Identification and Validation of Genomic Regions Associated with Pre-Harvest Sprouting Resistance in White-Grained Wheat (*Triticum aestivum* L.)

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ABSTRACT

Pre-harvest sprouting (PHS) in bread wheat (*Triticum aestivu*m L.) is one of the major abiotic constraints influencing the production of high quality grain. The flour milled from sprouted wheat grains has increased α-amylase activity as compared to non-sprouted grain. PHS negatively affects the properties of flour with deleterious effects on bread and noodle quality. White-grained wheat is generally more susceptible to PHS damage than red-grained wheat. The objectives of this study were to identify a suitable method for phenotyping PHS resistance and to identify PHS resistance genomic regions and markers that could be used for marker-assisted selection in wheat improvement programs. A doubled haploid (DH) mapping population from a cross between two white-grained spring wheat genotypes, Argent (non-dormant) and W98616 (dormant) was used in this study. Forty DH lines (20 dormant and 20 non-dormant) were evaluated for germination frequency, Falling Number, and α-amylase activity in dry and water-imbibed seeds and spikes. The germination test was the most reliable method for measurement of PHS resistance, whereas the Falling Number and α-amylase activity in dry harvested seeds could not be correlated to dormancy levels. However, a positive association (r = 0.60***) was detected between germination frequency and α-amylase activity in imbibed seeds. To identify the genomic regions associated with PHS resistance, a genetic linkage map with a total genome coverage of 2,577 cM was developed. The map was constructed from 913 scored markers (356 SSR, 290 AFLP, 258 DArT and 9 EST) with an average marker density of 3.7 cM/marker. Five genomic regions on chromosomes 1A, 3A, 4A, 7A and 7D were associated with PHS resistance by interval mapping and all regions were contributed by the dormant parent W98616. A total of 60 Canadian wheat cultivars and experimental lines were screened with three SSR markers, DuPw004, barc170 and wmc650, located under the major quantitative trait locus (QTL) on chromosome 4A. The SSR markers explained 60-75% of the total variation in germination frequency among different wheat genotypes. By using the DuPw004 marker in marker-assisted back crossing, the population size in the BC_1F_1 and BC_2F_1 generations were reduced by 41% and 59%, respectively. Thus, the 4A QTL markers have been proven useful for marker-assisted selection of PHS resistance for wheat improvement.

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LIST OF ABBREVATIONS

ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
bp	base pair
cM	centiMorgan
DArT	Diversity Array Technology
DH	Doubled haploid
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EST	Expressed sequence tag
GA	Gibberellic acid
LOD	Logarithm of odds
MAS	Marker-assisted selection
Mb	Megabase pair
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PHS	Pre-harvest sprouting
QTL	Quantitative trait locus
SSCP	Single strand conformation polymorphism
SSR	Simple sequence repeat

CHAPTER 1
INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple crops world-wide, with a total production of over 600 million tonnes annually. The wheat crop is exposed to several biotic and abiotic factors that contribute to losses in yield and grain quality. Pre- harvest sprouting (PHS) is one of the major abiotic constraints influencing the production of high quality grain. PHS is defined as in-spike germination of physiologically mature grain during unfavorable harvest conditions and occurs when harvest time coincides with relatively high humidity in the field due to untimely rainfall. It is a widespread response to wet harvest conditions and occurs in areas as diverse as Canada, USA, Australia, Europe, China and Japan. PHS reduces the functional quality of wheat flour and thus affects the economic value of the grain. In Western Canada during 1978-1988, downgrading due to PHS damage occurred in four years and was estimated to cost about US$100 million in each of those years (Derera 1990). The flour milled from sprouted wheat grain shows an elevated level of α-amylase activity, which negatively affects flour functionality with deleterious effects on bread and noodle quality (Edwards et al. 1989; Hatcher and Symons 2000; Mares et al. 2004). The flour from sprouted wheat produces sticky dough, and bread loaves with large holes, sticky crumb and dark-coloured crusts (Mares et al. 2004). Noodles made from sprouted wheat flour show higher levels of discoloration (spots) as compared to noodles made from sound flour (Hatcher and Symons 2000).

There is worldwide demand for white wheat because consumers in general prefer the taste and appearance of food products prepared from white wheat. The international market, particularly countries in Southeast Asia and the Middle East, often prefer white-grained wheat over red-grained wheat (Crosbie et al. 1998; Ambalamaatil et al. 2002). With fewer phenolic compounds and tannins in the bran, white wheat imparts a less bitter

taste and a more favorable appearance to the final products than red wheat. The demand for white wheat by the milling and baking industry has also increased in North America (Sosland 2005). Hard white wheat has a flour yield advantage over hard red wheat, as white wheat can be milled closer to the bran layer without negatively impacting flour colour or flavour. Canada Western Hard White Spring (CWHWS) wheat with a light-colored seed coat had improved milling yields (up to 2.6% higher) as compared to No.1 grade Canada Western Red Spring (CWRS) wheat (Ambalamaatil et al. 2006). Furthermore, flours from white wheat may be higher in protein and resulting in substantially lighter-colored end-products than CWRS wheat flours (Ambalamaatil et al. 2006). Australia is currently the largest producer of hard white wheat (Teetaert 2000), but if Canadian hard white wheat production increases to a significant level, then Canada may be in a position to maintain or capture a larger share of the global wheat market (Matus-Cádiz et al. 2003).

Recently efforts have been intensified to develop cultivars of hard white wheat suited for domestic and international markets, but PHS is a major constraint for white wheat cultivar improvement. White-grained wheat, on average, is more susceptible to sprouting than red-grained wheat, although both wheat groups vary in this respect (Bassoi and Flintham 2005). Red grain colour has long been recognised as a genetic marker for PHS resistance (Nilsson-Ehle 1914). However, it is now evident that red colour *per se* is not adequate to guarantee dormancy (Flintham et al. 1999). Breeding for PHS resistance based on conventional phenotypic evaluation is difficult because PHS is expressed as a quantitative character that is influenced by the environment as well as by genotype x environment interactions. Further, the PHS phenotype is complex in that genes involved may be expressed in one of three distinct tissues; the maternal plant, the endosperm, and/or the embryo, of which the latter two belong genetically to the next generation (Gale 1989). PHS is traditionally assayed by a germination test using threshed seeds or intact spikes that may be subjected to natural or artificial weathering (Mares 1989). Other indirect methods of PHS measurement are based on determination of α-amylase activity or Hagberg Falling Number (Hagberg 1960). Therefore, one objective of this study was to compare the different strategies to phenotype PHS in order to find a reliable, reproducible and simple method to determine PHS resistance.

The advent of environmentally insensitive DNA-based molecular markers during the last two decades has revolutionized the genetic analysis of quantitative traits. The DNA-based markers are not influenced by environmental factors and/or the developmental stage of the plant, and thus represent a promising, environment-insensitive tool for selecting genotypes with increased PHS resistance. QTL mapping requires the collection of genotypic (molecular marker) and phenotypic data from a segregating population, followed by statistical analysis to reveal all possible genetic loci where allelic variation correlates with the phenotype. The development of genetic linkage maps is a pre-requisite for dissection of complex traits such as PHS resistance through QTL analysis. Several detailed genetic maps based on DNA-based markers have been reported in wheat (Röder et al. 1998; Paillard et al. 2003; Sourdille et al. 2003; Somers et al. 2004; Quarrie et al. 2005; Akbari et al. 2006; Semagn et al. 2006; Torada et al. 2006; Båga et al. 2007; http://wheat.pw.usda.gov/ggpages/map_summary.html). Several quantitative trait loci (QTL) or genomic regions affecting PHS resistance or seed dormancy in wheat have been identified in different gene pools via linkage to molecular markers. In bread wheat, 20 chromosomes, with the solitary exception of chromosome 1D, have been reported to carry QTL/genes for PHS or dormancy (Anderson et al. 1993; Roy et al. 1999; Zanetti et al. 2000; Kato et al. 2001; Mares and Mrva 2001; Flintham et al, 2002; Groos et al. 2002; Mares et al. 2002; Osa et al. 2003; Kulwal et al. 2005; Mares et al. 2005; Mori et al. 2005; Tan et al. 2006). These large numbers of QTL suggest a complex trait controlled by numerous genes that are influenced by environmental conditions and genetic background. However, homoeologous chromosome group 3 and chromosome 4A carry major loci for PHS resistance as revealed in several studies (Mares and Mrva 2001; Groos et al. 2002; Osa et al. 2003; Kulwal et al. 2005; Mares et al. 2005; Mori et al. 2005; Tan et al. 2006). The majority of earlier studies were based on red-grained wheat, except those reported by Anderson et al. (1993), Mares and Mrva (2001) Mares et al. (2002), Mares et al. (2005) and Tan et al. (2006), which were based on white-grained wheat. The objective of this study was to construct a linkage map in a doubled haploid mapping population from a cross between two white-grained spring wheat genotypes, Argent (non-dormant) and W98616 (dormant), and to identify the genomic regions associated with PHS resistance in white-grained bread wheat in order to develop

molecular markers suitable for marker-assisted selection in wheat breeding programs. This research was carried out with the following objectives:

1. Comparison of different phenotyping methods for PHS resistance in white-grained wheat.
2. Identification of genomic regions associated with PHS resistance in white-grained wheat.
3. Validation of putative QTL for PHS resistance in different genetic backgrounds suitable for marker-assisted selection.

CHAPTER 2
LITERATURE REVIEW

2.1 Wheat: Major source of energy in the human diet

Wheat has accompanied humans since 3,000 to 4,000 BC. It has evolved in part by nature and in part by human manipulation from its primitive form (einkorn wheat) into the presently main cultivated species; bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*). Its wide adaptation to diverse environmental conditions, along with its unique characteristic of possessing a viscoelastic complex of storage proteins, glutenins and gliadins, which in dough form gluten, are the main factors that make wheat the most important food crop in the world (Pena 2002). Wheat is a staple food for nearly 35% of the world population (Mujeeb-Kazi and Villareal 2002), with a total production of over 600 million tonnes annually. One-fifth of the calories consumed by humans are derived from wheat products, which provide 521 and 652 calories per capita daily world-wide and in Canada, respectively (FAO Staistical Yearbook 2005-2006).

2.2 Wheat evolution and domestication

Bread wheat (*Trticum aestivum* L.), is an allohexaploid (2n = 6x = 42, AABBDD genomes) plant and has a genome size of 16,700 Mb/1C (Bennet et al. 2000) with about 90% repetitive DNA (Li et al. 2004). It originated from the hybridization of three different diploid progenitors belonging to the *Triticum* and *Aegilops* genera. The first step was the hybridization between *Triticum urartu* Thum. Ex Gandil. (AA genome) and *Aegilops speltoides* (Tausch) Gren. or a closely related species (BB genome). The result of this hybridization was tetraploid wheat, *Triticum turgidum* (AABB). Several distinct groups of this species exist, of which, *T. turgidum* var. *dicoccoides*, is believed to be most primitive type. One derivative of *T. turgidum* var. *dicoccoides* is *T. turgidum* var. *dicocccon*, which is the most likely progenitor of hexaploid bread wheat as a result of

hybridization with *Aegilops tauschii* Cross. (DD genome) (Feldman et al. 1995; Talbert et al. 1998).

The archeological records suggest that *T. aestivum* originated ~8000 years ago (Nesbitt and Samuel 1996), with the Fertile Crescent considered to be the birthplace. The principal area of the origin of *T. aestivum* is Armenia in Transcaucasia, but the southwest coastal area of the Caspian Sea in Iran and a corridor between the two areas may have played a role as well (Dvorak et al. 1998). In this region, *Aegilops tauschii* var. *strangulata* is predominant and may have hybridized with cultivated emmer to produce *T. aestivum*. Bread wheat has no wild hexaploid progenitor in nature and therefore it is a farming-associated natural hybrid that has since become the world's leading crop.

