16% and 0.02% in a receptor field width of 50 m and 400 m, respectively (Gustafson et al., 2005). Similarly, AP levels for triticale are expected to decrease as the receptor field width increases as would be the case in commercial production. The blended AP levels are below the threshold (0.9%) for labelling GM crops in the EU (European Union, 2003).

At similar distances (0.2 – 1.6 m), PMGF quantified from the large plot trials was approximately three times that of small plots (2.23% compared to 0.76%, respectively). Both the size of the pollen cloud and the relative amounts of pollen from each genotype changes with the relative size of the donor and

receptor plots. Pollen from smaller donor plot sizes may provide minor local contributions to pollen clouds that are unpredictable with distance (Treu and Emberlin, 2000; Eastman and Sweet, 2002). At low densities, pollen from donor populations may not be sufficient to out-compete pollen from within the recipient plot, particularly if the recipient plot is larger. Commercial scale fields have been shown to contribute to 'regional' pollen clouds that are able to travel greater distances at higher densities and are expected to have a greater contribution to PMGF.

Variation in PMGF quantified in small and larger scale experiments have been reported previously in the same cultivars of spring wheat (Table 5-2). PMGF of certain cultivars were reported to increase with plot size, while others decreased. Disparity may be due in part to comparative size of the donor and receptor plots, sample size, environmental variance and flowering synchrony. PMGF levels between experiment scale in other species has been reported, including, winter wheat (Gaines et al., 2007a) and canola (Beckie et al., 2003; Walklate et al., 2004; Damgaard and Kjellsson, 2005; Husken and Dietz-Pfeilstetter, 2007). Researchers must use caution and not extrapolate from smaller scale studies to predict PMGF at a commercial scale.

The maximum PMGF in triticale falls with the range reported in wheat cultivars collected at a similar scale (a maximum distance of 33 m) previously reported by (Hucl, 2001). However, the mean triticale PMGF of 3.4% was higher than most of the wheat cultivars tested (Table 5-2). Spring wheat PMGF has been reported as high as 6.7 % (Hucl, 1996; Lawrie et al., 2006) but other recent

studies recorded a maximum of <1% at closest distances (Matus-Cadiz et al., 2004; Hanson et al., 2005; Hucl, 2010; Beckie et al., 2011). The large differences in reported PMGF are mostly due to cultivar differences resulting in flower variability (Gatford et al., 2006; Matus-Cadiz et al., 2007; Beckie et al., 2011). Above average PMGF in triticale may be due to the presence of the rye genome in triticale that can lead to more floret opening and pollen transfer or may be a function of the cultivar chosen.

PMGF decreased in a leptokurtic pattern with increasing distance. The exponential decline was similar to observations in flax (Jhala et al., 2011), safflower (McPherson et al., 2008), and wheat (Matus-Cadiz et al., 2004; Hanson et al., 2005; Beckie et al., 2011). (Beckie et al. 2011) in commercial scale experiments using the wheat cultivar CDC Imagine as a pollen source and AC Barrie as a pollen receptor reported 0.2% PMGF closest to the donor, declining by 50% at 5 meters.

While there is no guarantee of a 0% PMGF risk when growing GM crops near conventional crop species, there are precautions that can be taken to reduce the probability of PMGF. Low levels at 50 m show that pollen can still be viable at increased distances. Pollen trap strips could assist in the reduction of PMGF and possible AP (Hucl, 2010). The most effective means to reduce outcrossing and PMGF in GM triticale may be developing cultivars that exhibit a lower propensity for floret opening.

Conclusions

PMGF in this triticale cultivar was similar to that reported for spring wheat at both small and medium scales. However, triticale cultivars may vary in their reproductive traits and future testing of additional triticale cultivars is recommended.

Table 5-1. Seeding, flower initiation, synchrony and harvest dates for triticale cultivars BA and AC Alta for all locations in 2007 and 2009. Flowering synchrony was assessed as the number of days the main head of the receptor cultivar flowered together with the donor cultivar divided by the total number of flowering days, multiplied by 100.

Site year	Cultivar	Seeding date	Flowering dates	Flowering synchrony	Harvest date
ElRS 2007	BA	May 13	July 16-26	-	Oct 3
	AC Alta	May 13	July 19-28	78%	Oct 3
ElRS 2009	BA	May 23	July 20-28	-	Sept 14
	AC Alta	May 18	July 20-27	100%	Sept 14
LRS 2007	BA	May 6	July 14-24	-	Sept 25
	AC Alta	May 6	July 18-26	75%	Sept 25
LRS 2009	BA	May 22	July 19-28	-	Sept 29
	AC Alta	May 18	July 19-27	100%	Sept 29

Table 5-2. Small-scale PMGF previously reported in spring wheat and experimental conditions; including the pollen donor, plot layout (PMGF measured between rows or between blocks and strips), pollen donor plot size, pollen receptor cultivars, maximum distance at which PMGF was measured, sample size and maximum PMGF (%) and the distance (m) at which maximum outcrossing occurred.

Pollen donor/study	Plot layout	Pollen donor size (m)	Pollen receptor	Max distance (m)	Sample size	Max % PMGF/m
T. aestivum 75% Konini:25% line 3496.3 Hucl, 1996	Measured between rows	6 x 0.4	*T. aestivum* – Biggar	0.2	500 spikes	1.15/0.2
			T. aestivum – CDC Makwa	0.2	500 spikes	0.30/0.2
			T. aestivum – Columbus	0.2	500 spikes	0.35/0.2
			T. aestivum – Genesis	0.2	500 spikes	0.65/0.2
			T. aestivum – Glenlea	0.2	500 spikes	1.10/0.2
			T. aestivum – Katepwa	0.2	500 spikes	0.55/0.2
			T. aestivum – Laura	0.2	500 spikes	0.95/0.2
			T. aestivum – Oslo	0.2	500 spikes	6.70/0.2
			T. aestivum – Roblin	0.2	500 spikes	1.65/0.2
			T. aestivum – Rongotea	0.2	500 spikes	2.40/0.2
			T. aestivum – Wildcat	0.2	500 spikes	1.65/0.2
T. aestivum – Purendo 38 Hucl, 2010	Measured between rows	10.4 x 7.2	*T. aestivum* – CDC Teal	3.6	6000 seeds	0.36/0.3
			T. aestivum – Glenlea	3.6	6000 seeds	0.63/0.6
			T. aestivum – Katepwa	3.6	6000 seeds	0.07/1.5
			T. aestivum – Roblin	3.6	6000 seeds	0.79/0.3
T. aestivum – Purendo 38 Hucl and Matus-Cádiz, 2001	Measured between block and strips	5 x 5	*T. aestivum* – Biggar	33	644 seeds	0.43/0
			T. aestivum – Katepwa	33	644 seeds	0.15/0
			T. aestivum – Oslo	33	644 seeds	3.78/0.5
			T. aestivum – Roblin	33	644 seeds	2.63/0

Table 5-3. Meteorological data for the triticale flowering period by site year. Mean maximum and minimum temperature (°C), total precipitation, prevailing wind direction with duration in parenthesis and average humidity is reported.

Site year	Temperature (°C)		Total Precipitation mm	Prevailing Wind Direction (%)	Humidity %
	Min	Max			
ElRS 2008	13.9	20.0	7.8	W (100)	81
ElRS 2009	15.5	21.3	4.2	SW,S (60,40)	74
LRS 2008	14.2	19.4	37.0	S,SW,W (25,25,50)	63
LRS 2009	14.9	21.8	4.0	S,SW,W (20,30,50)	74

Table 5-4. Actual PMGF (%) of triticale in the cardinal directions and mean PMGF for all directions at distances 0.2 – 50 m from the pollen source. The number of seeds screened and the number of blue seeds at each direction and the power of the test are included.

Mean distance (m)	N	E	S	W	Mean	Seeds screened[†]	Blue seeds detected	Power, (1-β); α=0.05%[‡]
	PMGF (%)							
0.2	3.21	5.07	3.35	1.92	3.4	162352	6121	0.95
0.4	3.42	4.15	2.65	2.12	3.2	130014	4234	0.95
0.6	2.54	3.23	1.83	1.47	2.3	157819	3869	0.95
0.8	1.89	2.90	1.43	0.95	1.8	180802	2853	0.95
1	1.93	2.23	1.40	0.73	1.6	196308	3209	0.95
1.6	1.42	1.53	1.00	0.45	1.1	215600	2377	0.95
2	1.15	1.51	0.95	0.51	1.0	133400	2321	0.95
2.4	1.20	1.38	0.72	0.50	1.0	233488	1901	0.95
3.25	0.73	0.99	0.63	0.36	0.7	312800	1684	0.95
4.75	0.31	0.64	0.49	0.16	0.4	462006	1487	0.95
6.25	0.35	0.44	0.30	0.14	0.3	808800	1716	0.95
7.75	1.89	0.37	0.29	0.13	0.2	886400	1745	0.95
10	0.14	0.29	0.24	0.16	0.2	1617200	2783	0.95
15	0.19	0.19	0.31	0.17	0.2	1846200	3588	0.95
20	0.14	0.20	0.21	0.11	0.2	2391785	3912	0.95
30	0.09	0.14	0.12	0.17	0.1	2308400	2154	0.95
40	0.10	0.11	0.10	0.11	0.1	2528967	2851	0.95
50	0.04	0.14	0.13	0.06	0.09	2740800	2315	0.95

[†] Total number of seeds screened from all directional blocks.

[‡] Power was calculated using a 95% confidence interval (α=5%) using equation (1).

Table 5-5. Directional effects of outcrossing averaged over sites and years. Parameter estimates are provided for distances where outcrossing was reduced by 50% (O_{50}) and 99% (O_{99}) and *a*, the intercept of the slope (*b*); for cardinal directions.

Direction	*Parameter*[†]	*Estimate*[‡]	*Standard error*	*Df*[§]	*95% Confidence Interval*	
					Lower	*Upper*
N	O_{50}	1.97	3.28	71	-4.5655	8.5009
	O_{99}	13.07	21.77	71	-30.3324	56.4789
	a	1.83E-6	3.55E-6	71	-5.25E-6	8.914E-6
	b	0.3523	0.5866	71	-0.8173	1.5218
E	O_{50}	2.35	3.31	72	-4.2578	8.9581
	O_{99}	15.61	22.02	72	-28.2883	59.5166
	a	2.25E-6	3.47E-6	72	-4.66E-6	9.166E-6
	b	0.2949	0.4160	72	-0.5343	1.1242
S	O_{50}	2.58	4.86	54	-7.1713	12.3240
	O_{99}	17.12	32.30	54	-47.6450	81.8787
	a	2.04E-6	4.80E-6	54	-7.59E-6	1.20E-5
	b	0.2690	0.5077	54	-0.7489	1.2870
W	O_{50}	2.11	5.03	71	-7.9081	12.1342
	O_{99}	14.04	33.39	71	-52.5403	80.6176
	a	1.14E-6	4.36E-6	71	-7.55E-6	9.83E-6
	b	0.3280	0.7802	71	-1.2277	1.8838
All Directions	O_{50}	2.62	1.88	366	-1.0873	6.3243
	O_{99}	17.40	12.52	366	-7.2239	42.0174
	a	1.57E-6	1.69E-6	366	-1.75E-6	4.883E-6
	b	0.2647	0.1905	366	-0.1099	0.6394

[†] Parameters *a* and *b* were estimated using equation (2). The distances (O_{50} and O_{99}) where outcrossing was reduced by 50 and 99% were estimated using equation (3) and (4), respectively.

[‡] Estimates of the parameters for intercept (a), slope (b) and the estimates of the distances where outcrossing was reduced by 50 and 99%.