As hexaploid wheat is a relatively young species, a low level of genetic variation may be expected. However, wheat exhibits abundant genetic variation for traits such as winter versus spring growth habit, response to day length, cold hardiness, disease and insect resistance, and other important characteristics. Because of this variation and its wide adaptability it has been suggested that hexaploid wheat arose more than once from crosses of different genotypes of its progenitor species (Vardi 1973; Talbert et al. 1998; Dubcovsky and Dvorak 2007).

Originating in the Fertile Crescent, wheat first spread to the Old World continents of Asia, Africa and Europe. Wheat cultivation was confined to these continents until the end of the fifteenth century and was brought to North America, South America and Australia in later centuries (Peterson 1965). Wheat was brought from England to New Zealand by Captain Cook in 1769 and the first records of wheat being grown in Australia and New Zealand date back in 1813 and 1788, respectively. Wheat was brought to South American countries by Spanish conquerors during the sixteenth centaury (Peterson 1965). In Canada, wheat was first grown by French settlers at Port Royal, Nova Scotia in 1605. The earliest attempts of wheat cultivation in Western Canada are associated with the Selkirk settlers in 1812 (Buller 1919). Red Fife was one of the first hard red spring wheat developed by an Ontario farmer, David Fife, from seeds received from Glasgow, Scotland (Buller 1919). The cultivar Marquis, from a cross between Red Fife and Hard Red Calcutta, was the cornerstone of wheat development in Western Canada (Buller 1919; DePauw and Hunt 2001).

2.3 Market classes of wheat in Western Canada

In Western Canada, wheat is grouped into eight market classes that are based on kernel characteristics and end-uses (Table 2.1). Each of the eight wheat classes has been assigned a combination of seed-coat colour and physical kernel configurations that are different and distinctive for each class. This Kernel Visual Distinguishability (KVD) system helps grain elevator agents and grain inspectors to readily distinguish one class of wheat from another as the grain moves from farms through the grain elevator system to customers. Complete separation of these classes of wheat ensures that buyers receive a product with known performance quality specifications required by end-use industries such as milling or baking companies.

Canada Western Hard White Spring wheat is a relatively new market class of wheat developed for production in Western Canada. White wheat has several advantages over red wheat. There is a world wide demand for white wheat because consumers in general prefer the taste and appearance of food products prepared from white wheat. With fewer phenolic compounds and tannins in the bran, white wheat also imparts a less bitter taste and confers a more favorable appearance to the final products. Hard white wheat also has flour yield advantage over hard red wheat when milled to a flour colour standard, as white wheat can be milled closer to the bran layer without negatively impacting flour colour or flavor. Generally, a flour miller can extract one to two percent more flour from a volume of white wheat as compared to red wheats (http://www.kswheat.com/). Hard white wheat is suitable for regular and whole wheat breads and buns, American and Middle Eastern flat breads (tortillas, pitas) and Chinese steamed breads. Hard white wheat is also suitable for ready-to-eat breakfast cereals, snacks and noodles (http://idahowheat.org/market/). The introduction of hard white wheat into the Canadian wheat classification system was done to increase Canada's competitiveness in the global wheat market. Development of improved hard white wheat cultivars would allow Canada to compete more effectively with Australian wheat producers, which solely grow white wheat and are the world's leading hard white wheat exporters.

Table 2.1. Western Canadian wheat market classes and their uses.

Market Class	Uses
Canada Western Red Spring Wheat (CWRS)	Pan bread, hearth bread, flat bread, steamed bread, noodles, common wheat pasta
Canada Western Extra Strong (CWES)	Blended to increase the flour strength of weaker wheat in production of pizza dough, whole wheat and frozen dough products.
Canada Prairie Spring Red Wheat (CPSR)	Hearth bread, flat bread, steamed bread, noodles
Canada Western Red Winter Wheat (CWRW)	French bread, flat bread, steamed bread, noodles
Canada Prairie Spring White Wheat (CPSW)	Flat bread, chapatis, noodles, crackers
Canada Western Soft White Spring Wheat (CWSWS)	Cookies, cakes, pastries, crackers
Canada Western Hard White Spring Wheat (CWHWS)	Noodle, flat bread, pan bread, buns
Canada Western Amber Durum Wheat (CWAD)	Semolina for excellent pasta-making quality

Source: Canadian Grain Commission (http://www.grainscanada.gc.ca/)

2.3.1 Hard white wheat: production and characteristics

About eight million hectares of spring bread wheat and two million hectares of durum wheat and 0.4 million hectare of hard red winter wheat are grown in Western Canada, generating four to five and half billion dollars in farm revenue. This production level supports the domestic flour, bread, confectionary and pasta industries and a substantial export industry that contributes to a trade surplus for Canada (Clarke et al. 2005). During 2005-2006, Canada exported 10.60 and 4.23 million tonnes of bread wheat and durum wheat grain, respectively, and 0.29 million tonnes of wheat flour. From a total of 14.83 million tonnes of wheat exports, the wheat classes CWRS and CWHWS constituted 7.42 million tonnes (50%) and 0.73 million tonnes (5%) of exports, respectively (www.grainscanada.gc.ca). The international market, particularly countries in Southeast Asia and Middle East often prefer white-grained wheat over red-grained wheat (Crosbie et al. 1998; Ambalamaatil et al. 2002). The demand for white-grained wheat by the milling and baking industries has also increased in North America (Sosland 2005). Australia is currently the largest producer of hard white wheat (Teetaert 2000), but if Canadian hard white wheat production increases to a significant level, then Canada is likely to capture a larger share of the global wheat market (Matus-Cádiz et al. 2003). Three cultivars, 'AC Snowbird' and 'Kanata' and 'Snowstar' are currently registered cultivars in the CWHWS wheat class. Under Western Grain Research Foundation's new wheat breeding agreements implemented in 2005, 15% of the allocated funds are currently invested in CWHWS wheat development. This recognizes the strong potential of CWHWS wheat, which is forecast to be grown on up to 20% of the Western Canadian wheat acreage in the next decade (http://www.westerngrains.com). The Canadian Wheat Board has an Identity Preserved Contract Program (IPCP) for 'Snowbird' and 'Kanata', which pays growers a $2.50 per tonne premium in 2006-2007 (http://www.cwb.ca/). Canadian hard white spring wheats with their light colored seed coat has improved milling yields (up to 2.6%) over No.1 grade CWRS wheat based on pilot-scale milling (Ambalamaatil et al. 2006). Also, white wheat flours have favorable higher protein concentration and substantially lighter-colored end-products than CWRS (Ambalamaatil et al. 2006). These advantages are important market characteristics that can lead to economic incentives for hard white wheat producers.

2.4 Pre-harvest sprouting (PHS) - a problem associated with white wheat

Pre-harvest sprouting refers to the precocious germination of the grain in the spike prior to harvest as a result of moist weather conditions at harvest time. Germination is initiated when conditions of moisture, temperature and oxygen occur that are conducive for embryo growth. In the field, grains in the spike usually experience oxygen levels and temperatures conducive for germination, but for PHS of wheat to occur, the trigger is usually the availability of moisture. The resistance to PHS is based on seed dormancy, i.e. the ability of the physiologically mature seed to withstand sprouting under conditions otherwise favourable for germination. Seed dormancy is generally an undesirable characteristic in agricultural crops, where rapid germination and growth are required. Extensive domestication and breeding of crop species have typically removed most dormancy mechanisms present in the seeds of their wild ancestors (Bewley 1997). This has led to a shorter dormancy period in domesticated winter wheat, as compared to its wild relatives (MacKey 1989). However, some degree of dormancy is required during seed development in cereal crops to prevent germination of grain while still in the spike. White grained wheat has been reported to be more susceptible to PHS than red grained wheat, although both groups vary in PHS (Bassoi and Flintham 2005). However, it is now apparent that red colour *per se* is inadequate to guarantee dormancy (Flintham et al. 1999).

2.4.1 Occurrence of PHS in Canada and worldwide

Pre-harvest sprouting affects many wheat producing regions of the world including Canada, Australia, South Africa, USA, Central Asia and Europe. Despite being a major problem in wheat producing countries, there is very little published data documenting the extent of PHS in terms of incidence and economic loss. The only available published document about the PHS worldwide dates to 1990, where incidence of sprout damage was reported in three or four years during the 1978-1988 (Derera 1990). Since that time, very little information has been published regarding the prevalence and economic impact of PHS. In China, 24.91 million hectares in the northeastern spring wheat region and northern winter wheat region, which constitute 83% of the wheat planting area, was affected by PHS in 1997 (Xiao et al. 2002). In the Western Australian wheatbelt, PHS occurs once out of every four years (Biddulph et al. 2007).

Based on grain prices in the 2004/2005 season, farmers in Australia lost about 22% of their grain revenue due to sprouting, which downgraded Australian Standard White wheat to feed grades (Biddulph et al. 2007).

In Canada, during 1978-1988, downgrading due to PHS damage occurred in four of ten years and was estimated to cost about $100 million in each of these years (Derera 1990). The Canadian wheat crop value loss in 2000 was estimated at greater than $100 million due to PHS damage causing elevated α-amylase activity and unacceptable Falling Number values (Clarke et al. 2005). In 2002, PHS, loss of colour and test weight were also major problems in wheat production (Clarke et al. 2005).

2.4.2 Grading regulation of PHS in wheat by Canadian Grain Commission

Canada is known worldwide as a supplier of high quality wheat as a result of consistency in grain quality over the years. The responsibility for monitoring wheat quality lies with the Canadian Grain Commission (CGC), a federal government agency that operates under the authority of the *Canada Grain Act*. To assure grain quality with regards to PHS, CGC has set limits for total sprouted grain for different wheat grades and classes (Table 2.2). For example, a severely sprout-damaged and total sprouted kernel frequency of more than 0.5 % and 5%, respectively, in CWHWS wheat results in downgrading to feed grade wheat (Official Grain Grading Guide 2007). The Canadian Grain Commission defines the sprouted kernels and severely sprouted kernels as following:

Kernels are sprouted if one of the following conditions exists:

• Kernels show clear evidence of growth in the germ area.

• The bran is noticeably split over the germ from apparent growth.

• The germ is missing and there is apparent greyish discolouration normally attributable to sprouting.

• The germ, though intact, appears distinctly swollen as a result of sprouting activity.

Kernels are assessed as severely sprouted when:

• The sprouts extend beyond the normal contour of the germ.

• The kernels are severely degenerated as an apparent result of advanced sprouting.

• The sprout has been clearly broken and only a portion remains.

• The sprout is completely gone and there is evidence showing that there was extension

Table 2.2. Sprouted kernel frequency limit for primary and export grades for different wheat market classes.

Wheat class	Grade	Sprouted kernels (%)	
		Severely sprouted	Total
CWRS	No.1 CWRS	0.1	0.5
	No.2 CWRS	0.2	1
	No.3 CWRS	0.3	3
	No.4 CWRS	0.5	5
	CW Feed	No Limit	No Limit
CWHWS	No.1 CWHWS	0.1	0.5
	No.2 CWHWS	0.2	1
	No.3 CWHWS	0.3	3
	No.4 CWHWS	0.5	5
	CW Feed	No Limit	No Limit
CWAD	No.1 CWAD	0.1	0.5
	No.2 CWAD	0.2	2
	No.3 CWAD	8	8
	No.4 CWAD	12	12
	No.5 CWAD	No Limit	No Limit
CWRW	No.1 CWRW	0.1	0.5
	No.2CWRW	0.3	2.5
	CW Feed	No Limit	No Limit
CWSWS	No.1 CWSWS	0.1	1
	No.2 CWSWS	0.3	5
	No.3 CWSWS	0.5	8
	CW Feed	No Limit	No Limit
CWES	No.1 CWES	0.1	0.5
	No.2 CWES	0.3	2
	CW Feed	No Limit	No Limit
CPSW	No.1 CPSW	0.1	0.5
	No.2 CPSW	0.3	2
	CW Feed	No Limit	No Limit
CPSR	No.1 CPSR	0.1	0.5
	No.2 CPSR	0.3	2
	CW Feed	No Limit	No Limit

Source: Official Grain Grading Guide, 2007. Canadian Grain Commission.

of the sprout outside the normal contour of the germ.

2.5 Effect of PHS on grain and end-product quality

Wet weather prior to harvest is a major abiotic stress contributing to loss of quality and grade in different wheat classes. Wet pre-harvest weather conditions reduce the grain value to the producers by impacting four primary grade determinants; (1) test weight (bulk density), (2) vitreousness (translucent properties of kernel), (3) degree of soundness (overall visual grain quality), and (4) percent sprouted kernels (Canadian Grain Commission, 2005; McCaig et al. 2006).

Starch accounts for 64-74% of the total dry weight of wheat grains (Hucl and Chibbar 1996) and starch properties are important for determining the end-use quality of wheat flour. PHS has been closely associated with elevated levels of α-amylase, an enzyme that catalyzes endohydrolysis of α-1, 4-glucan bonds in starch molecules in wheat grain. Degradation of native starch granules negatively affect quality of various products made from wheat flour such as breads, cookies and noodles. There are four known possible sources of α-amylase activity in grains. The primary reason for α-amylase accumulation in the grain is delayed harvest due to wet weather causing breakdown of grain dormancy (Flintham and Gale 1988). Another major cause of excess α-amylase activity is the deposition of α-amylase in the endosperm cavity (Flintham and Gale 1988; Evers et al. 1995). The third source of elevated α-amylase activity is associated with pre-maturity sprouting and involves germination during early grain development when kernels are still at high moisture content (Flintham and Gale 1988; Lunn et al. 2001a; Lunn et al. 2001b). The fourth possible cause of α-amylase activity has been identified as retained pericarp α-amylase in grains and is associated with non-uniform maturity of the wheat crop (Olered 1975; Lunn et al. 2001a; Lunn et al. 2001b). The pre-maturity α-amylase and retained pericarp α-amylase are also known as late maturity α-amylase (LMA) (Mares and Mrva 2007) and are not directly associated with PHS, as LMAs are often triggered by low temperatures (12-18˚C) during the second half of the grain filling period (25-35 days after flowering) (Mrva and Mares 2001). Rainfall at harvest, however, is the main cause of PHS inducing α-amylase activity. During the early phase of grain filling low-isoelectric point (pI) isozymes are present in the pericarp

tissue under control of the *α-Amy-2* genes located on long arms of group 7 chromosomes. The high pI isozymes that are product of *α-Amy-1* gene family located on group 6 chromosomes are synthesized during later stages of grain development and also during PHS (Lunn et al. 2001a; Lunn et al. 2001b; Wrigley 2006; Mares and Mrva 2007).

Starches from sprout damaged wheat grain exhibit a lower swelling power, gelatinize at a lower temperature and over a narrow temperature range than starches from sound grain. A lower peak viscosity, as determined by Rapid Visco-Analyzer tests and increased digestibility by glucoamylase is noted for starch from sprouted wheat grain (Noda et al. 2004).

Breads baked from hard wheats are affected more than other wheat products by PHS (Figure 2.1). Bread production is complicated by increased stickiness of the dough, which necessitates special handling in small bakeries and can disrupt operations of large bakeries. Even minor sprout damage can cause significant reductions in gluten strength of wheat flour making it unsuitable for bread making (Barbeau et al. 2006).

Sprout damage affects both the processing and quality of different kinds of noodles. High α-amylase activitiy in dry noodles weakens the dough so that noodles cannot support their own weight and break during the dehydration process (Nagao 1995). As noodle appearance is the first critical judgement made by consumers when evaluating noodle quality, any change to noodle colour, brightness or appearance of undesirable discolorations (spots) will render the noodles less attractive. Alkaline noodles made from severely sprouted wheat flour may show up to a five-fold higher number of spots as compared to products made from sound flour (Hatcher and Symons 2000).

Sprout damage also affect the products made from soft white wheat. A reduced thickening power of sprout damaged soft wheat flour results in poor cake baking quality, resulting in cakes with low volume, and a dip in the centre (Lorenz and Valvano 1981).

2.6 Methods for measuring PHS

Two basic approaches have been used to identify genotypes with improved PHS resistance based on seed germination. PHS can be measured by sprouting test of spikes subjected to artificial or natural wetting treatment or germination test of threshed seeds (Mares 1989). Artificial wetting treatment is intended to parallel field conditions but with greater control than rainfall-induced weathering immediately following physiological

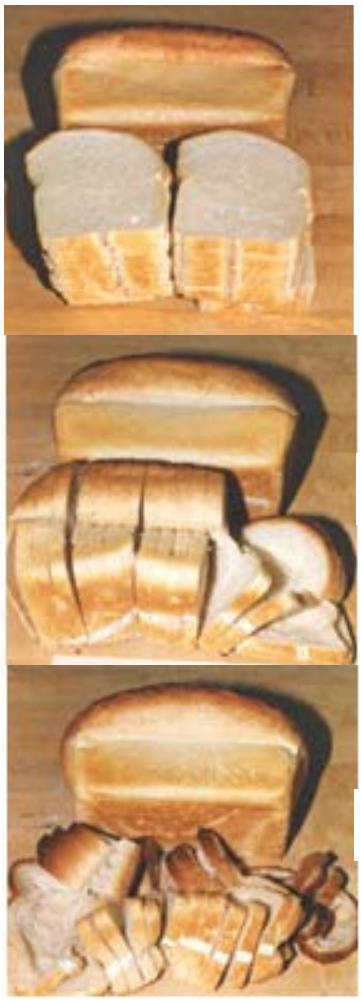


Figure 2.1. Loaves of bread made from sound and sprouted grains of wheat.

Source: http://www.grainscanada.gc.ca

maturity (Paterson et al. 1989). The germination test of threshed seeds provides a direct measure of seed dormancy and sprouting test of spikes may include other mechanisms associated with the intact spikes such as germination-inhibitors present in the hull (Kato et al. 2002) or spike morphology affecting the wetting efficiency (King and Richards 1984).

Seed dormancy can be estimated by a germination index ranging from 0 to 1.0 (Reddy et al. 1985), germination resistance index ranging from 0 to 50 (Gordon 1971) or germination percentage ranging from 0 to 100%. All seed dormancy values are calculated on threshed grains imbibed on filter paper. The germination index is a weighted index which gives maximum weight to grains which germinate early and less weight to grains which germinate later. Thus, germination index can differentiate between lines which germinate rapidly from lines which germinate at a slower rate. Germination resistance index measures the relative rate of germination, by estimating the time to 50% germination. Germination index may give an important differentiation in a relatively small rainfall event; a slower germination rate will result in a lower proportion of germinated grain and thus more sprouting tolerance (Biddulph et al. 2007). Shorter et al. (2005) reported that germination index is consistent and repeatable across years (R^2 = 64%) compared to visible sprouts (R^2 = 46%), sprout index (R^2 = 38%) and Falling Number (R^2 = 8%) under artificial weathering conditions.

With the advent of highly automated food production plants, such as bakeries, variation in α-amylase activity levels in the grain has become more unacceptable (Mares and Mrva 2007). The Hagberg Falling Number method (Hagberg 1960; Hagberg 1961; Perten 1964) is a simple and rapid technique to determine α-amylase activity in grain and is widely used in classification, quality control and marketing of wheat. The Falling Number method is a viscometric assay that involves rapid gelatinization of a flour or meal, water suspension by boiling, with subsequent measurement of the starch liquification process caused mainly by α-amylase activity. There is an inverse curvilinear relationship between α-amylase activity and Falling Number (Mares 1987b), but at low α-amylase activity levels, the overall starch quality is the determining factor for Falling Number (Ringlund 1983). Falling Number measures the endosperm quality at harvest time (Hagemann and Ciha 1984) but Falling Number values fluctuate widely depending

on the degree of ripening and the amount of rainfall prior to harvest (Mares 1993). Furthermore, sample weight may affect Falling Number test precision, reproducibility and predictability of α-amylase activity (Finney 2001). Humphreys and Noll (2002) found that some genotypes with high sprouting scores had high Falling Number indicative of low α-amylase activities.

While Falling Number is used universally at grain receival to access grain quality and α-amylase activity, the Rapid Visco Analyzer (RVA) is more commonly used by the milling, baking and grain export industries (Mares and Mrva 2007). An ELISA method using polyclonal antibodies to both high and low pI α-amylase and monoclonal antibodies to high pI α-amylase have also been developed for on-farm detection of pre-harvest sprouting in wheat (Verity et al. 1999; Skerritt and Heywood 2000)

2.7 Factors associated with PHS resistance

The interaction between plant growth regulators, particularly gibberellins (GA) and abscisic acid (ABA) is an important factor controlling the transition from embryogenesis to germination in seed (Figure 2.2). In the cereal grain aleurone layer, the expression of genes encoding starch hydrolytic enzymes such as α-amylases that are needed for seed germination, are induced by GA but suppressed by ABA (Lovegrove and Hooley 2000; Brady and McCourt 2003; Ho et al. 2003).

2.7.1 Abscisic acid

Abscisic acid (ABA) is a plant growth regulator that plays important roles during many phases of the plant life cycle, including seed development, dormancy, and responses to environmental stress conditions such as drought, cold and salinity (Zeevaart and Creelmann 1988; Seo and Koshiba 2002; Seki et al. 2007). During seed development, ABA content increases and regulates key processes involved in the imposition and maintenance of dormancy (Bewley 1997). This is illustrated by ABA-deficient mutants of maize that display *vivipary* (McCarty 1995) and by ABA-deficient mutants of Arabidopsis that can germinate in the absence of GA (Koornneef et al. 1982). Upon imbibition, the high concentration of ABA is reduced in order for seed to germinate, and studies have shown that this occurs when dormancy is broken by after-ripening, stratification or darkness (Gubler et al. 2005). In after-ripened non-dormant Arabidopsis seeds, ABA concentration declines rapidly upon imbibition, after which germination

proceeds. In contrast, imbibed dormant seed show a low level of ABA decline and only transiently, with ABA content again increasing to a level similar to that observed in the non-imbibed seeds (Ali-Rachedi et al. 2004). Similar to Arabidopsis, the dormant or non-dormant dry seeds or embryos of barley (*Hordeum vulgare*) grains contain high concentrations of ABA, that decreases rapidly upon imbibation of grains with a more prominent reduction in non-dormant than dormant seeds (Millar et al. 2006).