[§] Degrees of freedom.

Figure 5-1. Temperature (°C) and precipitation (mm) monthly averages for ElRS (A & C) and LRS (B & D) during 2008 and 2009. The 30 year average is indicated.

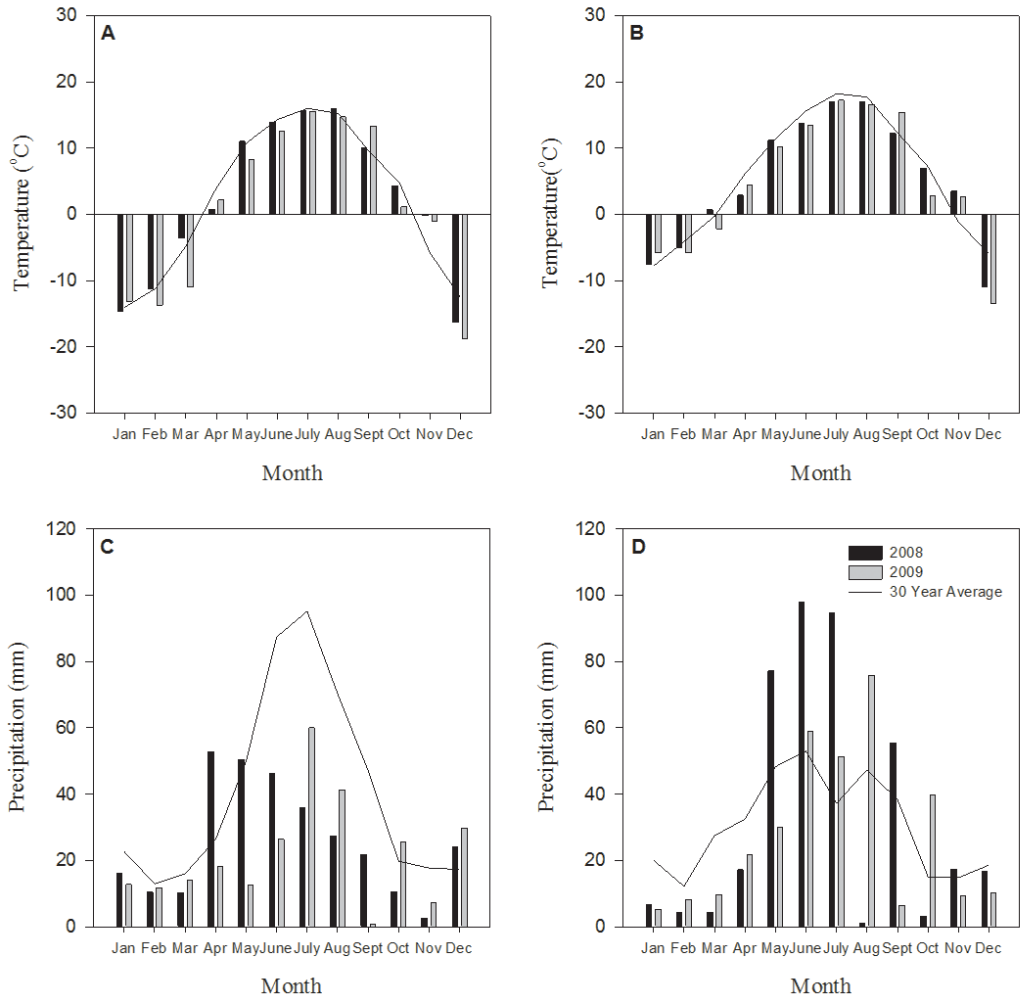


Figure 5-2. Directionality of triticale PMGF (%) in samples taken from between 0.2 and 7.75 m (A) and between 10 and 50 m (B) averaged across all sites and years.

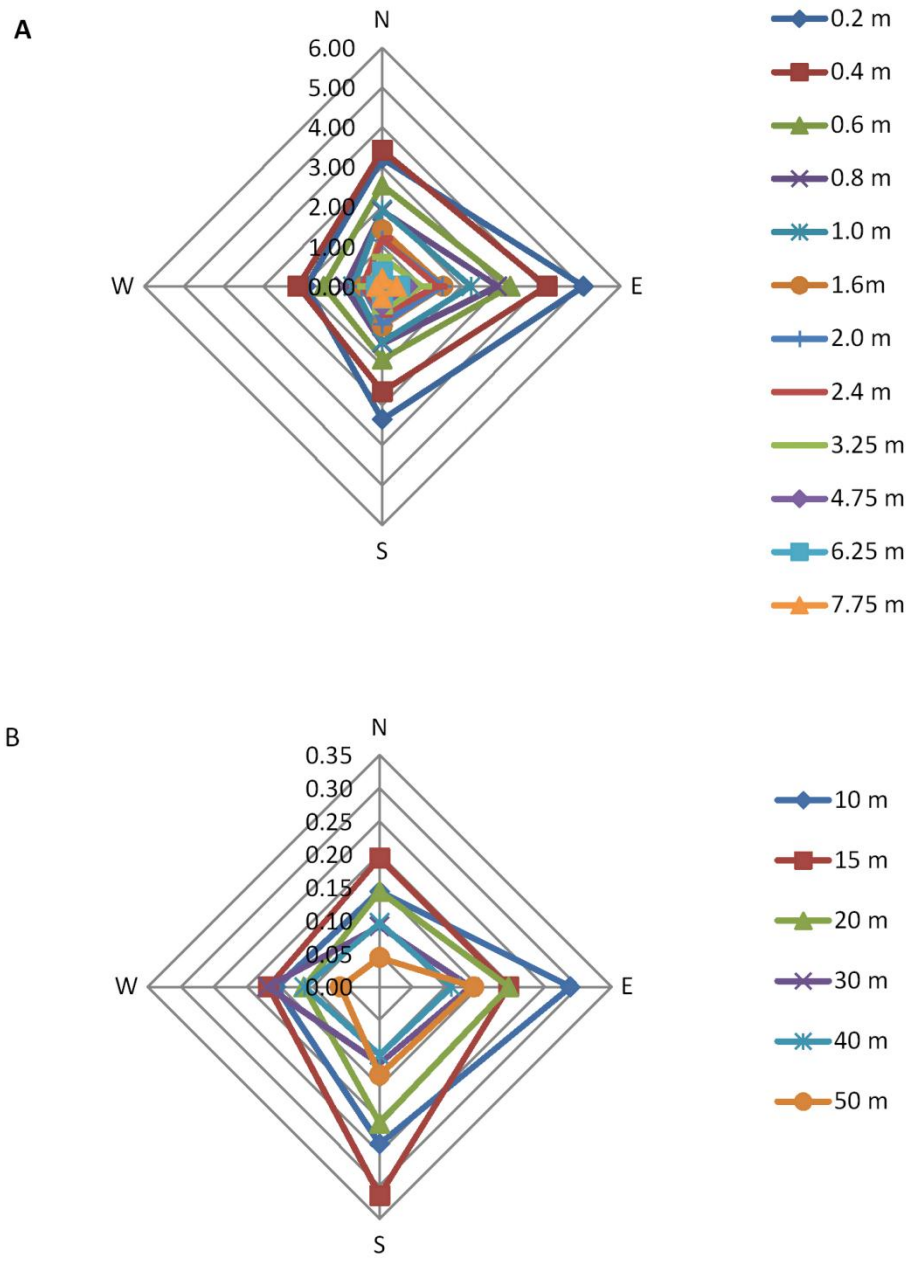
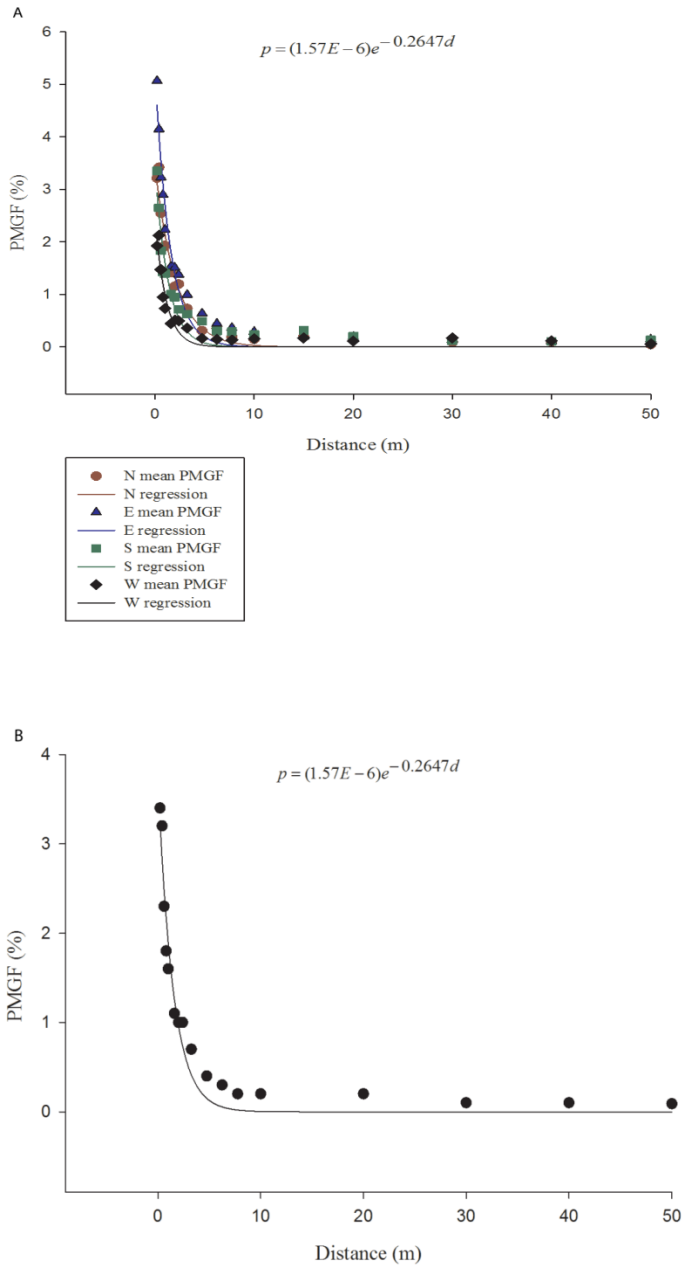


Figure 5-3. PMGF (%) as a function of distance from the blue aleurone triticale (BA) pollen donor to AC Alta. Lines are the fitted model and points the PMGF means at each distance. Data was combined for each direction from 2 locations/years (A) and over all directions, two years and two locations (B). Refer to Table 5-5 for parameter estimates and statistical significance.



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Chapter 6. General Discussion and Conclusions

Triticale is a minor crop in Canada with 30,000 tonnes produced each year (FAOSTAT, 2011). It has been used as a feed for ruminant animals where it exhibits superior digestibility qualities (Hill, 1991; Bird et al., 1999). Triticale has higher biomass than other cereal crops and chemical properties that may make it beneficial as a cereal platform for biofuel and bioproducts such as adhesives, plastics and building materials through the use of genetic modification (Canadian Triticale Biorefinery Initiative, 2010). However, genetically modified (GM) triticale may pose risks to the environment through pollen-mediated gene flow (PMGF) to wild relatives that may affect population dynamics (Andow and Zwahlen, 2006; Warwick et al., 2009) or to the economy via PMGF to conventional triticale or wheat leading to AP that surpasses international thresholds for approved GM traits (Chapotin and Wolt, 2007; Gealy et al., 2007). Before GM triticale can be approved for commercial release and sale, environmental biosafety assessments are performed as part of the pre-commercialization risk assessment. Determining risk of PMGF from GM triticale to conventional cultivars and relatives fulfills a component of this assessment.

A literature review identifying potentially compatible wild relatives of triticale was performed with emphasis on at-risk Canadian species in **chapter 3**. Field experiments were conducted to quantify PMGF from triticale with a blue aleurone trait to wheat in data **chapter 4** and conventional triticale in data

chapter 5. Finally in this chapter best management practices will be recommended for GM triticale cultivation to mitigate potential PMGF and AP of transgenes in conventional products.

Triticale is a recent (1800`s) hybrid of cultivated wheats (*Triticum aestivum* L. & *T. durum* Desf.) and rye (*Secale cereale* L.) and has potential to cross with related Poaceae species. To assess PMGF probability and focus research further, we reviewed the phylogeny of triticale, wheat and rye, species distribution, outcrossing barriers and reported crosses. An important finding of this review was that triticale PMGF candidates were distributed throughout the entire triticeae tribe rather than restricted to parental genera and close relatives. This is contrary to other crop species (i.e. flax; Jhala et al., 2008) and in relation to gene flow has only been reported one other time in *Prunus* (Cici and Van Acker, 2010). Cleistogamy and the presence of genetic barriers (i.e. *Ph*1, *Ph*2 & *Kr* gene family) that inhibit crossing with other species suggest risk for PMGF may be low. Outside of parental wheat and rye species, the highest risk for PMGF is with wild species intermediate wheatgrass (*Agropyron intermedium*) and jointed goatgrass (*Aegilops cylindrica*). While of interest, PMGF between these species and triticale was outside the mandate of this thesis.

Interspecific PMGF between triticale and spring and durum wheat was assessed through small strip plots (1.4*50 m) in four site years in **chapter 4**. PMGF was greater between spring wheat x triticale (0.0006%) than durum wheat x triticale (0.0008%). Seed morphology was highly shriveled and viability was

low. Low PMGF and subsequent viability indicate risks GM triticale cultivation may not interfere with spring or durum wheat markets.

Small and large plot experiments were conducted to quantify intraspecific triticale PMGF rates in **chapter 5**. PMGF from small strip plots (1.4*50 m) from 0.2-1.4 m was 0.76% averaged over four site years. Large plot experiments were conducted at two locations in both 2008 and 2009 using a concentric donor (20*20 m) and receptor (120*120 m). PMGF declined with distance from the donor and best fit an exponential decay model. The highest average PMGF (3.4%) was adjacent the donor crop and rapidly declined to 0.09% by 50 m from the donor. From 0.2-1.4 m average PMGF in the large plot was 2.23%. Lower levels of PMGF in smaller plots were unexpected and may be due to lower localized pollen cloud contribution (Treu and Emberlin, 2000; Eastman and Sweet, 2002). Directional differences associated with prevailing winds at flowering were reported and underscore additional environmental parameters need to be considered when formulating mitigation strategies. Harvest blending of field plot (100*100 m) lowered PMGF to 0.22%. Based on this conservative field-scale assessment, PMGF alone in triticale may not prevent the coexistence of GM and conventional triticale using the EU threshold (0.9%). Employment of best management practices highlighted below may reduce rates further and ease GM triticale cultivation concerns should it be accepted by international markets.

Field-scale intraspecific gene flow trials suggest removal of the first 3.25 or 4.75 m of a conventional triticale crop directly adjacent a GM source may reduce

maximum PMGF from 3.4% to below 0.7% and 0.4% respectively. However, caution must be exercised when applying field trials to commercial scale cultivation. The rapid PMGF decline suggests a 3 m conventional triticale buffer crop surrounding a GM field may reduce AP in nearby conventional fields below the EU AP threshold. Results from this experiment indicate required triticale intraspecific isolation distances of 1 m for certified seed in Canada is not sufficient to eliminate the possibility of maximum PMGF below 0.9% EU thresholds levels.

This biosafety risk assessment to quantify triticale PMGF affirms that if best management practices are followed GM triticale PMGF may be below conservative international thresholds and may not cause market disruption. Although triticale is unlikely to hybridize with wild species in Canada, monitoring for rare events should be conducted if GM cultivars are approved.

Field experiments quantified PMGF from a simulated transgenic triticale to other triticales and durum and common wheat. It established PMGF base-lines previously unknown in triticale, but essential for GM triticale commercialization. Stewardship and best management strategies provided can be a tool to reduce gene flow and AP in exports and the environment.

Suggested Future Research

- Identify differences in intraspecific PMGF at the same distance with different scales of pollen donor size. Extrapolating commercial-scale PMGF rates from a field study may be misleading. Differences in PMGF

between field- and commercial-scale cultivation have been reported in spring wheat (Beckie et al., 2011) and may hold true for triticale. Commercial-scale triticale outcrossing trials should be conducted to more precisely capture intraspecific PMGF and better characterize economic risks.

- Cultivars of wheat exhibit large differences in PMGF (Hucl, 1996; Hanson et al., 2005). This thesis examined PMGF when only one cultivar of triticale was the pollen source and another cultivar was the pollen receptor. PMGF of the other Canadian registered triticale cultivars should also be examined and selection of triticale cultivars for production of bioproducts should include screening for PMGF propensity.

- Two wild species (*Agropyron intermedium* and *Aegilops cylindrica*) were identified as potentially at-risk for outcrossing with triticale. They are distributed throughout the United States and parts of Canada near agricultural areas. Tiered experiments should be completed to establish natural PMGF risk starting with Tier 1 – worst case scenario greenhouse experiments to assess compatibility. In addition, life-cycles should be compared to determine flowering synchrony and likelihood of pollen transfer should compatibility be confirmed.

- Finally, only plants sympatric to Canada were the focus of the interspecific triticale PMGF literature review. However, because of international grain trade, GM triticale may be a concern in Europe and parts of Asia where triticale cultivation is common. Seed loss and gene movement during transport can result in volunteers in agricultural areas and transportation corridors where PMGF to wild relatives may affect population dynamics, or GM triticale PMGF to crop relatives can lead to AP in conventional grain affecting markets. Further research should be directed towards compatibility between European and Asian wild and weedy triticale relatives.

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Appendix 1

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Evaluation of crossability between triticale (X *Triticosecale* Wittmack) and common wheat, durum wheat and rye

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Development of transgenic triticale as a platform for novel bio-industrial products is predicated on an environmental biosafety assessment that quantifies the potential risks associated with its release. Pollen-mediated gene flow to related species and conventional triticale varieties is one pathway for transgene movement. A tier 1 quantification of triticale hybridization was conducted by emasculating and hand pollinating flowers under greenhouse conditions. Approximately 2000 manual pollinations were conducted for each cross and its reciprocal between two triticale genotypes: a modern triticale cultivar (AC Alta) and primary triticale (89TT108), and common wheat, durum wheat and rye. The frequency of outcrossing, hybrid seed appearance and weight, and F_1 emergence and fertility were recorded. Outcrossing, F_1 emergence and fertility rates were high from crosses between triticale genotypes. Outcrossing in inter-specific crosses was influenced by the species, and the genotype and gender of the triticale parent. In crosses to common and durum wheat where triticale was the male parent, outcrossing was ≥ 73.0% and ≥ 69.5%, respectively, but ≤ 23.9% and ≤ 3.0% when triticale was the female parent. Overall, outcrossing with rye was lower than with common and durum wheat. F_1 hybrid emergence was greater when triticale was the female parent. With the exception of a single seed, all wheat-triticale F_1 hybrid seeds were non-viable when triticale was the male parent in the cross. Only seven durum wheat-triticale F_1 hybrids emerged from 163 seeds sown, and all were produced with triticale 89TT108 as female parent. With rye, 8 F_1 hybrids emerged from 38 seeds sown, and all were produced from crosses to AC Alta; five with AC Alta as the female parent and three as the male. Interspecific F_1 hybrids were self-sterile, with the exception of those produced in crosses between common wheat and triticale where triticale was the female parent. Tier 2 hybridization quantification will be conducted under field conditions.

Keywords: triticale / outcrossing / risk assessment / biosafety / hybridization / rye / wheat / crossability

INTRODUCTION

Triticale (X *Triticosecale* Wittmack) is being evaluated as a candidate for the production of novel bioproducts in Canada. Improvement of triticale through genetic modification necessitates that potential risks associated with the release of transgenic cultivars be assessed. Triticale is an intergeneric hybrid between wheat (*Triticum* ssp.) as the female parent and rye (*Secale* ssp.) as the male parent. Reduced fertility and shriveled kernels were common in early triticale, but breeding programs were successful in improving these traits (Oettler et al., 2005). The first commercial triticale cultivars were released in the late 1960s. Triticale production increased almost 50% worldwide from 1991 to 2001, and in 2004 triticale was grown on over 3 million ha in 28 countries (http://faostat.fao.org/). In Canada, triticale acreage reached a maximum in 2002 with 87 000 ha. It is used primarily for animal feed, but small amounts enter the human food chain either intentionally or unintentionally through mixing with other grains. Triticale has many favorable agronomic qualities for production in Canada: it is more productive than other cereals under abiotic stress conditions, less susceptible to most diseases, and is competitive with other cereals in terms of grain yield (Alberta Agriculture, Food and Rural Development, 2005). Furthermore, triticale has excellent potential for improvements through breeding. Recent studies suggest that significant improvements in yield

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are possible through hybrid breeding (Oettler et al., 2003; 2005) and breeding programs are underway to improve additional traits including disease and lodging resistance and starch profile (Green, 2007). There is also the potential to enhance breeding programs using existing genetic markers (Gonzalez et al., 2005; Kuleung et al., 2006; Tams et al., 2004; 2005; Zhang et al., 2005). Successful transformation of triticale was achieved using microprojectile bombardment and *Agrobacterium*-mediated transformation (Nadolska-Orczyk et al., 2005; Zimny et al., 1995). Currently, the traits expressed by transgenic triticale in the published literature are limited to reporter genes, such as antibiotic resistance, used to identify successful transformation events. Development of transformation protocols for triticale allows the introduction of a number of novel traits.

Triticale may be a valuable platform for bioproducts. Bio-industrial farming opportunities encompass existing and emerging markets including bioenergy, biorefining and biomaterials. Conventional triticale has already been identified as a suitable resource for biofuel production (Plochl and Heiermann, 2006; Rosenberger, 2005; Rosenberger et al., 2002; Wang et al., 1997). Biotechnology may provide opportunities to improve triticale as a biofuel resource and to introduce new traits, expanding its applications with bio-industrial farming.

The use of triticale as a platform for bioproducts requires an environmental risk assessment. The current small acreage of triticale reduces the opportunities for transgenic, or novel, triticale to outcross with conventional triticale. In addition, triticale has limited use in products destined for human consumption, reducing the challenges associated with segregating bioproducts from conventional products. One concern regarding the introduction of a transgenic crop is gene flow to related species. If pollen from transgenic triticale can successfully produce hybrid seed with similar or related conventional crops (*i.e.* triticale, wheat, or rye) and transgene introgression occurs, the transgene could enter the conventional food chain. This could result in market harm to conventional crops. The potential for pollen-mediated gene flow from triticale to other crops, and the potential impact on their weediness or invasiveness needs to be assessed.