Changes in ABA content of imbibing seeds following dormancy release are likely to reflect changes in the balance between ABA synthesis and catabolism, with synthesis dominating in dormant seeds and catabolism dominating in non-dormant seeds. Dormancy release by after-ripening and stratification causes a switch to ABA catabolism, resulting in a decrease in ABA content in the embryo and a corresponding increase in inactive ABA metabolites such as phaseic acid (PA) and dihydrophaseic acid (Gubler et al. 2005). The ABA and PA content in embryos of after-ripened barley grains decrease and increase, respectively, rapidly after hydration. In dry dormant grains, the ABA content of the embryo is similar to that of after-ripened grains and although it also decreases for the first 12 hr and then increases, indicating that ABA is both synthesized and catabolized. Thus, the ABA content might be controlled by the balance between catabolism dominating in non-dormant grain and synthesis in dormant grain (Jacobsen et al. 2002).

Several alternative catabolic pathways exist for the inactivation of ABA (Zhou et al. 2004; Nambara and Morion-Poll 2005). Hydroxylation of ABA at the 8'-position to produce 8'-hydroxyABA, which spontaneously isomerizes to phaseic acid, is considered to be the predominant pathway in ABA catabolism (Figure 2.2) (Nambara and Morion-Poll 2005). This reaction is catalyzed by a cytochrome P450 monooxygenase known as ABA 8'-hydroxylase (Krochko et al. 1998). Molecular and genetic analysis of the ABA 8'-hydroxylase gene family (*CYP707A*) indicates that the *CYP707A2* gene is more active in non-dormant than dormant seeds of Arabidopsis (Millar et al. 2006). Similarly, a barley CYP707 homologue (*HvABA8'OH-1*) is expressed at much higher levels in embryos from non-dormant grains than from dormant grains (Millar et al. 2006).

2.7.2 Gibberellins

Gibberellins (GAs) are a large family of tetracyclic diterpenoid compounds, some of which are bioactive growth regulators associated with diverse plant growth and developmental processes such as seed germination, stem elongation, flowering and fruit development (Davies 1995). To date, 126 different GA molecules have been identified in higher plants, fungi and bacteria. However, relatively few GAs have intrinsic biological activity and therefore possess a growth regulator function. (Hedden and Phillips 2000).

Upon germination, GA released from the embryo triggers the aleurone cells to secrete hydrolytic enzymes, particularly α-amylases, which mobilize the endosperm reserves that fuel the germination process (Figure 2.3). GA not only stimulates the secretion of hydrolytic enzymes but, in combination with internally generated reactive oxygen species, also triggers the onset of programmed cell death in aleurone cells (Bethke et al. 2001). GA-treated aleurone layer protoplasts undergo cell death within 5-8 days, whereas ABA-treatment can keep protoplasts alive for up to six months (Fath et al. 2000). Inhibitors of GA biosynthesis do not inhibit the increase in α-amylase production during germination, suggesting that rather than GA being *de novo* synthesized, stored GA precursors are mobilized (Groselindemann et al. 1991). Released GA from the embryo triggers several responses ranging from gene induction and repression and up-regulation of secretory responses. The earliest event following GA treatment of the aleurone is an increase in cytoplasmic Ca^{2+} concentration (Figure 2.2). Hetero-trimeric G protein is involved in transducing the GA signal in the aleurone layer (Lovegrove and Hooley 2000). The signal transduction pathway leading to hydrolase synthesis utilizes changes in cytosolic Ca^{2+}, calmodulin (CaM), cGMP and the trans-activating protein GAMyb (Figure 2.2). In intact wheat aleurone cells, a response is observed within 2-5 min of GA-treatment (Bush 1996). The alteration in Ca^{2+} concentration indicates a potential signaling role and calmodulin protein levels are stimulated two- to four-fold by GA and reduced by ABA in barley (Schuurink et al. 1996). Changes in cytosolic Ca^{2+} and CaM also play a role in programmed cell death (Levine et al. 1996; Groover and Jones 1999). In barley aleurone, GA induces a two- to three-fold increase in cGMP level (Penson et al. 1996). An inhibitor of guanylyl cyclase prevents the GA-induced increase in cGMP and inhibits GA-induced α-amylase synthesis and secretion. The inhibitor also prevents GA-

induced accumulation of α-amylase and GAMyB mRNA (Penson et al. 1996). The expression of α-amylase is transactivated by the transcription factor GA-induced Myb-like protein (GAMyb) that binds specifically to the GARE box of an α-amylase promoter (Gubler et al. 1995). ABA and an ABA-induced protein kinase, PKABA1, repress the GA induction of GAMyb. In the barley *slender* mutant, GAMyb and α-amylase are highly expressed, even in the absence of GA. However, ABA, PKABA1 and inhibitor of cGMP inhibit the constitutive expression of GAMyb and α-amylase (Gómez-Cadenas et al. 2001). ABA also represses the expression of a GA 20-oxidase gene, suggesting that ABA might block the germination process by repressing GA biosynthesis (Pérez-Flores et al. 2003). Gibberellins appear not to be involved in the control of seed dormancy *per se* but rather, are important in the promotion and maintenance of germination, and act after the ABA mediated inhibition of germination has been overcome (Bewley 1997).

2.7.3 Starch hydrolysis

Starch represents a major source of calories in human food and animal feed and a vital storage compound in plants (Chibbar et al. 2004). Starch is composed of two distinct glucan polymers; amylose and amylopectin. During germination, degradation of starch in the cereal endosperm occurs in a nonliving tissue that is effectively an acidic, apoplastic environment in which no intracellular or intercellular compartmentation remains (Smith et al. 2005). Among the hydrolytic enzymes active during starch breakdown, α-amylases play a major role in degrading native starch granules. Up to 70% of newly synthesized and secreted enzymes from the aleurone are α-amylases (Ritchie et al. 2000). Only through the concerted action of α-amylase, β-amylase, debranching enzyme, and α-glucosidase can starch be completely hydrolyzed (Figure 2.3) (Sun and Henson 1991).

α-Amylase, an endoamylase, carries out the first step in starch hydrolysis, which involves cleavage of α,1-4 glucosidic linkages in the inner part of the amylose and amylopectin chains. Three classes of α-amylase genes; *α-Amy1*, *α-Amy2* and *α-Amy3* have been identified in wheat. In hexaploid wheat, 12-14 *α-Amy1* genes reside on homoeologous chromosomes 6A, 6B and 6D, whereas 10-11 *α-Amy2* genes are found on chromosomes 7A, 7B and 7D. Three to four *α-Amy3* genes are located on homoeologues of chromosome 5 (Baulcombe et al. 1987; Huttly et al. 1988). The three classes of α-amylase genes are differentially expressed. The *α-Amy1*and *α-Amy2* genes are active in

the aleurone cells of germinating wheat grain. However, the *α-Amy1* isozymes are more prevalent following the immediate onset of germination (at days 1-2), while *α-Amy2* isozymes increase later (at day 3) (Sargent 1980). The *α-Amy3* is only expressed in developing grains (Baulcombe et al. 1987).

β-Amylase, an exoamylase, exclusively cleaves α,1-4 glycosidic bonds at the non-reducing ends of starch-glucan polymers. β-Amylase accumulates during grain development in two forms, soluble and bound (Forsyth and Koebner 1992; Ziegler 1999). A large portion of β-amylase in starchy endosperm is in the bound form, where the enzyme forms disulphide linkages to protein such as glutenins. Bound β-amylase is inactive and deposited on the periphery of the starch granules during grain development and is probably synthesized as a mature protein. The enzyme is a component of the protein matrix which covers the starch and might protect starch from premature attack by α-amylase (Ziegler 1999). Zhang et al (2005) demonstrated that nitric oxide is able to induce a rapid response in β-amylase in wheat seeds within 12 hr of germination. Nitric oxide can be produced enzymatically or non-enzymatically from nitrite at low pH in plants. β-Amylase has been reported to be encoded by multiple loci on chromosomes 4A, 4D, 5A and 5B in hexaploid wheat (Ainsworth et al. 1983).

The third group of starch-converting enzymes is the debranching enzymes represented by isoamylases and pullulanases that exclusively hydrolyze α,1-6 glycosidic bonds. The major difference between pullulanase (limit dextrinase in plants) and isoamylase is their ability to hydrolyze pullulan, a polysaccharide with a maltotriose repeating unit of that is α,1-6 linked. Limit dextrinases hydrolyze α,1-6 glycosidic bonds in pullulan and amylopectin, whereas isoamylase can only hydrolyze α,1-6 bonds in amylopectin (van der Maarel et al. 2002). Overexpression of thioredoxin *h*, a 12-kDa extraplastidic protein containing a redox-active disulfide group, in the endosperm of germinated grain in barley increases the activity of limit dextrinase (four-fold) and gibberellin A1 content (1.6 to 2.8-fold) (Cho et al. 1999). This suggests that the endosperm communicates directly with the embryo and the aleurone, respectively, to accelerate germination and the accumulation of α-amylase activity (Wong et al. 2002).

α-Glucosidase catalyzes the hydrolysis of α-1,4 glucan bonds at the non-reducing ends of dextrins and maltose to produce glucose. In barley, this enzyme is synthesized *de*

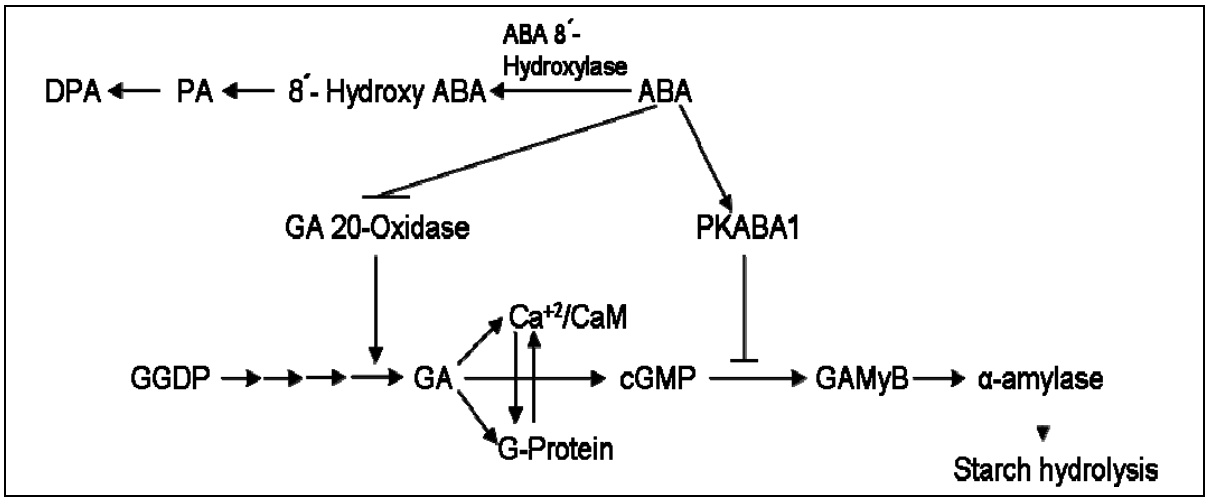


Figure 2.2. ABA and GA interactions during seed germination and dormancy. ABA- abscisic acid, CaM- calmodulin, cGMP- cyclic guanosine monophosphate, DPA- dihydrophaseic acid, GA- gibberellic acid, GAMyB- GA-induced Myb-like protein, GGDP- geranylgeranyl diphosphate, G-protein- GTP-binding protein, PA- phaseic acid, PKABA1- ABA-induced protein kinase (refer to the text for references).