Triticale may be tetraploid, hexaploid or octoploid, but hexaploid varieties are the most commercially successful. Hexaploid hybrids of Triticale ($2n = 6x = 42$; AABBRR) may be developed by crossing durum wheat (*Triticum turgidum* ssp. *durum*; $2n = 4x = 28$; AABB) and rye (*Secale cereale*; $2n = 2x = 14$; RR). Additionally, octoploid triticales often revert to the hexaploid level (Oettler, 2005). Although triticale is primarily selfing, considerable outcrossing may occur, and fertilization was detected as far as 30 m from a pollen source (Yeung and Larter, 1972). The potential for pollen-mediated gene flow over long distances in triticale is unknown; however, a recent publication examining pollination from common wheat to neighboring fields within a 10 km radius of a central pollinator field detected gene flow at distances up to 2.75 km (Matus-Cadiz et al., 2007). Triticale was previously reported to outcross with common wheat (*Triticum aestivum*; $2n = 6x = 42$; AABBDD) and rye (Chaubey and Khanna 1986; Guedes-Pinto et al., 2001; Lelley, 1992), though crossability was higher with wheat than with rye (Lelley, 1992). Crosses between triticale and common wheat where triticale was the female parent (ABR × ABD) produced viable F_1 seed, but the reciprocal cross (ABD × ABR) produced non-viable seed (Bizimungu et al., 1998; Chaubey and Khanna, 1986; Khanna, 1990; Nkongolo et al., 1991). Although the seed is non-viable, common wheat × triticale (ABD × ABR) hybrids can be obtained using *in vitro* embryo rescue (Kapila and Sethi, 1993). Hybridization was influenced by the genotypes of the parents used in the interspecific cross (Bizimungu et al., 1998; Guedes-Pinto et al., 2001; Lelley, 1992; Lima-Brito and Guedes-Pinto, 1998; Nkongolo et al., 1991). In addition to genetic factors, observations of variation in hybridization success in different years and at different temperatures suggest an environmental influence on hybridization (Guedes-Pinto et al., 2001; Nkongolo et al., 1991).

In order to quantify the potential for pollen-mediated gene flow, we are conducting a tiered evaluation of outcrossing in triticale. This evaluation will include the potential for outcrossing between triticale varieties, as well as between triticale and related crops. Assessing the potential for hybridization in a tiered manner was described by Raybould and Cooper (2005). This approach involves several tiers of testing, the first tier involving a simple study in the laboratory or greenhouse under conservative "worst case" assumptions. The results of tier 1 testing determine if testing moves to a second tier under more complex and environmentally realistic conditions. In testing for potential hybridization, tier 1 testing is carried out under optimal controlled greenhouse conditions where self-fertilization is prevented by emasculation and pollen is transferred manually. The removal of pollen competition through emasculation enhances the potential for hybridization. As a result, hybridization is a very conservative measure of potential gene movement. In the case of successful hybridization at the tier 1 level of testing, tier 2 assessment is recommended. Tier 2 testing includes tests for "spontaneous" hybrid production in the lab or field (Raybould and Cooper, 2005).

This research documents the results of tier 1 testing on the intra- and inter-specific outcrossing frequencies between triticale cultivars and between triticale and its relatives: common wheat, durum wheat and rye.

The frequency of outcrossing, hybrid seed production and weight, and F_1 emergence and fertility were quantified.

RESULTS

Outcrossing frequency

Approximately 2000 flowers were emasculated and hand pollinated (data not shown) under greenhouse conditions for each intra- and inter-specific cross and its reciprocal cross. The study used two triticale genotypes (cv. AC Alta and 89TT108, ABR), common wheat (AC Barrie, ABD), durum wheat (Kyle, AB) and rye (Rogo, R). Crosses were carried out over a period of 10 months. The mean outcrossing for the results obtained at each time point was determined and standard error calculated. Seasonal environmental fluctuations were anticipated for conditions such as light quality, and all representative crosses were performed at regular intervals. In addition to the inter-specific crosses, crosses between triticale genotypes and crosses between plants of a single triticale genotype were carried out. Common wheat, durum wheat and rye were also crossed to themselves to evaluate the success of emasculation and pollination. Percentage outcrossing (*OC*) was determined for each intra- and inter-specific hybridization, correcting for unsuccessful hybridizations due to factors such as mechanical damage during emasculation and pollination. In the inter-specific crosses not involving triticale, *OC* was highest in crosses between durum and common wheat where durum wheat was the female parent (52.0% ± 2.6%) (Tab. 1). In crosses involving rye *OC* was highest in crosses to durum wheat (15.3% ± 2%) where rye was the female parent.

The *OC* for reciprocal crosses between the two triticale genotypes and between triticale and common wheat, durum wheat, and rye were analyzed (Fig. 1). Crosses between the two triticale genotypes AC Alta and 89TT108 demonstrated that *OC* was not reduced compared to crosses between plants of the same genotype ($p < 0.05$), and *OC* was not affected by which triticale was used as the female parent ($p < 0.05$). In crosses between triticale and both common and durum wheat, the direction of the crosses affected the number of F_1 hybrid seeds produced, with *OC* higher when triticale was the male parent for both triticale genotypes ($p < 0.05$). A significantly higher *OC* was obtained when triticale AC Alta was the male parent in crosses with common wheat (86.0% ± 4.0%) compared to when it was the female parent (20.9% ± 1.4%). Similar results were obtained with triticale 89TT108. *OC* was higher when triticale AC Alta was the male parent in crosses with durum wheat (89.4% ± 5.7%) than when it was the female parent (1.4% ± 0.5%). Again, similar results were obtained with triticale 89TT108. In crosses between triticale 89TT108 and rye, outcrossing was higher when triticale was the male parent (21.1% ± 1.7% *vs.* 4.7% ± 0.9%). However, no difference in *OC* for reciprocal crosses between triticale AC Alta and rye was observed ($p < 0.05$) with an *OC* of 15% for crosses in both directions.

In general, the *OC* for crosses between triticale and common wheat, durum wheat, and rye were significantly lower than the *OC* obtained when plants of a triticale genotype were crossed to each other ($p < 0.05$), with the exception that the *OC* of crosses between triticale AC Alta and common wheat and durum wheat, when AC Alta was the male parent, were not significantly lower.

Seed weight and appearance

F_1 hybrid seed was harvested from all species, photographed (Fig. 2), and weighed to determine thousand kernel weight (TKW) (Tab. 2). When plants of a cultivar were crossed to the same cultivar, the TKWs were as follows: triticale AC Alta 47.6 g, triticale 89TT108 46.3 g, common wheat AC Barrie 24.5 g, durum wheat Kyle 26.6 g, rye Rogo 39.4 g. Crosses between triticale genotypes resulted in plump seed with a TKW of ≥ 45.7 g. Crosses between common or durum wheat and triticale, when wheat was the female parent, produced F_1 hybrid seeds that appeared shriveled, reflected in the low TKW. F_1 seeds produced from triticale × common wheat had a TKW of ≥ 18.5 g and appeared healthy. Seeds from the reciprocal cross (ABD × ABR) had a TKW of ≤ 5.9 g and appeared shriveled. F_1 seeds produced from durum wheat (AB) and triticale (ABR) had a TKW of ≤ 2.8 g from crosses in both directions, and all seeds appeared shriveled. The TKW of F_1 seeds for crosses between rye and triticale were also low, with a maximal TKW of 7.3 g obtained with AC Alta × Rogo, and seeds appeared shriveled.

F_1 hybrid emergence

Sprouting resistance was overcome and F_1 seed was planted at uniform depth. Emergence was recorded as a percentage of the total seeds planted (Tabs. 1 and 3). The results for emergence when plants of a cultivar were crossed to each other were as follows: triticale AC Alta 99%, triticale 89TT108 100%, common wheat AC Barrie 97%, durum wheat Kyle 88%, rye Rogo 100%. F_1 hybrid emergence was variable for inter-specific crosses between common wheat, durum wheat and rye (Tab. 1), and emergence was highest for the F_1 hybrids produced by inter-specific crosses where rye was the female parent in crosses to both common and durum wheat. F_1 emergence from crosses between the two triticale cultivars was 100% (Tab. 3). F_1 emergence for triticale × common

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Table 1. Results of inter-specific crosses between common wheat, durum wheat and rye.

♀ Parent	♂ Parent	Outcrossing (%)	F_1 hybrid emergence (%)	F_1 hybrid fertility (%)
Common wheat (ABD)	Durum wheat (AB)	12.0 (± 1.6)	45	0
	Rye (R)	0.6 (± 0.3)	0	-
Durum wheat (AB)	Common wheat (ABD)	52.0 (± 2.6)	12	0
	Rye (R)	1.0 (± 0.4)	25	0
Rye (R)	Common wheat (ABD)	4.0 (± 0.9)	67	0
	Durum wheat (AB)	15.3 (± 2.0)	88	0

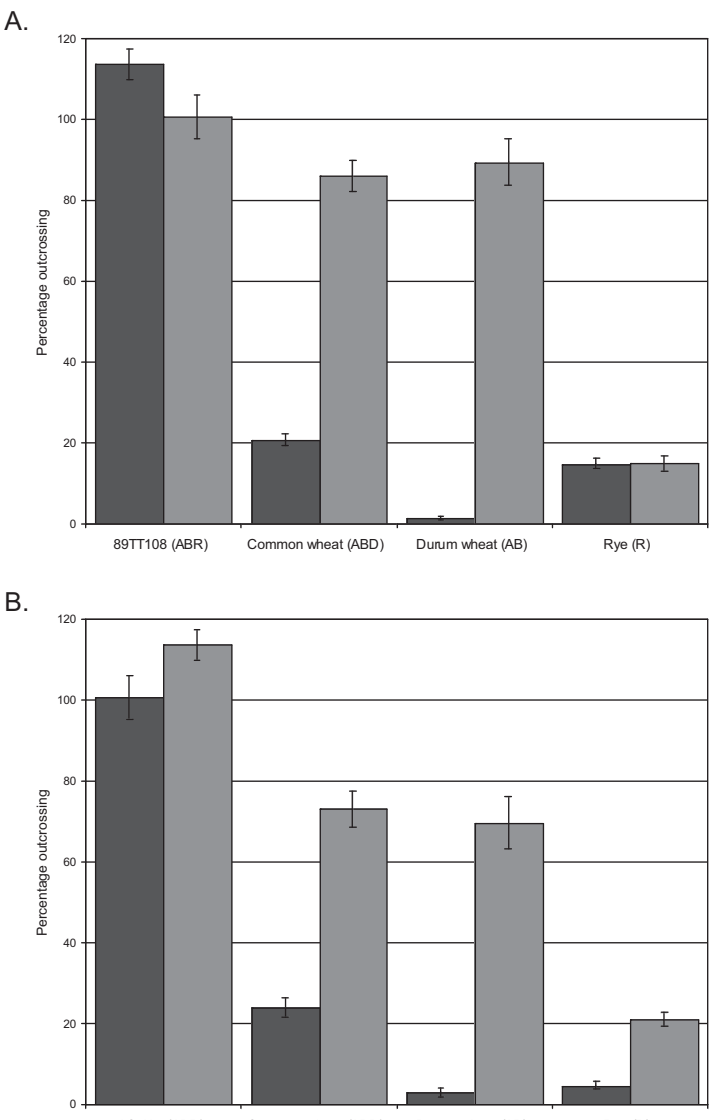

Figure 1. Percentage outcrossing (*OC*) for triticale inter- and intra-specific crosses. Outcrossing between two triticale varieties, AC Alta (A.) and 89TT108 (B.), common wheat, durum wheat and rye, was quantified for each cross and its reciprocal. Dark bars depict results obtained when triticale was the female parent in the cross, and light bars when triticale was the male parent. Mean *OC* and the corresponding standard error bars are shown.

Appendix 1

Quantifying crossability between triticale, wheat, durum wheat and rye

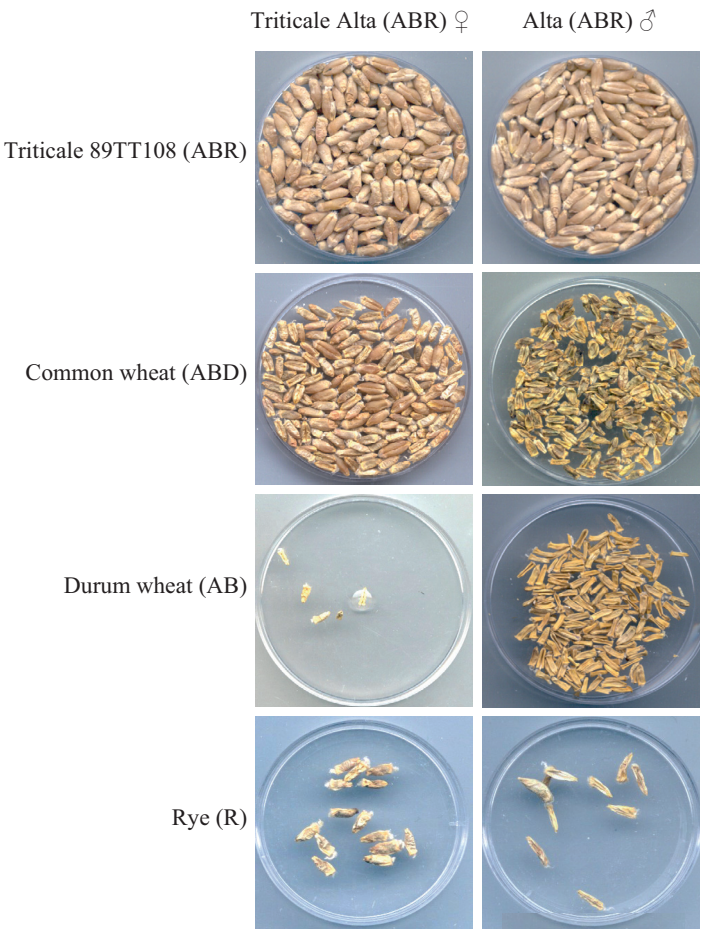

Figure 2. Appearance of F1 hybrid seeds at maturity.

wheat was >90%, while emergence of the reciprocal cross was ≤ 1% with only one F_1 plant emerging. Seed weight and appearance were good predictors of emergence for crosses between common wheat and triticale (Fig. 2, Tab. 2). F_1 emergence for durum wheat × triticale was 0%. The reciprocal cross resulted in 0% F_1 seed emergence with AC Alta as the female parent, and 41% with 89TT108 as the female parent. F_1 emergence was 0% in crosses between rye and 89TT108 in both directions. In contrast, F_1 seed produced from crosses between rye and AC Alta where AC Alta was the female parent or male parent showed emergence of 50% and 38%, respectively. TKW was not a good indicator of emergence for any crosses between triticale and durum wheat or rye.

F_1 hybrid fertility

When the F_1 hybrid plants flowered, spike sterility was recorded. Only F_1 hybrids formed from the triticale × common wheat crosses were self-fertile, with 95% and 90% fertility recorded for the 89TT108 and AC Alta crosses, respectively. Spikes of all other hybrids did not produce seed (data not shown and Tab. 1).

DISCUSSION

This study initiates the quantification of triticale crossability through measurements of intra- and inter-specific outcrossing frequency, hybrid seed production, and F_1 emergence and fertility. Tier 1 tests of hybridization under conservative conditions, where self-fertilization is prevented by emasculation and pollen is transferred manually, provides information to make informed decisions regarding the need for further testing. Hybrid seed formation does not equate to transgene introgression, or potential gene flow, but does suggest a need for further testing. If introgression can occur and the F_1 hybrid is sterile, gene flow is prevented, but the transgenic seed

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Table 2. Thousand kernel weight (TKW) of F_1 hybrid seed.

Parent 1	Parent 2	TKW (g)	
		♀ Triticale	♂ Triticale
Triticale AC Alta (ABR)	Common wheat (ABD)	20.6	5.5
	Durum wheat (AB)	2.7	2.8
	Rye (R)	7.3	3.8
	Triticale 89TT108 (ABR)	50.9	45.7
Triticale 89TT108 (ABR)	Common wheat (ABD)	18.5	5.9
	Durum wheat (AB)	2.6	0.3
	Rye (R)	3.5	4.0
	Triticale AC Alta (ABR)	45.7	50.9

Table 3. Percentage of F_1 hybrid seed emergence.

Parent 1	Parent 2	♀ Triticale	♂ Triticale
Triticale AC Alta (ABR)	Common wheat (ABD)	91	1
	Durum wheat (AB)	0	0
	Rye (R)	50	38
	Triticale 89TT108 (ABR)	100	100
Triticale 89TT108 (ABR)	Common wheat (ABD)	97	0
	Durum wheat (AB)	41	0
	Rye	0	0
	Triticale AC Alta (ABR)	100	100

could potentially be harvested with conventional food crops. However, hybrid seed carrying a transgene, even if non-viable, may be detectable and pose a regulatory concern despite its inability to contribute to transgene spread. Seed size becomes critical in this instance, because small, light seed may be left in the field as a result of the seed size and weight selectivity of harvesting machinery. If a fertile transgenic hybrid is formed, gene flow may occur *via* volunteers produced from seed lost during harvest and/or replanting of contaminated seed lots.

As expected, significant outcrossing was observed between the two triticale cultivars, indicating the potential for outcrossing between transgenic and conventional triticale crops. Observations of hybrid emergence and fertility further emphasize the potential for temporal and spatial transgene movement. The rate and distance of triticale outcrossing will be further investigated in tier 2 studies under field conditions.

Common wheat is the most widely grown and exported crop in Canada with approximately 25.0 million acres of common wheat grown annually in Canada. A high percentage of inter-specific outcrossing was observed between common wheat and triticale (Fig. 1). Outcrossing was significantly higher when triticale acted as the male parent ($\geq 73\%$) than observed when triticale was the female parent ($\leq 23\%$). Although crossing between wheat and triticale with triticale as the male parent produced seed, only a single F_1 plant emerged and was not fertile. Therefore, although outcrossing with common wheat where triticale was the male parent occurred at a higher frequency, these outcrosses rarely produced viable seed. With the exception of the single emergent F_1, these results support previous observations that hybrid seed produced from crosses between triticale and common wheat are only viable when triticale acts as the female parent in the cross (Bizimungu et al., 1998; Chaubey and Khanna, 1986; Khanna, 1990; Nkongolo et al., 1991). While crossability between triticale and common wheat is minimal, the scale of wheat acreage and the importance of this crop suggest testing should continue.

Approximately 5.6 million acres of durum wheat are grown in Canada annually and exported worldwide. Durum wheat is grown in similar areas to triticale, and flowering periods are likely to be synchronous. Outcrossing between durum wheat and triticale was primarily observed with triticale as the male parent, with a total of 509 seeds produced from 2471 pollinated and emasculated flowers; these seeds were not viable. F_1 emergence was only observed from crosses with triticale as the female parent. None of the F_1 hybrids that emerged were self-fertile. Tier 2 tests will continue to quantify seed production in the field.

Outcrossing between triticale and rye was low for crosses in both directions, indicating that the potential for outcrossing with rye is limited. Crossability was previously shown to be lower between triticale and rye than between triticale and common wheat (Lelley, 1992). The moderate emergence observed for the F_1 hybrids indicates the potential for successful hybrid production between triticale and rye, but the hybrids were infertile

indicating that transgene spread as a result of hybridization with rye is unlikely. With the exception of the single emergent common wheat-triticale F_1 hybrid, rye was the only species to produce viable, though infertile, F_1 hybrids with triticale as the male parent. This observation illustrates the potential for pollen-mediated gene flow from triticale to rye. However, the low rate of outcrossing and the lack of fertile F_1 hybrids suggest there is minimal risk for triticale pollen-mediated gene flow to rye. Only 0.5 million acres of rye grown annually in Canada, and the majority is winter rye. Flowering synchrony between winter rye and spring triticale is unlikely. Spring rye, which may flower synchronously with spring triticale, is rarely grown, reducing the potential for gene flow under field conditions.

Triticale is a potential platform for bio-industrial products. In order to grow transgenic triticale in the field, the risk of pollen-mediated gene flow to conventional triticale and its related crops must be quantified. Because of the frequency of cereals in the western Canadian crop rotation, there is a high probability that transgenic triticale could be grown in proximity to a related crop or conventional triticale if it is released. The lack of self-fertile inter-specific F_1 hybrids produced with triticale as the male parent suggest that significant transgene spread through triticale pollen-mediated gene flow to related species is unlikely. However, we did not evaluate the mechanism of sterility and cannot reject the possibility that these self-sterile F_1 hybrids might act as female parents in outcrosses. Similarly, the hybrid pollen was not tested for its ability to successfully fertilize non-hybrid plants. Transgene movement between triticale cultivars is of a greater concern due to the production of fertile intraspecific hybrids.

AC Alta is an elite hexaploid cultivar and has been the subject of extensive breeding efforts, whereas 89TT108 is a primary triticale synthesized as an octoploid and then selfed for five generations. During this process, 89TT108 reverted to the hexaploid level. Differences were observed in the results obtained with the two triticale genotypes, AC Alta and 89TT108. For example, when AC Alta was the female parent in crosses to common and durum wheat, outcrossing was higher than that observed with 89TT108. Furthermore, AC Alta-Rye F_1 hybrid emergence was $\geq 38\%$, but no 89TT108-Rye F_1 hybrid plants emerged. In contrast, emergence of 89TT108-durum wheat F_1hybrids was 41% where triticale was the female parent, but no AC Alta-durum wheat F_1 hybrids emerged. A genetic component to crossability has been established in previous research (Bizimungu et al., 1998; Guedes-Pinto et al., 2001; Lelley, 1992; Lima-Brito and Guedes-Pinto, 1998; Nkongolo et al., 1991) and these results are not unexpected. Observations that outcrossing is influenced by differences in cultivars as well as environmental factors suggest that it may be advisable to conduct tier 2 testing with more cultivars in several environments. Furthermore, future decisions regarding the genetic background to use for transgenic applications should take into consideration the outcrossing potential of the genotype. Tier 2 testing in the field will accommodate the need to observe hybridization in a field environment with environmental fluctuations. Analysis of how the results of tier 1 risk assessment reflect on those at the tier 2 level will be valuable in making future risk assessment decisions, as well as providing valuable data on the potential for triticale pollen-mediated gene flow.

MATERIALS AND METHODS

Plant material

Plant materials used were as follows: triticale cultivar AC Alta (6x, ABR); triticale 89TT108 from CIMMYT which was synthesized from a wheat × rye cross and then selfed through five generations and confirmed to be $2n = 6x = 42$ (ABR) including 14 intact rye chromosomes (George Fedak, personal communication); common wheat cultivar AC Barrie (ABD); durum wheat cultivar Kyle (AB); and rye cultivar Rogo (R). Plants were grown in 1-gallon pots filled with soil-less Cornell mix (Boodley and Sheldrak, 1977) in a single greenhouse at AAFC Lethbridge, Alberta (49° 41' 51.91" N 112° 46' 24.82" W; elevation 909 m). Temperature was maintained at 21/18 °C and humidity was 40–50%. Photoperiod (18 h light/6 h dark) was provided by natural light supplemented with electronically determined amounts of artificial light to reduce crossing variability from September to June. Plants were staked at 3 weeks of age to prevent slumping and breaking of stems. Watering was carried out daily according to need and plants were fertilized every second week with liquid 20-20-20 fertilizer. Plants received a preventative propiconazole (Tilt®) and imidacloprid (Impower®) treatment at the 5–7 leaves stage to prevent leaf disease and insect damage.