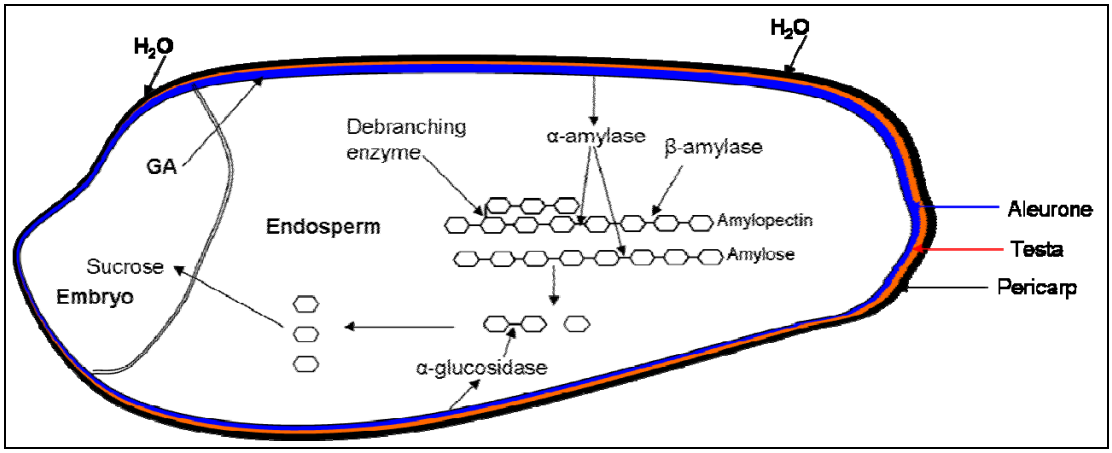


Figure 2.3. Starch hydrolysis during cereal grain germination. The ring structure symbolizes the glucose moiety.

novo in response to GA in de-embryonated half-seeds (Hardie 1975) and is secreted from isolated aleurone layer in the presence of GA and Ca^{+2} in a manner similar to α-amylase (Sun and Henson 1990). In rice, α-glucosidase is present in dry seeds, however, dry seeds of barley and wheat do not contain α-glucosidase, but the enzyme is rapidly induced during germination and *in vivo* starch metabolism (Guglielminetti et al. 1995). Starch and maltose hydrolysis in germinating wheat seeds is reduced in the presence of the α-glucosidase inhibitor 'Bay m 1099', which causes a reduction in glucose levels and inhibits plant growth (Konishi et al. 1994). Two forms of barley α-glucosidase (101 and 95 kDa) are present early in seed germination, but their concentrations decline sharply later in germination, to be replaced by an 81 kDa α-glucosidase. These isoforms may represent α-glucosidases in different states of glycosylation and/or proteolytic processing. A 14-fold induction of these isoforms also occurs in isolated aleurones after treatment with gibberellin (Tibbot et al. 1998).

2.7.4 Phenolics

A positive correlation between length of dormancy and the content of phenolic compounds is found for developing and ripening barley caryopses (Weidner et al. 1993). In wheat, rye and triticale, genotypes with deeper dormancy possess a high concentration of phenolic acids in the form of soluble esters. For all three cereals, the concentrations of ferulic and sinapic acids, and in case of wheat and rye also *p*-coumaric, are higher in caryopses with deeper dormancy than caryopses with shallow dormancy (Weidner et al. 1999). The concentration of the soluble esters of ferulic acid for wheat cv. Elena with deeper dormancy is almost twice as high as that of wheat cv. Alba with shallow dormancy. A high level of phenolic acids liberated from soluble glycosides also correlates with dormancy in wheat. However, the differences in the concentration of free phenolic acids are less marked for cultivars with different levels of dormancy (Weidner et al. 1999). A study indicated that phenolic acids in the form of esters most probably dictate dormancy, which is gradually lost by the process of after-ripening. After six months of dry storage, seed dormancy is generally gone and the levels of free and conjugated forms (esters and glycosides) of phenolic acids are reduced (Weidner et al. 1996).

2.8 PHS resistance - a quantitatively inherited trait

PHS is expressed as a quantitatively inherited trait and is strongly affected by environmental factors (Anderson et al. 1993). Traditionally, PHS resistance is known to be associated with red grain colour, whereas susceptibility is associated with white grain colour (Nilsson-Ehle 1914). The association between PHS and kernel colour may be due to either tight genetic linkage between genes affecting the two traits or due to pleiotropic effect by the gene controlling kernel colour (DePauw and McCaig 1983; Soper et al. 1989; McCaig and DePauw 1992). White-grained wheat has been reported to be more susceptible than red-grained wheat, although both groups vary in PHS (Bassoi and Flintham 2005). However it is now clear that red colour *per se* is not sufficient to guarantee dormancy (Flintham et al. 1999). Combining dominant *R* alleles (*R-A1, R-B1* and *R-D1*) at two or three loci confer an additive effect on dormancy (Flintham 1993). PHS tolerance in red grained wheats has been attributed to hypersensitivity to abscisic acid in developing embryos (Walker-Simmons 1987), reduced α-amylase activity in the grain (Bhatt et al. 1976), the presence of compounds in the bract that inhibit germination (Derera and Bhatt 1980) and slower water uptake (King 1984). PHS is also influenced by spike morphology (King and Richards 1984). One study has demonstrated that the hulls of wheat seeds possess several kinds of inhibitors, which become synergistically operative as germination inhibitors. In case of Lancer and RL4137 (dormant varieties), the germination of intact seeds is restrained by the presence of husk. In contrast, existence of husk for Menyou (non-dormant variety) did not cause germination restraint. The water-soluble extracts; 2-phenylethylalcohol, dihydroactinidiolide, 4-vinylphenol and its 2-methoxy derivative extracted from the husks of a dormant variety (Kwankei W421) show a clear inhibition of germination at 500 mg/L (Kato et al. 2002).

2.9 Genetic mapping of Quantitative Trait Loci (QTL)

There are three basic requirements for the genetic mapping of QTL; genetic markers with genotypic data for population, mapping population showing segregation for trait valves and reliable phenotype data for population.

2.9.1 Genetic markers

A marker used for genetic mapping corresponds to a certain locus on the genome. In plant genetics, the most common marker types are morphological, biochemical

(enzyme and protein) and molecular (DNA) markers. The morphological markers are limited in number and are expressed mostly in adult plants. Biochemical markers (isoenzymes) were introduced to plant genetics in the 1960's but these markers are also limited in number and rarely used today. Biochemical markers may show tissue specific expression and may be influenced by environmental factors. In the last two decades, the DNA based markers (molecular markers) have become the dominant marker system for genetic analysis. Molecular markers are numerous and show higher polymorphism than morphological and biochemical markers. DNA markers are not influenced by environmental factors or the developmental stage of the plant. The different types of molecular markers have been extensively reviewed in earlier publications (Gupta et al. 1999; Gupta and Rustgi, 2004).

2.9.1.1 Simple sequence repeat (SSR)

The genomes of all eukaryotic organisms contain a class of repetitive sequences called as microsatellites or simple sequence repeat (SSR) (Litt and Lutty 1989; Tautz et al. 1986). SSR consist of 1-6 bp long monomer sequences that exist in tandem repeats. SSR polymorphisms derive mainly from variability in number of repeats rather than in the primary sequence. The length changes in microsatellite DNA are generally thought to arise from slippage by DNA polymerase during DNA replication (Levinson and Gutman 1987). When the nascent strand realigns out of register, renewed replication will lead to the insertion or deletion of repeat units relative to the template strand (Ellegren 2004). Wheat SSR markers show a high level of polymorphism (Plaschke et al. 1995; Röder et al. 1995) and are generally chromosome-specific and inherited in a co-dominant manner. Several detailed genetic maps based on SSR markers have been reported in wheat (http://wheat.pw.usda.gov/ggpages/map_summary.html; Röder et al. 1998; Somers et al. 2004; Båga et al. 2007). The increasing availability of expressed sequence tags (ESTs) in wheat and related cereals provides another valuable source of markers in wheat. EST-based markers are physically associated with coding regions of the genome, and play a role in the genetic analysis of the transcribed region of the genome. A substantial portion of ESTs contain microsatellites, which has led to the development of EST-SSR markers (Eujayl et al. 2002; Nicot et al. 2004; Yu et al. 2004). However, when compared to

genomic SSRs, EST derived SSRs (EST-SSRs) are less polymorphic (25%) in hexaploid wheat (Eujayl et al. 2002).

2.9.1.2 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a technique based on selective PCR amplification of restriction fragments from a total digest of genomic DNA of any origin or complexity (Vos et al. 1995). This technique shows an ingenious combination of RFLP and PCR. The fingerprints are produced without prior sequence knowledge using a limited set of generic primers. This technique is robust and reliable because stringent conditions are used for primer annealing. The AFLP procedure mainly involves three steps: (i) restriction of DNA using a rare cutting and a frequent cutting restriction enzyme (e.g. *Eco*R1 and *Mse*1) and ligation of oligonucleotide adapters to generated ends, (ii) selective amplification of sets of adapter ligated fragments, and (iii) gel analysis of amplified fragments. AFLP analysis has been conducted in a number of crops including wheat (Barrett and Kidwell 1998; Barrett et al. 1998) to access the genetic diversity. Although few maps have been developed using AFLP markers (http://wheat.pw.usda.gov/ggpages/map_summary.html; Semagn et al. 2006), a wheat molecular map using AFLP and SSR markers has recently been constructed in winter wheat (Båga et al. 2007).

2.9.1.3 Diversity Array Technology (DArT®)

Diversity Array Technology (DArT®) is a microarray hybridization-based technique that enables the simultaneous genotyping of several hundred polymorphic loci spread over the genome without prior sequence information (http://www.diversityarrays.com/; Jaccoud et al. 2001; Wenzl et al. 2004). DArT generates whole-genome fingerprints by scoring the presence versus the absence of specific DNA fragments in samples of digested genomic DNA. DArT has recently been used in genetic mapping and fingerprinting studies in *Arabidopsis* (Wittenberg et al. 2005), barley (Wenzl et al. 2004), rice (Jaccoud et al. 2001), cassava (Xia et al. 2005), and wheat (Akbari et al. 2006, Semagn et al. 2006). DArT has the potential to increase the marker density on a map within a short time and at a 10-fold lower low cost than SSR markers (Xia et al. 2005).

2.9.1.4 Expressed Sequence Tags (ESTs)

Expressed sequence tags (EST) are short cDNA sequences that serve to tag a gene from which the messenger RNA (mRNA) originated. They represent a snapshot of genes expressed in a given tissue and/or at a given developmental stage. Typically, ESTs are 200-700 bp single pass sequences that can be used to search DNA and protein databases for similar genes. The information from the search can be used to determine if a specific gene (or sequence motif) is present in the same or other organisms and if its function is known (Lazo et al. 2004). ESTs have been developed for numerous organisms and sequences are deposited in GenBank and in species-specific databases. Among plants, *Triticum aestivum* currently has 1,051,146 ESTs deposited in the GenBank database as of 19 October 2007 (http://www.ncbi.nlm.nih.gov/projects/dbEST). National Science Foundation Wheat EST Project has developed 90,016 ESTs (http://wheat.pw.usda.gov/NSF/), out of which 8,241 were physically mapped to chromosomes or chromosome bins using wheat aneuploid stocks and Southern hybridization (Lazo et al. 2004; http://wheat.pw.usda.gov/NSF/). These ESTs could lead to the development of markers associated with a specific function and used for direct gene selection of target traits in plant breeding.

2.9.1.5 Advantages and disadvantages of different markers

Molecular markers can be broadly classified in three groups: hybridization-based, PCR-based and sequence-based DNA markers (Gupta et al. 1999). Each marker system developed in higher plants during the last two decades is associated with some advantages and disadvantages (Table 2.3), and the choice of marker system is dictated to a large extent by the intended application, convenience and the cost involved. Most of the markers developed and used in the past, related to genomic DNA (gDNA) and therefore could belong to either the transcribed region or the non-transcribed region of the genome. However, during the last few years, emphasis has shifted towards the development of molecular markers from the transcribed region of the genome, and both wet lab and *in silico* approaches have been used for this purpose. This has become possible, firstly, due to availability of a large number of cDNA clones in a variety of plant systems and secondly, due to accumulation of a large number of expressed sequence tags (ESTs) in public databases (Gupta and Rustgi 2004).