Hybridization

Cultivar AC Alta and CIMMYT genotype 89TT108 (ABR) were crossed to Canadian cultivars of related species; AC Barrie (ABD), Kyle (AB), and Rogo (R). Similar numbers of reciprocal crosses were made with a target of 2000 emasculated and hand pollinated flowers (data not shown) for each intra- and inter-specific cross and its reciprocal cross. Inter-specific crosses among the wheat and rye species were made as well (Tab. 1). Emasculation was begun when plants were 8 weeks old

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(± 1 week) and/or just prior to anthesis: common wheat (Barrie) was emasculated when the spike was 4–7 cm out of boot; durum wheat (Kyle) at 6–9 cm; triticale (AC Alta) at 7–10 cm; triticale (89TT108) at 7–10 cm; spring rye (Rogo) at 3–5 cm. Following emasculation, 4–6 days were allowed for flowers to mature to the receptive stage where they begin to open. Scar tissue caused by the emasculation process was clipped to relieve tension on the flower and allow it to open fully. Flowers were pollinated with a spike of the desired male plant. Spikes for pollinating were collected at the following stages of development; common wheat when fresh yellow anthers were showing from centre flowers along 1/2 the length of the spike, durum wheat when pollen was showing along 3/4 of the length of spike and flowers had begun to swell slightly, triticale AC Alta when pollen was showing on no more than 1/3 the length of the spike; triticale 89TT108 when pollen was showing on only 6–8 flowers; rye when pollen was showing on 1/4 to 1/3 the length of the spike. Each week approximately 30 crosses were carried out between plants of the same cultivar to document manual pollination efficiency. Glassine bags (5 cm × 19 cm), surgical scissors, tweezers and # 1 paper clips were used for manual emasculation and pollination (Allen, 1980). In addition, emasculated spikes were bagged and not manually pollinated to document the frequency of selfing. Bags were removed 10 days after pollination. The spike was allowed to mature for 6 weeks and then dry off for 2 weeks, after which it was cut and allowed to dry for 1 week prior to threshing by hand. Seeds were collected at full maturity, counted, and weighed to determine the thousand kernel weight. Percentage outcrossing (*OC*) was determined using the following equation:

$$OC = \left[\frac{HS/F}{P} \right] \times 100$$

where HS = the number of F_1 hybrid seeds produced, F = the number of flowers emasculated and pollinated, and P is the percentage outcrossing of the female parent genotype when plants of this genotype were crossed to each other and was determined using the following equation, where S is the number of seeds produced:

$$P = \frac{S}{F}.$$

χ^2 analysis was used to determine if there was a significant effect of *OC* when triticale was the female parent compared to when triticale was the male parent. χ^2 analysis was also used to determine whether *OC* differed significantly in the intra- and inter-specific crosses when compared to the *OC* obtained when crosses were carried out between plants of a single triticale genotype. The number, quality, and thousand kernel weight of seeds were then recorded.

F_1 emergence and fertility

Sprouting resistance was overcome by placing F_1 hybrid seed in alternating refrigerator (4 °C) and room temperatures for 2 days at each temperature for 7 cycles. Seeds were placed in RootTrainers (Nursery Supplies Inc.) with one seed per cell planted at a uniform depth covered with 1 cm of cornell mix (Boodley and Sheldrak, 1977). Approximately 70 F_1 hybrid seeds were sown for each cross and its reciprocal where possible. Where crossability was low, fewer seeds were sown. Seeds were watered as required. The percentage emergence was determined as:

$$Emergence\ (\%) = \frac{F_1\ plants\ emerged}{seeds\ planted} \times 100.$$

At four weeks, plants were transferred into 1-gallon pots. When plants flowered, sterility was observed and recorded.

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Appendix 1

Quantifying crossability between triticale, wheat, durum wheat and rye

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Appendix 2. Transferability and Polymorphism of Rye and Wheat SSR Markers among Wheat, Rye and Triticale*

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To expedite the submission of this paper, the writing of this research paper was lead by Dr. Melissa Hillls. Vanessa Kavanagh established the methods, in collaboration with Drs. Harpinder Randhawa, Aakash Goyal, Francois Eudes and Linda Hall, conducated the research at AAFC Lethbridge and used the markers to complete the research described in **chapter 4. To ensure transparence of the process and provide recognition for the roles played by the research team, this research was placed in the appendix.*

Introduction

SSR (simple sequence repeat), or microsatellite, markers are abundant and highly polymorphic and are useful tools for assaying genetic variation, facilitating plant breeding, gene mapping and identification, and establishing genetic and evolutionary relationships between species (Kalia et al., 2011). The ability to adapt SSR markers developed in one species for applications in another has potential to hasten development of molecular maps, accelerate gene discovery and complement conventional breeding programs in minor crops (Varshney et al., 2010).

Triticale (x*Triticosecale* Wittmack) is a man-made inter-generic hybrid of wheat and rye. Triticale is being investigated as a platform for bioindustrial products and is amenable to genetic engineering (Fengying et al., 2010). Triticale is only grown on ~4.2 M ha worldwide (FAOSTAT, 2010), making it a minor cereal crop compared to wheat. Triticale generally displays the resistance to disease and abiotic stress observed in rye combined with the high yield potential and grain protein content observed in wheat (Oettler, 2005). Triticale is used primarily as an animal feed crop (reviewed by McGoverin et al., 2011); however, the strong agronomic qualities of triticale, in addition to its limited use in human food products, make it a promising platform for bioproducts through genetic engineering.

Triticale has a limited ability to outcross to wheat or rye (Hills et al., 2007). Differences in outcrossing rates were observed between different species and cultivars in greenhouse studies, but field studies are required to quantify the potential for pollen-mediated gene flow from triticale to wheat species. Identification of suitable phenotypic markers to visually identify hybrids is difficult, and such traits may be subject to incomplete penetrance or variable expression. The use of molecular markers in environmental biosafety evaluations could overcome this limitation.

The tribe Triticeae of the subfamily of grasses Pooideae includes many agronomically important species such as wheat, rye and barley. Wheat is grown on over 225 M ha worldwide (FAOSTAT, 2010). The majority of wheat

production is of common wheat (*Triticum aestivum*) which is a hexaploid wheat with the genome composition AABBDD and a total of 42 chromosomes. Durum wheat (*Triticum turgidum*) is a tetraploid with 28 chromosomes and the genome composition AABB. Rye (*Secale cereale*) is grown on over 6.5 M ha worldwide (FAOSTAT, 2010) and is a diploid with 14 chromosomes and the genome composition RR.

Primary triticales, created from inter-specific crosses between wheat and rye, suffer initially from genetic instability and reduced fertility (Oettler, 2005) and loss of entire chromosomes can occur (Dou et al., 2006; Ma and Gustafson, 2006; Ma and Gustafson, 2008). Stable triticales can be tetraploid, hexaploid or octoploid. Most triticale cultivars are hexaploid; created from crossing tetraploid wheat (AABB) to rye (RR) to synthesize the AABBRR genome, or produced spontaneously from chromosomal reductions in octoploid cultivars. Octoploid cultivars are created by crossing common wheat and rye to synthesize the AABBDDRR genome and reduction to a hexaploid genome will result in a mixture of R and D chromosomes remaining (AABBR/D) with some chromosomes being preferentially retained (Dou et al., 2006). Most commercial cultivars of triticale are further developed through crosses between triticale cultivars, or between triticale and wheat.

Molecular mapping efforts in wheat, rye and triticale reflect their economic importance. Considerable effort has been made to identify molecular markers in wheat and map their position in the genome for use in various

applications (Landjeva et al., 2007; Varshney et al., 2007). Recently, four independent genetic maps were combined to create a high density SSR consensus map of hexaploid wheat (Somers et al., 2004). SSR identification and mapping in rye is less advanced, but progress has been made (Bolibok et al., 2006; Bolibok-Bragoszewska et al., 2009; Hackauf and Wehling, 2002; Kofler et al., 2008; Korzun et al., 2001; Saal and Wricke, 1999). As a minor crop, there has been relatively little investment in molecular marker development in triticale, with notable exception (Badea et al., 2011). Transferability, SSR markers detectable in one species also detectable in a second, has been reported for wheat and rye SSRs, to each other, and to triticale (Kuleung et al. 2004, Kuleung et al. 2006). The ability to use wheat SSRs in triticale may accelerate the development of molecular maps in triticale for use in breeding programs and accelerate the improvement of key traits such as disease and insect resistance (Mergoum et al., 2009). In addition, SSR polymorphisms, detectable markers in one species or cultivar that produce different products in a second, can be used to differentiate cultivars (Kuleung et al., 2006, Vyhnanek et al., 2009, Mangini et al., 2010) and hybrids (Nair et al., 2006, Aitken et al., 2007, Asif et al., 2009).

Two common wheat cultivars, one durum wheat cultivar, one rye cultivar, and two triticale cultivars were screened with 235 hexaploid wheat and 27 rye SSR markers in order to quantify:

1) the transferability of molecular markers to each species and between cultivars;

2) the level of polymorphism between species (inter-specific polymorphism) and between two different cultivars of wheat and triticale (intra-specific polymorphism);

3) the number of alleles detected and polymorphic information content (PIC) of the SSR markers.

Materials and Methods

Plant Materials

Two common wheat cultivars (AC Barrie and AC Crystal), one durum wheat cultivar (AC Avonlea), one rye cultivar (Rogo), and two triticale cultivars (AC Alta and blue aleurone triticale) were selected. AC Barrie is a hard red spring wheat (McCaig et al., 1996); AC Crystal is a Canada prairie spring wheat (Fernandez et al., 1998); AC Avonlea is a durum wheat (Clarke et al., 1998) and AC Alta is a spring triticale (McLeod et al., 1996). These four cultivars were developed at the Agriculture and Agri-Food Canada Research Centre, Swift Current, Saskatchewan. The blue aleurone line (BC_4F_4), The pollen donor was a blue aleurone line (BC_4F_4), from a cross between AC Alta/Purendo-38 with 4 subsequent backcrosses to AC Alta (BA; developed at Lethbridge by Agriculture and Agri-Food Canada). Purendo-38 is an experimental wheat line containing a blue aleurone as a visual marker (Abdel-Aal and Hucl, 2003).

SSR Markers

A total of 235 published genomic SSR markers developed for use in hexaploid wheat were selected to screen for polymorphism between the different species and cultivars. Marker selection was based on genome mapping position and at least four markers were tested per chromosome (two for each arm) to provide greater genome coverage. In total, 188 BARC (USDA-ARS Beltsville Agricultural Research Center, USA, (Song et al., 2002; Song et al., 2005) common wheat SSR markers were screened. Twenty-three GWM (Gatersleben wheat microsatellite) common wheat SSR markers were screened. These markers were developed at IPK Gatersleben (Institute of Plant Genetics, Germany; Roder et al., 1998). Six WMC (wheat microsatellite consortium) common wheat markers were screened. The markers were developed through an international collaboration out of the Long Ashton Research Station in England (Isaac, 2004). Fourteen CFD and four CFA markers were screened. These markers were developed at IRNA Blermont Reffand (France, Guyomarc'h et al., 2002; Sourdille et al., 2003). The CFD markers were originally developed using *Aegilops tauschii* (the D-genome of common wheat) genomic library and then screening the markers using hexaploid wheat to identify polymorphic wheat markers. A high density microsatellite consensus map for common wheat was recently published that incorporated CFA, CFD, GWM and BARC primers (Somers et al., 2004).

All 27 rye markers tested were *Secale cereale* microsatellite (SCM) markers derived from publicly available rye cDNA sequences and developed by the Institute of Agricultural Crops (IAC) from the Federal Centre for Breeding

Research on Cultivated Plants (Hackauf and Wehling, 2002; Saal and Wricke, 1999).

DNA Extraction and SSR Marker Analysis

DNA was extracted from leaf tissue as previously described using a modified CTAB method with phenol-chloroform purification (Randhawa et al., 2009). The extracted DNA was dissolved in 500 µl TE buffer, and stored at −20°C.

Forward primers incorporated a 5' M13 tail (CACGACGTTGTAAAACGAC) as did three fluorescently labeled (6-FAM, VIC and PET) universal primers (Invitrogen, Burlington ON, Canada) for detection with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCR was performed in a PX2 Thermal cycler (Thermo Fisher Scientific Inc. Waltham, MA, USA). The PCR reactions contained 25–100 ng of genomic DNA, 1· PCR buffer, 2.5 mM dNTPs, 50 mM $MgCl_2$, 1 pmol of dye M-13 forward tail primer, 1 pmol each of forward and reverse primers and 0.1 U Taq DNA polymerase (New England Biolabs, Beverly, MA, USA) in a 10 µL volume. The PCR program was run with an initial denaturation temperature of 94°C for 3 min, followed by 35-40 cycles of denaturation at 94°C for 1 min, annealing for 1 min at either 50, 55 or 60°C depending on the individual primers, 2 min extension at 72°C and a final extension for 7 min at 72°C. Amplification was confirmed by electrophoresis on a 2% (w/v) agarose gel in 1.0×TBE buffer. Dye-labeled PCR products from the 6 cultivars were triplexed and analyzed on an automatic capillary array based ABI 3100-Avant Genetic Analyzer. The product was diluted

with TE buffer at 1/10 before loading and mixed with 9.0 µl of the loading dye Hi-Di formamide and 0.05 µl of Liz500 size standard (Applied Biosystems), denatured at 94°C for 5 min and chilled on ice for 5 min. GeneScan® and Genotyper® Software (Applied Biosystems) used to extract the data. Markers indicating polymorphism between species were retested to replicate results and reduce error.

Data Analysis

Polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a population. This is determined with consideration of the number of detectable alleles and the distribution of their frequency for a specific marker. PIC for each SSR marker was determined using the equation:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the jth allele for the ith marker summed over n alleles (Anderson et al., 1993). Null alleles were not included.

The percentage transferability between cultivars was calculated as the number of SSR markers that produced an amplified product in both of the cultivars being compared, expressed as a percentage of the total 218 markers. The transferability of the 218 markers to each species was assessed by genome (A, B and D). Genome transferability was calculated as the number of markers for a particular genome that produced an amplified product, expressed as a percentage

of the total number of markers screened for that genome. Markers that represented more than one genome linkage group were not included in the analysis.

The percentage polymorphism between cultivars was calculated as the number of polymorphic markers expressed as a percentage of the total markers that produced an amplified product in both cultivars being compared. Markers that produced a product in only one of the two cultivars were not included in the pair-wise comparison.

Results and Discussion

Six cultivars representing four species were screened with 235 hexaploid wheat SSRs and 218 of these produced an amplified product in at least one of the six cultivars. High levels of transferability were observed (Table A-1). Ninety-five and 96% of the markers produced an amplified product in each of the two common wheat cultivars tested, 85% in the durum wheat cultivars, 87% and 85% in the two triticale cultivars and 48% in the rye.

The observation that 48% of the wheat markers were transferable to rye reflects the common evolutionary origin and genome conservation of wheat and rye. Of the species tested, rye is the most distantly related to wheat, reflected by the lower marker transferability observed. Wheat SSRs have been previously shown to be transferable to rye (Fu et al., 2010; Kuleung et al., 2004; Roder et al., 1995; Zhang et al., 2007). Roder et al. (1995) tested 15 markers on four accessions of rye and nine produced amplification products. In a larger study, 111 wheat SSR markers were tested on five lines of rye and observed 17% of the

markers produced an amplified product in at least three of the five lines (Kuleung et al. 2004). Zhang et al. (2007) found transferability of wheat markers to rye to be 73%. This last study used EST-SSRs, rather than genomic SSRs, which have been found to be more highly conserved, but less polymorphic, than genomic SSRs (Gupta et al., 2003; Zhang et al., 2007).

High levels of transferability of the wheat markers to the two triticale cultivars were exhibited (87 and 85%). This result supports previously published data demonstrating that wheat markers are suitable for use in triticale. As wheat is a parental species of triticale, it was expected that wheat SSRs would show a high degree of transferability to triticale. Wheat SSRs have been previously used in studies on triticale, but few studies have specifically reported the percentage of transferability observed. da Costa et al. (2007) demonstrated that wheat SSRs could be used to estimate the molecular diversity of 54 genotypes of triticale. Forty-two SSRs were screened achieving 71.42% transferability. In another study, five lines of triticale were screened with 148 wheat markers and observed 58% transferability to triticale (Kuleung et al. 2006). In this study markers were only considered transferable if they produced an amplified product in at least three of the five lines tested which accounts for the lower transferability reported.

The transferability of markers for genomes A, B and D were compared (Table A-2). Apart from rye, which exhibited lower levels of transferability (43 – 55%), the lowest levels of transferability were observed for the D genome markers in durum wheat and triticale (70-77%). Durum wheat (AABB) lacks a D

genome, therefore, the 70% of D-genome markers that produced amplified products in the durum wheat reflect sequence conservation between the D genome of common wheat and the A and B genomes of durum wheat. Tams et al. (2004) observed very low levels of transferability of wheat D genome markers to triticale. Only three amplified products were detected from the 39 D genome specific markers screened. The authors attributed this result to the use of winter triticale cultivars which are assumed to have AABBRR genomes without D chromosome substitutions. Dou et al. (2006) analysed hexaploid cultivars of triticale occurring spontaneously from octoploid cultivars and observed preferential loss of chromosomes from the wheat D genome with the exception of chromosome 2D. It is possible that AC Alta and Blue Aleurone triticale have similarly lost some of the D-genome chromosomes accounting for the lower transferability observed for this genome. Because 50% of the D genome markers also amplified products in rye it is not possible to conclude whether successful D genome amplification in triticale was associated with the presence of D genome chromosomes, or due to amplification of D genome markers on rye (R) chromosomes due to sequence conservation between the related species. Caution should be exercised when using information about the genomic position of markers in hexaploid wheat to prescribe a markers genomic position in the triticale genome or in other related species.

Wheat SSR marker transferability between the four different species was quantified (Table A-3). 83 – 89% of markers were transferable between common wheat, durum wheat and triticale. The lowest levels of wheat SSR transferability

were observed between rye and the other species (51 – 62%). Although wheat SSR transferability between rye and common wheat was low (51 and 52%), the percentage polymorphism of the transferable markers was high (63 and 57%; Table A-4). Inter-specific polymorphism between the four species ranged from 40 – 63%. The number of alleles and polymorphic information content (PIC) for each wheat marker was determined (Table A-7, Supplemental Table A-1). The mean number of alleles detected was 2.2 and ranged from 1 to 6. The PIC values ranged from 0 – 0.8 with a mean of 0.4.

A total of 27 rye SSR markers were screened and 56 – 76% of these produced an amplified product in the wheat and triticale cultivars tested (Table A-1). Inter-specific transferability of the rye SSR markers between common wheat, durum wheat and triticale ranged from 56 – 86% (Table A-5). Inter-specific polymorphism between each species ranged from 53% - 100% (Table A-6). All of the markers (100%) that amplified a product in both rye and triticale were polymorphic. The number of alleles and polymorphic information content (PIC) for each rye marker was determined (Table A-7, Supplemental Table A-2). The mean number of alleles detected was 3.1 and ranged from 1 to 5. The PIC values ranged from 0 – 0.8 with a mean of 0.5.

High levels of transferability and polymorphism suggest that wheat and rye markers may be useful for research and breeding efforts in triticale and rye and in distinguishing between triticale and its parent species in outcrossing studies. Molecular markers have been used for assessing hybrid seed purity

(Astarini et al., 2008; Liu et al., 2007; Naresh et al., 2009; Pallavi et al., 2011) and differentiating between selfed and hybrid seed (Gomez et al., 2008). In a study on *Haliotis* (Abalone) species, SSRs were used to identify interspecific hybrids and distinguish them from parental species (Lafarga de la Cruz et al., 2010; Luo et al., 2010). Use of molecular markers to identify hybridization has potential in an environmental risk assessment where the potential for pollen mediated gene flow must be quantified in order to predict the potential for gene flow from genetically engineered plants to conventional crops or related species. Currently, these studies rely on suitable phenotypic markers which can be difficult to identify and results from such trials may be impacted by variable expressivity and reduced penetrance of the identifying trait.

In conclusion, wheat and rye genomic SSR markers demonstrated a high level of transferability and polymorphism in our analyses. Wheat and rye genomic SSR markers are suitable for use in triticale studies. The levels of transferability observed for the wheat SSR markers in rye suggest that information regarding the genomic position of particular markers in wheat should not be relied on for conclusions regarding the genome position, or genome specific transferability, of these markers in triticale.

Table A-1. Amplification of wheat and rye SSR markers in common and durum wheat, rye and triticale.

SSR donor Species	Total markers	# Amplified Markers (% amplification)					
		Common wheat		Durum wheat	Rye	Triticale	
		AC Barrie	AC Crystal	AC Avonlea	Rogo	AC Alta	BA
Wheat	218	208	209	186	105	190	186
		(95%)	(96%)	(85%)	(48%)	(87%)	(85%)
Rye	27	17	19	22	27	20	15
		(63%)	(70%)	(76%)	(100%)	(74%)	(56%)

Table A-2. Percentage transferability of wheat SSR markers within the A, B and D genomes*.

Genome	Total markers	Common wheat		Durum wheat	Rye	Triticale	
		AC Barrie	AC Crystal	AC Avonlea	Rogo	AC Alta	BA
A	82	93	99	88	55	90	88
B	67	96	91	96	43	94	88
D	61	98	97	70	44	77	74

*****markers that detected alleles on more than one chromosome were not assigned to a specific genome.

Table A-3. Percentage of wheat SSR marker transferability observed between cultivars.

Cultivar	Common wheat		Durum wheat	Rye	Triticale	
	AC Barrie	AC Crystal	AC Avonlea	Rogo	AC Alta	BA
AC Barrie	-	94	83	51	87	84
AC Crystal	94	-	85	52	87	85
AC Avonlea	83	85	-	62	89	86
Rogo	51	52	62	-	58	61
AC Alta	87	87	89	58	-	88
BA	84	85	86	61	88	-

Table A-4. Percentage of polymorphism for wheat SSR markers observed between cultivars.

Cultivar	Common wheat		Durum wheat	Rye	Triticale	
	AC Barrie	AC Crystal	AC Avonlea	Rogo	AC Alta	BA
AC Barrie	-	32	54	61	53	55
AC Crystal	32	-	47	57	52	48
AC Avonlea	54	47	-	47	50	49
Rogo	61	57	47	-	40	40
AC Alta	53	52	50	40	-	14
BA	55	48	49	40	14	-

Table A-5. Percentage of rye SSR marker transferability observed between cultilvars.

	Common wheat		Durum wheat	Rye	Triticale	
Cultivar	**AC Barrie**	**AC Crystal**	**AC Avonlea**	**Rogo**	**AC Alta**	**BA**
AC Barrie	-	89	77	63	77	70
AC Crystal	89	-	86	70	70	62
AC Avonlea	77	86	-	81	86	70
Rogo	63	70	81	-	74	56
AC Alta	77	70	86	74	-	77
BA	70	62	70	56	77	-

Table A-6. Percentage of polymorphism for rye SSR markers observed between cultivars.