Table 2.3. Advantages and disadvantages of different marker systems.

Features	Isozymes	RFLP	RAPD	AFLP	SSR	DArT	SNP	STS	SSCP
Quantity of DNA required	-	High	Low	Low	Low	Low	Low	Low	Low
Hybridization/ PCR based	-	Hybridization	PCR	PCR	PCR	Hybridization/ PCR based	PCR	PCR	PCR
Level of polymorphism	Low	Medium	Medium	High	High	High	High	Low	Low
Reproducibility	High	High	Low	High	High	High	High	High	Medium
Dominant/co-dominant	Codominant	Codominant	Dominant	Dominant	Codominant	Dominant	Dominant	Dominant	Codominant
Sequence information required	-	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Amenability to automation	No	No	No	Yes	Yes	Yes	Yes	Yes	No

RFLP- Restriction Fragment Length Polymorphism, RAPD- Random Amplified Polymorphic DNA, AFLP- Amplified Fragment Length Polymorphism, SSR- Simple Sequence Repeat, DArT- Diversity Array Technology, SNP- Single Nucleotide Polymorphism, STS- Sequence Tagged Site, SSCP- Single Strand Conformation Polymorphism

2.9.2 Mapping populations

The identification of QTL linked to a desired trait requires a segregating population derived from parents that differ in the trait of interest. Different types of mapping populations may be used for mapping depending on the genetics of the trait of interest. F_2 populations and backcross populations are the simplest type of mapping populations for self-pollinated species like wheat. The main advantages of these populations are that they are easy to develop and can be obtained in a short time. These populations are used for mapping traits having Mendelian inheritance, but are unsuitable for quantitative traits. Recombinant inbred lines (RILs) and doubled haploid (DH) lines represent permanent and immortal mapping populations as their genotypes are stable over generations. Inbreeding from individual F_2 plants derived from F_1 hybrids allows the development of RILs, consisting of near homozygous lines having a unique combination of chromosomal segments. The main disadvantage of producing RILs is time for production, which requires six or more generations. Haploid production with maize pollen procedure (Kisana et al. 1993; Knox et al. 2000) and anther culture (Gustafson et al. 1995; Fedak et al. 1997) followed by chromosome doubling results in creation of genetically pure DH lines within a relatively short period of time. The major advantage of RILs and DH populations are that they are true breeding lines. This allows replicated trials across different locations and years, which is a major requirement for phenotyping a quantitative trait influenced by environment.

2.9.3 Genetic linkage maps

Genetic dissection of complex agronomic traits through QTL analysis requires the development of molecular-marker linkage maps. A linkage map is a road map of chromosomes derived from two parents (Paterson 1996). Linkage maps indicate the position and relative genetic distance between markers along the chromosomes. Linkage maps are based on recombination fractions during meiosis and the frequency of recombinant genotypes is used to calculate the genetic distance between markers and relative order of markers on the chromosomes (Paterson 1996; Collard et al. 2005). Mapping functions are required to convert the recombination fractions into centiMorgan (cM) distances because the recombination frequency and frequency of crossing-over during meiosis are not linearly related (Hartl and Jones 2001). Two different mapping

functions are used in linkage analysis; Kosambi and Haldane mapping functions (Haldane 1919; Kosambi 1944). The Kosambi mapping function assumes interference between crossover events, whereas, the less used Haldane mapping function assumes no interference (Hartl and Jones 2001). Linkage between markers is calculated using odds ratios (i.e. the ratio of linkage versus no linkage) (Collard et al. 2005). This ratio is expressed as logarithm of odds (LOD score) (Risch 1992). Software packages, such as MAPMAKER/EXP 3.0 (Lander et al. 1987) and JoinMap® 3.0 (van Ooijen and Voorrips 2001) are available for genetic linkage analysis.

Initially, genetic maps were mainly developed from interspecific and wide crosses using RFLP markers (Chao et al. 1989; Cadalen et al. 1997; Messmer et al. 1999). The International Triticeae Mapping Initiative (ITMI) population ('Synthetic'/ 'Opata') is one of the most polymorphic wheat mapping population, that has been extensively mapped with RFLP, AFLP and SSR markers (http://wheat.pw.usda.gov). Although the ITMI map has high marker density, it is primarily made up of RFLP markers, which are not amenable to high-throughput molecular breeding strategies. RFLP analysis requires large quantities of DNA and is technically demanding, expensive and laborious, and the most common detection method is based on radioisotopes. However, mapping of agronomically important genes or QTL is a major goal of plant breeding and requires informative markers in an intraspecific context. Less than 10% of RFLP markers are polymorphic in intraspecific molecular analysis (Röder et al. 1998). In contrast, microsatellite markers are abundant, highly polymorphic and require only a small amount of DNA for analysis. The first linkage map of wheat based on 214 SSR markers was constructed by Röder et al. (1998). Paillard et al. (2003), Sourdille et al. (2003), Quarrie et al. (2005), Torada et al. (2006) and Båga et al. (2007) constructed intraspecific genetic maps covering genetic lengths of 3,086, 3,685, 3,522, 3,441 and 2,873 cM, respectively. A consensus map of 1,235 SSR markers, covering a genetic length of 2,569 cM, was constructed by joining four independent genetic maps (Somers et al. 2004). The entire genetic distance of the hexaploid wheat genome is considered to correspond to about 4,000 cM (Sourdille et al. 2003).

2.9.4 QTL mapping of traits

A quantitative trait locus (QTL) is a region on the genome that contributes to trait value. QTL mapping is a means to estimate the locations, numbers, magnitude of phenotypic effects, and modes of gene action of individual determinants that contribute to the inheritance of a continuously variable trait (Paterson 2002).

The statistical analysis of associations between phenotype and genotype in a population to detect quantitative trait loci includes single-marker mapping, interval mapping and composite interval mapping (Tanksley 1993; Liu 1998). The simplest method for detecting QTL is single-marker analysis based on *t*-test, analysis of variance (ANOVA) and simple linear regression, which assess the segregation of a phenotype with respect to a marker genotype. Linear regression is most commonly used because the coefficient of determination (R^2) from marker explains the phenotypic variation arising from QTL linked to the marker (Collard et al. 2005). This method does not require a complete linkage map and can be performed with basic statistical software programs such as SAS and Minitab**.** The main weakness of single-marker analysis is the failure to provide an accurate estimate of QTL location or recombination frequency between the marker and the QTL, because the evaluation of individual markers is conducted independently, and without reference to their position or order (Doerge 2002). These situations can be addressed by fixing the location of the QTL and estimating the QTL effect between intervals of markers, known as interval mapping (Lander and Botstein 1989).

The simple interval mapping (SIM) uses an estimated genetic map as framework for location of QTL and analyzes intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing each single marker separately (Lander and Botstein 1989). The principle behind interval mapping is to test a model for the presence of a QTL at several positions between two mapped marker loci. The model is fit, and its goodness is tested using the method of maximum likelihood. Maximum likelihood involves searching for QTL parameters which give the best approximation for quantitative trait distributions observed for each marker class. Models are evaluated by computing the likelihood of the observed distributions with and without fitting a QTL effect. The results of test are expressed as LOD (logarithm of the odds) scores, which

compare the evaluation of the likelihood function under the null hypothesis (no QTL) with the alternative hypothesis (QTL at the testing position). The best estimate of the location of the QTL is given by the chromosomal location that corresponds to the highest significant likelihood ratio. Software packages, such as MAPMAKER/QTL (Lincoln et al. 1992), QGene (Nelson 1997) and MapQTL® 5 (van Ooijen 2004) are available for QTL mapping.

There are two problems with the single interval mapping (SIM) method resulting from a single QTL model as mentioned above. One is that the effects of additional QTL will contribute to sampling variance. The other is that combined effects of two linked QTL will cause biased estimates. Composite interval mapping (CIM) combines the interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to adjacent pairs of linked markers for interval mapping (Jansen 1993; Zeng 1993; Jansen and Stam 1994; Zeng 1994). CIM gives more power and precision than SIM because the effects of other QTL are not present as residual variance. Furthermore, CIM can remove the bias that would normally be caused by QTL that are linked to the position being tested. However, a large number of potential QTL and their interactions will lead to innumerable statistical models and heavy computational demands in comparison to statistical approaches to locate multiple QTL. Software packages, such as QTL Cartographer (Basten et al. 1999), MapManager/QTX (Meer et al. 2004) and MultiQTL (Mester et al. 2004) are available to locate multiple QTL.

2.9.5 Chromosomal regions associated with PHS resistance

In bread wheat, 20 chromosomes with the solitary exception of chromosome 1D have been identified to carry QTL for PHS resistance /dormancy based on germination of grains in the spike using artificial rain simulation, germination test, grain colour, Falling Number and α-amylase activity (see Figure 2.4 for QTL locations and references). The large numbers of QTL reported suggests PHS is a trait controlled by numerous genes, influenced by environmental conditions and genetic background. RFLP analyses revealed that eight regions of the wheat genome were associated with PHS-resistance (Anderson et al. 1993). Based on multiple regression analysis, specific sets of markers and their interaction accounted for 44% of the genetic variance for PHS in the population CC/NY18 and 51% in the population NY18/NY10. Roy et al. (1999) associated PHS

with a microsatellite marker and a STS marker on chromosomes 6B and 7D, respectively, indicating that perhaps two genes exhibiting complementary interaction govern the PHS tolerance in cultivar SPR8198. However, in an inheritance study using the cross SPR8198/WL711, PHS tolerance in PSR8198 was shown to be controlled by a single dominant gene (Sharma et al. 1994).

Homoeologous chromosome group 3 and chromosome 4A have been found to carry loci for PHS resistance in several studies (Mares and Mrva 2001; Groos et al. 2002; Osa et al. 2003; Kulwal et al. 2005; Mori et al. 2005; Mares et al. 2005; Tan et al. 2006). Flintham et al. (1999) reported a *Phs* locus on chromosome 7D based on bulk segregant analysis of DH lines derived from the cross Boxer/Soleil. Subsequently analysis using RILs, the *Phs* locus was relocated to the long arm of chromosome 4A. The *Phs* locus cosegregated with marker *Xpsr* 1327 in both the DH and RIL populations, placing it in the region of an ancestral translocation/inversion point between chromosomes 4AS and 5AL (Flintham et al. 2002). A major QTL (*QPhs.ccsu-3A.1*) was detected on 3AL at a genetic distance of ~183 cM from the centromere in a RIL population developed from the cross SPR8198/HD2329 (Kulwal et al. 2005). This QTL explained 24.68% to 35.21% of the phenotypic variation in six different environments and 78.03% of the variation across environments (pooled data). This QTL was not associated with red grain colour, nor did it show a pleiotropic effect of the *R* gene.

The transcription factor VIVIPAROUS-1 encoded by the *Vp1* gene plays a critical role in maintenance and induction of dormancy in maize (McCarty et al. 1991). Maize mutants lacking VP1 activity are *viviparous*, i.e. the immature embryos germinate precociously on the cob. Genes orthologous to maize *Vp1* have been cloned from rice (Hattori et al. 1994), wild oat (Jones et al. 1997), sorghum (Carrari et al. 2001) and *Craterostigma plantagineum* (Chandler and Bartels 1997). Bailey et al. (1999) identified the *taVp1* gene, an orthologue of the *Vp1* gene, at 30 cM from the *R* locus in the distal region of long arm of group 3 chromosomes of wheat. The level of expression of the *Vp1* correlates with the level of seed dormancy in Minamino (dormant) and Tozan 18 (non-dormant) cultivars (Nakamura and Toyama 2001). However, McKibbin et al. (1999) reported that the *taVp1* transcript abundance was similar in developing embryos of dormant and non-dormant genotypes and not associated with the level of dormancy of the

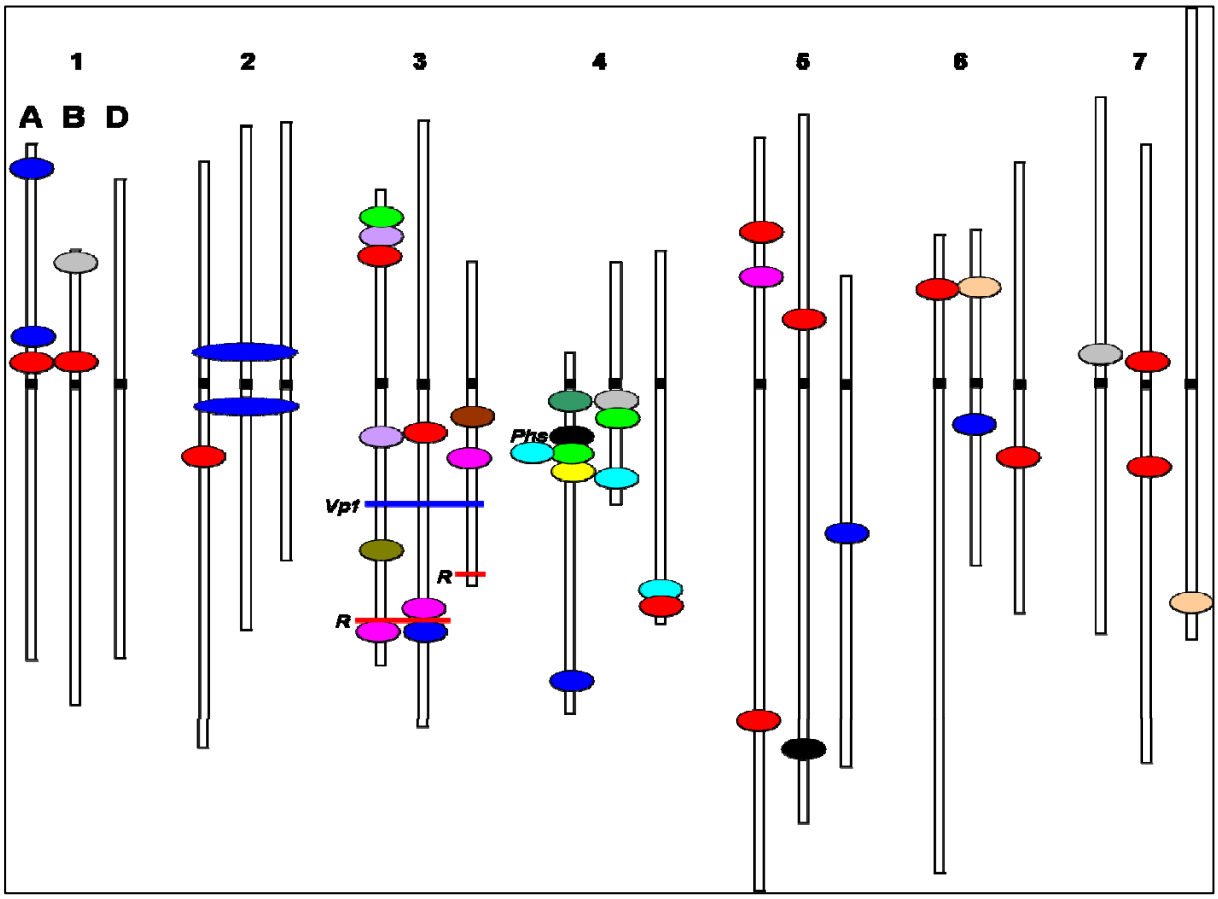


Figure 2.4. Summary of genomic locations of QTL associated with PHS resistance. *Vp1* – transcription factor VIVIPAROUS-1; *R* – Red grain colour loci; *Phs* – Pre-harvest sprouting locus; approximate locations of QTL in following studies: – Anderson et al. 1993; – Roy et al. 1999; – Zanetti et al. 2000; - Mares and Mrva 2001; - Kato et al. 2001; – Flintham et al. 2002; - Groos et al. 2002; – Mares et al. 2002; - Osa et al. 2003; - Kulwal et al. 2005; – Mares et al. 2005; – Mori et al. 2005; – Tan et al. 2006. The chromosome length and centromere positions are based on a wheat consensus map (Somers et al. 2004).

wheat grain. Later studies showed that mis-splicing of the *VP1* gene contributes to the susceptibility to PHS of modern hexaploid wheats (McKibbin et al. 2002). Analysis of *VP-1* transcript structure in wheat embryos during grain development showed that each homoeologue produces mRNA of different sizes. A majority of *VP-1* transcripts are mis-spliced and do not have the capacity to encode full length proteins. Embryos of closely related tetraploid species (*Triticum turgidum*) and ancestral diploids also produce mis-spliced *VP-1* transcripts similar to modern hexaploid wheat suggesting that compromised structure and expression of *VP-1* transcripts in modern wheat are inherited from ancestral species (McKibbin et al. 2002).

2.9.6 Validation of molecular markers associated with a trait

Before a marker can find wide application in crop improvement programs, it is important to validate its usefulness for marker assisted breeding outside the mapping population. Generally, markers should be tested for their effectiveness in determining the target phenotype in independent mapping populations and different genetic backgrounds. However, there is no guarantee that markers identified in one population will be useful in other populations. Parker et al. (1998) identified a marker for wheat flour colour on chromosome 7A based on the cross Schomburgk/Yaralinka and later confirmed it's usefulness in the Cranbrook/Halberd and Sunco/Tasman crosses (Mares and Campbell 2001). But the same marker was not applicable to yellow colour characteristics of lines such as Cunningham and Janz, but was applicable to material with Schomburgk-type yellow flour colour (Sharp et al. 2001). Sharp et al. (2001) also identified a marker linked to stem rust *Sr2* gene in Chinese Spring x Chinese Spring (Hope3B) on chromosome 3B, but the polymorphism was not diagnostic when assayed in a wide range of CIMMYT, Australian and other cultivars of known *Sr2* genotypes.

To date, marker-assisted selection (MAS) has been effective for relatively simple traits governed by single genes (qualitative traits) but less effective for complex traits controlled by many genes (quantitative traits) that are under the influence of gene x gene and gene x environment interactions. A further complication is that estimates of quantitative trait loci (QTL) effects are biased by the necessity of working with limited sets of genotypes in a limited set of environments and hence application of these

estimates may not be as effective as expected when used more broadly within a breeding program (Podlich et al. 2004).

One of the most critically anticipated and most often cited benefits of genetic markers for plant breeding has been their use to facilitate MAS as an indirect selection tool in crop improvement programs (Koebner and Summers 2003). MAS allows breeders to conduct early generation selection for a trait or combination of traits on a single plant basis. This is particularly valuable in situations where a trait is under multigene control, and/or environmental variation has a significant influence on trait expression or the trait is inherited recessively (Koebner and Summers 2002; Koebner and Summers 2003). MAS is also useful in accumulating multiple genes for resistance to specific pathogens and pests within the same cultivar, a process known as gene pyramiding (Huang et al. 1997; Barloy et al. 2007). An additional benefit of MAS is that it can be performed on DNA extracted from small leaf tissue samples and consequently provides a non-destructive, seed quantity independent alternative to phenotypic based selection. Thus, MAS can be used at any stage of a breeding program (Kuchel et al. 2007).

The selection for recombination between target gene and flanking markers is highly effective even when a marker is rather distant from the target gene. MAS can be used for such large distance as recombination occurs with increasing distances with higher probability. The larger the distance between the marker and the gene, the smaller the population size can be chosen (Frisch 2005; Wenzel 2006). However, linkage drag is one of the main concerns in marker-assisted backcrossing if the desired trait is linked to an undesirable character. In introgression of target alleles from unadapted germplasm, linkage drag is the main cause for the differences between the recipient line and converted line. Tightly linked flanking markers can be used for substantial reduction of linkage drag (Frisch et al. 1999).

Recently, a report was published detailing computer simulation based analysis of a specific wheat breeding strategy designed to employ MAS to select for multiple genes (Kuchel et al. 2005). The detailed economic analysis showed that incorporation of marker assisted selection at the BC_1F_1 followed by doubled haploid technology not only increased the genetic gain over the phenotypic alternative but also reduced the overall cost by 40%. The results of practical validation agreed with those of the simulation study

(Kuchel et al. 2007). Introgression of *Lr34*/*Yr18* or *Lr46*/*Yr29* loci in a susceptible recurrent parent resulted in an improvement in leaf rust and stripe rust resistance. Selection for favorable glutenin alleles significantly improved dough resistance and dough extensibility. However, grain yield was improved marginally at one of the five sites used for grain assessment (Kuchel et al. 2007).

Evaluation of markers linked to specific traits in different cultivars could also reveal the origin of superior allele by tracing back to a specific genotype and introduction of new germplasm in that breeding program could significantly contribute to the development of improved genotypes (Malysheva et al. 2004).

2.10 Hypothesis for thesis

Pre-harvest sprouting in wheat is a worldwide problem resulting in substantial economic losses to producers, processors and allied industries because PHS reduces the functional quality of wheat flour and limits the end-use applications. In Canada, such losses from PHS in wheat have been calculated to be $100 million annually in the years favorable for PHS (Derera 1990; Clarke et al. 2005). Therefore, PHS resistant wheat cultivars have the potential to deliver significant savings to producers, processors and industries. Breeding for PHS resistant cultivars is challenging because PHS in wheat is a complex trait controlled by numerous genes and influenced by environmental conditions. In bread wheat, 20 chromosomes with the solitary exception of chromosome 1D are now known to carry QTL for PHS resistance /dormancy (Anderson et al. 1993; Roy et al. 1999; Zanetti et al. 2000; Kato et al. 2001; Mares and Mrva 2001; Flintham et al, 2002; Groos et al. 2002; Mori et al. 2005; Tan et al. 2006). However, a critical analysis of published literature, leads to following hypothesis of my research:

Genomic regions on wheat chromosomes 4A and homoeologous group 3 play major roles in PHS resistance in white-grained bread wheat.

The present investigation was conducted to identify the genomic regions associated with PHS resistance in white-grained wheat and validate the putative QTL for PHS resistance in different genetic backgrounds suitable for marker-assisted selection in Canadian wheat breeding programs.

CHAPTER 3
COMPARISON OF DIFFERENT METHODS FOR PHENOTYPING PHS IN WHITE-GRAINED WHEAT

3.1 ABSTRACT

The objective of this study was to identify a suitable method for phenotyping pre-harvest sprouting (PHS) resistance in white-grained bread wheat. Forty doubled haploid (DH) lines derived from a cross between two white-grained spring wheats (*Triticum aestivum* L.) cultivar 'Argent' (non-dormant) and wheat breeding line 'W98616' (dormant), were evaluated for germination frequency, Falling Number, and α-amylase activity in dry and water-imbibed seeds and spikes. The α-amylase activity in dry seeds or spikes did not differ significantly between parent lines, or lines of the DH population. Wetting of seeds or spikes for two days caused a five- to seven-fold increase in α-amylase activity, but only in 'Argent and the 'non-dormant' sub-group (49-100% germination) of the DH lines. A positive association (r = 0.60***) was detected between germination frequency and α-amylase activity in imbibed seeds and spikes. The germination frequency could not be correlated to Falling Number or α-amylase activity in dry harvested seeds. Falling Number showed a strong correlation (r = -0.83***) to α-amylase activity in dry harvested seeds, but could not be correlated to α-amylase activity in imbibed seeds. In conclusion, the germination test was the most reliable method for measurement of PHS resistance, because seed dormancy provides potential resistance to PHS, whereas high α-amylase activity may occur in grains with or without PHS.