	Common wheat		Durum wheat	Rye	Triticale	
Cultivar	**AC Barrie**	**AC Crystal**	**AC Avonlea**	**Rogo**	**AC Alta**	**BA**
AC Barrie	-	24	59	94	100	93
AC Crystal	24	-	53	95	94	92
AC Avonlea	59	53	-	95	94	93
Rogo	94	95	95	-	100	100
AC Alta	100	94	94	100	-	40
BA	93	92	93	100	40	-

Table A-7. Number of alleles and polymorphic information content (PIC) of SSR markers.

		Number of Alleles			PIC		
Markers	**Total Markers**	**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**
Wheat SSRs	218	2.2	2	1- 6	0.4	0.44	0 – 0.80
Rye SSRs	27	3.1	3	1 - 5	0.5	0.63	0 – 0.80

Supplemental Table A-1. Allele number and polymorphic information content of wheat SSR markers in common wheat, durum wheat, triticale and rye. Asterisk indicates marker used in Tier II putative hybrid testing in **chapter 4**.

Marker	Chr	# Alleles	PIC
BARC1	5A	1	0.00
BARC3	6A	1	0.00
BARC5	2A	2	0.48
BARC6	3D	1	0.00
BARC7	2B	2	0.48
BARC8	3D	4	0.72
BARC10	2B, 4B	4	0.72
BARC11	2D	1	0.00
BARC12	3A	1	0.00
BARC13	2B	2	0.48
BARC15	2A	1	0.00
BARC17*	1A	4	0.72
BARC19	3A	5	0.78
BARC21	5B	1	0.00
BARC23	6D	2	0.32
BARC24	6B	1	0.00
BARC25	3A	1	0.00
BARC26	7D	1	0.00
BARC28*	1A	4	0.72
BARC29*	7A	4	0.72
BARC32	5B	2	0.63
BARC37	6A	1	0.00
BARC40	5A	2	0.28
BARC42	3D	1	0.00
BARC44	5D	3	0.61
BARC45	3A	1	0.00
BARC50	7B	1	0.00
BARC51*	3A	2	0.48
BARC52	4A	2	0.28
BARC53	7D	1	0.00
BARC54*	3A	2	0.50
BARC56	5A	2	0.68
BARC57	3A	1	0.00
BARC59*	5B	3	0.61
BARC60	4B	1	0.00
BARC61	1B	2	0.68
BARC62*	1D	4	0.81
BARC64	7A	1	0.00
BARC66*	1D	3	0.39
BARC67	3A	1	0.00
BARC68	3B	2	0.28
BARC70	4A	4	0.67
BARC71	3D	1	0.00
BARC72	7B	2	0.68
BARC73	3B	2	0.44
BARC74	5B	1	0.00
BARC75	3B	1	0.00
BARC77	3B	3	0.44
BARC78	4A	3	0.50
BARC79	6B	2	0.68
BARC80	1B	1	0.00
BARC81	1B	4	0.72
BARC82	7B	3	0.50
BARC83	1A	4	0.72
BARC84	3B	3	0.56
BARC85	7B	1	0.00
BARC86	3A	2	0.68
BARC87	3B	2	0.32
BARC88	5B	1	0.00
BARC89	5B	1	0.00
BARC90	7B	2	0.32

BARC92	5A	1	0.00
BARC93	4D, 5D	2	0.48
BARC94*	5A	4	0.72
BARC95	2D	1	0.00
BARC96	6D	1	0.00
BARC98	4D	2	0.44
BARC99	1D	1	0.00
BARC101	2B	4	0.75
BARC105	7D	2	0.50
BARC106	4A	1	0.00
BARC107	6A	1	0.00
BARC108	7A	2	0.44
BARC109*	5B	2	0.52
BARC110	5D	1	0.00
BARC111*	7D	2	0.44
BARC117	5A	1	0.00
BARC119	1A, 1D	2	0.32
BARC122	5A	3	0.44
BARC123	6D	1	0.00
BARC126*	7D	3	0.61
BARC127	7A	2	0.28
BARC130	5D	1	0.00
BARC134	6B	1	0.00
BARC137	1B	2	0.32
BARC138	4A	4	0.67
BARC139	3B	1	0.00
BARC140	5B	2	0.44
BARC141	5A	2	0.32
BARC142*	5B	5	0.78
BARC143	5D	1	0.00
BARC144	5D	2	0.38
BARC146	6D	3	0.36
BARC149*	1D	2	0.48
BARC151*	5A	4	0.72
BARC152*	1D	1	0.00
BARC153	4A	3	0.67
BARC159	2D	3	0.64
BARC162	1A, 1D	1	0.00
BARC164	3B	2	0.38
BARC167*	2B	2	0.48
BARC168	2D	1	0.00
BARC169	1D	3	0.61
BARC170*	4A	3	0.56
BARC171	6A	2	0.44
BARC172	7D	2	0.38
BARC173	6D	3	0.63
BARC174*	1B	4	0.72
BARC175	6D	1	0.00
BARC177	5D	1	0.00
BARC178	6B	2	0.38
BARC180	5A, 6B	4	0.72
BARC181	1B	3	0.64
BARC182*	7B	2	0.44
BARC183	6D	2	0.50
BARC184*	4A	4	0.72
BARC186	5A	1	0.00
BARC187	1B	3	0.67
BARC188	1B	3	0.44
BARC192*	7A	3	0.63
BARC194	1B	1	0.00
BARC196	6D	1	0.00
BARC197	3A	1	0.00
BARC199*	4B	2	0.48
BARC200*	2B	3	0.61
BARC201	2A	3	0.56
BARC202	6D	2	0.38
BARC203	3B	1	0.00
BARC204	6A, 6D	1	0.00

BARC206	4A	2	0.32
BARC207	5A	2	0.44
BARC208	2A	2	0.44
BARC210	1D, 2B	4	0.67
BARC211	6B	1	0.00
BARC212	2A	2	0.38
BARC214	7D	3	0.61
BARC216	5B	3	0.64
BARC220	2A	2	0.38
BARC222*	7A	3	0.64
BARC223	6B	2	0.28
BARC225	4D	1	0.00
BARC228	2D	1	0.00
BARC229	1D	1	0.00
BARC232*	5D	2	0.48
BARC240*	1B	3	0.63
BARC243	5B	2	0.38
BARC263	1A	1	0.00
BARC267	7B	1	0.00
BARC1015	1B	1	0.00
BARC1022	1A	1	0.00
BARC1025	7A	1	0.00
BARC1030	6D	1	0.00
BARC1032	5B	1	0.00
BARC1033	7D	2	0.32
BARC1040	3D	1	0.00
BARC1044	3B	2	0.44
BARC1045*	4B	3	0.64
BARC1046	7D	1	0.00
BARC1047	4A	2	0.50
BARC1048	1A	1	0.00
BARC1052	4A	1	0.00
BARC1060	3A	1	0.00
BARC1061	5B	1	0.00
BARC1069*	4D	2	0.48
BARC1073*	7B	4	0.72
BARC1075	7D	1	0.00
BARC1077*	3B	2	0.48
BARC1088*	7A	2	0.28
BARC1095	1A, 2D	2	0.50
BARC1096	4B	2	0.32
BARC1099	3A	1	0.00
CFA2129	1A	5	0.78
CFA2134*	3A	3	0.61
CFA2170	3B	3	0.61
CFA2256	4A	1	0.00
CFD2	1B	5	0.78
CFD15	1D	2	0.32
CFD23*	4D	4	0.72
CFD28	3B	2	0.44
CFD36*	2A	5	0.78
CFD39	4D	4	0.72
CFD65	1D	4	0.72
CFD79*	3A	4	0.72
CFD84*	4D	3	0.64
CFD88*	4A	6	0.83
CFD106*	4D	2	0.48
CFD168*	2A	3	0.56
CFD257	4A	2	0.28
CFD282	1D	2	0.63
GWM16*	2B	5	0.78
GWM18	1B	2	0.32
GWM47	2A	1	0.00
GWM95	2A	1	0.00
GWM99	1A	1	0.00
GWM102	2D	1	0.00
GWM106*	1D	2	0.44
GWM120*	2B	4	0.67

GWM124	1B	1	0.00
GWM148	2B	1	0.00
GWM157*	2D	3	0.61
GWM165	4A	1	0.00
GWM191*	1D	3	0.50
GWM210*	2D	3	0.50
GWM232	1D	1	0.00
GWM257*	2B	4	0.67
GWM339*	2A	3	0.61
GWM349*	2D	4	0.67
GWM359	2A	1	0.00
GWM372*	2A	3	0.50
GWM382	2A	4	0.75
GWM497*	1A	4	0.67
GWM512	2A	1	0.00
WMC201*	6A	5	0.80
WMC254*	6A	4	0.67
WMC398	6A	4	0.67
WMC417	6A	4	0.67
WMC553	6A	4	0.67
WMC580*	6A	5	0.80

Supplemental Table A-2. Allele number and polymorphic information content of rye SSR markers in common wheat, durum wheat, triticale and rye.

Marker	# Alleles	PIC
SCM9	4	0.72
SCM28	4	0.72
SCM39	5	0.78
SCM43	4	0.72
SCM65	4	0.67
SCM86	5	0.78
SCM101	3	0.63
SCM102	2	0.38
SCM109	2	0.28
SCM180	1	0.00
SCM242	2	0.44
SCM268	1	0.00
SCM304	4	0.81
SCM306	2	0.44
SCM307	4	0.72
SCM6297	4	0.63
SCM6423	4	0.63
SCM6538	1	0.00
SCM6665	1	0.00
SCM6690	3	0.67
SCM6707	4	0.67
SCM6813	4	0.72
SCM6939	5	0.80
SCM7032	3	0.56
SCM7048	3	0.63
SCM7364	1	0.00
SCM8566	3	0.64

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M.Sc. St. Mary's University, Department of Biology, Halifax, Nova Scotia, Canada (May 2005-May 2008), Assessment of the symbiotic efficiency of *Bradyrhizobium japonicum* L. for the development of a soybean inoculant for western Canada.

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Employment

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Laboratory Instructor – Weed Science **2008, 2010**

Prepared plant materials, lectures, and examinations for labs.

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Term Instructor – Introductory Plant Biology **2009**

Responsible for lectures and examinations, supervised field trip.

Saint Mary's University, Halifax, Nova Scotia Canada

Teaching Assistant – Cell biology, General plant biology, **2005 - 2007**

General Biology, Primitive Plants

Created materials for labs, led and supervised laboratory sessions.

Saint Mary's University, Halifax, Nova Scotia Canada

Research Assistant **2005**

Employed various laboratory techniques for microbial isolation and culturing, performed greenhouse trials, assisted in different molecular techniques.

Dalhousie University, Halifax, Nova Scotia Canada

Assistant **2004 – 2005**

Assisted in herbarium curation, including acqusition and new speicimen processing. Performed vegetation surveys, developed botanically related activities for various organizations, helped to develop course materials for plant science classes.

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Field Assistant **2003 – 2004**

Performed quadrat and soil sampling (also along transects), plant identification, interpretation of ecological habitats across different environmental settings.

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Laboratory Instructor – Flora of Nova Scotia **2003-2005**

Created lectures and examinations relevant to Nova Scotia major habitat niches. Prepared and supervised field trips and labs and managed herbarium collections.

Academic Awards

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Publications

Kavanagh, V.B., M.J. Hills, F. Eudes, K. Topinka, R.-C. Yang and L. M. Hall. 2011. Pollen-mediated gene flow in triticale (x*Triticosecale* Wittmack). (Submitted).

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