3.2 INTRODUCTION

In-spike germination of physiologically mature grain is denoted as pre-harvest sprouting (PHS) and is a serious problem in susceptible lines of bread wheat (*T. aestivum* L.). The condition occurs when harvest time coincides with relatively high humidity due to untimely rains. The immediate consequences of PHS are reduced yield, decreased

kernel quality and downgrading of the grain, leading to economic losses for the producer. During the period 1978-1988, the estimated loss due to PHS in Western Canada was valued at US $100 million (Derera 1990).

Sprouted grain and flour have increased levels of α-amylase and protease activities with deleterious effects on production of bread and noodle (Edwards et al. 1989). The flour from sprouted wheat will yield sticky dough which, upon baking, produces loaves with large holes, sticky crumb and dark-coloured crusts (Mares et al. 2004). Noodles made from sprouted wheat flour show higher levels of discoloration (spotting) as compared to noodles made from sound flour, and thus are less attractive to the consumer (Hatcher and Symons 2000). Pasta made from semolina of sprouted wheat negatively affects product texture. To assure grain quality with regards to PHS, the Canadian Grain Commission sets limits for total sprouted grain for different wheat grades and classes. For example, severely sprout-damaged and total sprouted kernel frequencies of more than 0.5% and 5%, respectively, in Canada Western Hard White Spring Wheat results in downgrading to Feed Wheat (Official Grain Grading Guide 2007).

Grain sprouting becomes visible when the bran layer surrounding the embryo ruptures and the cotyledon emerges. One of the first biochemical changes associated with germination is increased activities from starch hydrolytic enzymes such as α-amylases, which play an important role in starch degradation. The α-amylases (EC 3.2.1.1) belong to the glycoside hydrolase family 13 (http://www.cazy.org/; Coutinho and Henrissat 1999) and catalyze endohydrolysis of α-(1→4) glycosidic bonds present in amylose and amylopectin chains of starch (van der Maarel et al. 2002). There are four possible sources of α-amylase activity in wheat grains. The primary reason for α-amylase accumulation in the grain is delayed harvest due to wet weather causing breakdown of grain dormancy followed by sprouting (Flintham and Gale 1988). Another major cause of excess α-amylase activity is the deposition of α-amylase in the endosperm cavity (Flintham and Gale 1988; Evers et al. 1995). The third route of α-amylase activity is termed pre-maturity sprouting and involves germination in early development when grains are still at a high moisture content (Flintham and Gale 1988; Lunn et al. 2001a; Lunn et al. 2001b). The fourth possible reason is the retained pericarp α-amylase in grains that has been

associated with non-uniform maturity of the wheat crop (Olered 1975; Lunn et al. 2001a; 2001b).

The resistance to PHS is based on seed dormancy, i.e. the ability of the physiologically mature seed to withstand sprouting under conditions otherwise favourable for germination. Seed dormancy is affected by both genetic and environmental factors and, therefore, PHS in wheat is expressed as a quantitatively inherited trait (Anderson et al. 1993; Tan et al. 2006). Seed germination and dormancy are influenced by a wide range of plant growth regulators, of which abscisic acid (ABA) plays a key role in the maintenance of dormancy (Walker-Simmons 1987; McCarty 1995; Kawakami et al. 1997; Koornneef et al. 2002; Gubler et al. 2005). The red grain colour is a traditional marker for sprouting resistance in wheat, and may act by increasing the sensitivity of embryos to ABA (Himi et al. 2002).

A reliable, reproducible and simple method to determine susceptibility to PHS is a pre-requisite for wheat improvement programs aimed at developing new lines with PHS resistance (Humphreys and Noll 2002; Mrva and Mares 2002; Shorter et al. 2005). PHS measurements are traditionally done by a germination test using threshed seeds or intact spikes that may have been subjected to field or artificial weathering (Mares 1989). The germination test provides a direct measure of seed dormancy, whereas sprouting tests of spikes may include other mechanisms associated with the intact spikes such as germination-inhibitors in the hull (Kato et al. 2002) and spike morphology (King and Richards 1984). Other more indirect methods for PHS estimation rely on determinations of grain α-amylase activity or Hagberg Falling Number (Hagberg 1960; Hagberg 1961). The Falling Number can serve as a gauge for starch degradation and is inversely related to α-amylase activity by a curvilinear relationship (Hagberg 1960; Hagberg 1961). In order to evaluate the different assays for PHS, this study compared different methods using a doubled haploid mapping population for which PHS had been evaluated in a series of field trials. To eliminate the confounding effects of grain colour on PHS, the DH lines used were derived from white-grained spring wheat cultivars.

3.3 MATERIALS AND METHODS

3.3.1 Plant materials and field trials

A population of 151 doubled haploid (DH) lines was used for phenotyping of PHS. The DH lines were developed from a cross between a white-grained spring wheat cultivar 'Argent' (non-dormant) and a white-grained line 'W98616' (dormant). The 'W98616' parent was selected from the AUS1408 (white-grained) / RL4137 (red-grained) cross (Hucl and Matus-Cádiz 2002b), where both parents are considered to be good sources of PHS resistance.

The initial replicated (n=2) field trials involving the whole DH population of 151 lines and parent lines were conducted at the Seed Farm and Kernen Farm, University of Saskatchewan, Saskatoon, in the years 2002 and 2003. In 2005, a four replicate trial in a Randomized Complete Block Design (RCBD) with 40 DH lines (20 dormant and 20 non-dormant lines selected based on the 2002 and 2003 trials), parents ('Argent' and 'W98616') was conducted at the Seed Farm, University of Saskatchewan, Saskatoon, Canada. A total of 50 spikes per line were harvested when the plants had reached the Zadoks growth stage 92, which represents the physiological maturity stage of wheat development (Zadoks et al. 1974). The spikes were air dried for one week and stored at -20°C until analysis. Wetting of spikes and threshed seeds was done by incubation in distilled water for 48 hr at 20°C. Thereafter, an aliquot of the wetted spikes and threshed seeds were separately dried at 35°C for 48 hr.

3.3.2 Germination test

Fifty seeds were placed in Petri dishes (8 cm diameter) containing a Whatman #1 filter paper soaked with 3.0 mL of distilled water. The Petri plates were placed in a large plastic container holding a relative humidity level of >90 % and incubated at 20°C. The germination count was done after seven days. Non-germinated seeds were treated in a Petri-dish with 0.05% w/v gibberellic acid (GA_3) and incubated at 12°C to break dormancy. The final germination count was done after seven days and seed viability of the sample was calculated. Germination tests were performed on four biological replications for each line tested.

3.3.3 Extraction of soluble proteins from flour

Grain samples were milled in a cyclone mill (Udy Corp., Fort Collins, CO) and sifted through a 0.5 mm sieve. Extraction of proteins from the meal was done following the procedure of McCleary and Sheehan (1987). Briefly, 1.0 g of meal and 7.0 ml of malate extraction buffer (50 mM Malic acid, 87.5 mM sodium hydroxide, 50 mM sodium chloride, 2 mM calcium chloride, 3 mM sodium azide, pH 5.2) were added to a 50 mL centrifuge tube, followed by vigorous stirring and incubation at 40°C for 20 min with occasional mixing. The slurry was centrifuged at 1,000 x *g* (Allegra^{TM} Centrifuge, Beckman Coulter, Inc.) for 10 min at room temperature and the supernatant was stored on ice until analysis. The protein concentration in the cell extract was determined by Coomassie blue dye-binding assay (Bradford 1976) using the Quick Start^{TM} Bradford Protein Assay Kit from Bio-Rad Laboratories, Inc., USA.

3.3.4 Determination of α-amylase activity

The α-amylase enzyme activity in the soluble protein extract was analyzed within 2 hr of preparation and was done essentially as described by McCleary and Sheehan (1987). The enzyme extract and substrate mixture of 2 mM blocked *p*-nitrophenyl maltoheptaoside and 1.25 U thermostable α-glucosidase (Megazyme International Ireland Ltd., Ireland) were pre-warmed to 40°C for 5 min and the reaction was initiated by adding 0.2 mL of enzyme extract to 0.2 mL substrate mixture. After 20 min incubation at 40°C, the reaction was stopped by adding 3.0 mL 1% w/v tri-sodium phosphate pH~11 followed by vigorous stirring. The absorbance from released *p*-nitrophenol was measured at 400 nm using a DU® 800 Spectrophotometer (Beckman Coulter, Inc.). The specific α-amylase activity was defined as the amount of enzyme that released one μmole of *p*-nitrophenol from blocked *p*-nitrophenyl maltoheptaoside min^{-1} mg^{-1} protein, when thermostable α-glucosidase is present in excess. For each line tested, the α-amylase assays were performed on four biological replications with three experimental assays per replication.

3.3.5 Determination of Falling Number

Grain samples were milled in a cyclone mill and sifted through a 1-mm sieve. The moisture content in 3.0 g meal samples was determined following the standard AACC Method 44-15A (American Association of Cereal Chemists, 2000). Falling Number was

determined from 7.0 g meal at 14% moisture basis according to the standard AACC Method 56-81B (American Association of Cereal Chemists, 2000) using a FN 1700 instrument (Perten Instruments). Falling Number determinations were performed on four biological replications for each line.

3.3.6 Statistical analysis

Results were expressed as least square (LS) means of three or four replicates ± standard error. The data were compared by Duncan's test at a significance level of 0.05, while relationships between measured parameters were assessed by Pearson's test using Statistical Analysis System software (SAS Institute, Cary, NC).

3.4 RESULTS AND DISCUSSION

3.4.1 Predictability of PHS from germination frequency

Based on germination tests performed on seeds from the entire mapping population of 151 DH lines, a subgroup of 20 'dormant' (8-28% germination) and 20 'non-dormant' (83 to 97 % germination) lines were selected for further evaluation of PHS in 2005. Seeds for laboratory analyses were obtained by growing the lines in a Randomized Plot Design and collecting the spikes when plants had reached physiological maturity. Included in the fields tests were the parent lines 'Argent' (non-dormant) and 'W98616' (dormant). Similar to previous field tests performed in 2002 and 2003, the dormant parent (W98616) and non-dormant (Argent) parent showed large differences in germination percentage, 4% and 100%, respectively, in the 2005 field trial. Among the DH lines, the germination percentage for the 'dormant sub-group' was 2-31% and the 'non-dormant sub-group' showed germination frequencies of 49-100%. The germination frequencies separated the dormant and non dormant DH lines in all three trials (Figure 3.1), which indicated that the germination test is a reliable predictor for PHS resistance and seed dormancy in white-grained bread wheat. Among the three years of field trials, the dormancy levels were higher overall in 2003, as compared to 2002 and 2005. This suggests the influence of environment on seed dormancy.

3.4.2 Predictability of PHS from α-amylase activity levels in pre-wetted seeds

Three different types of samples from harvested seeds and spikes of the dormant line 'W98616' and the non-dormant line 'Argent' were analyzed for α-amylase activity.

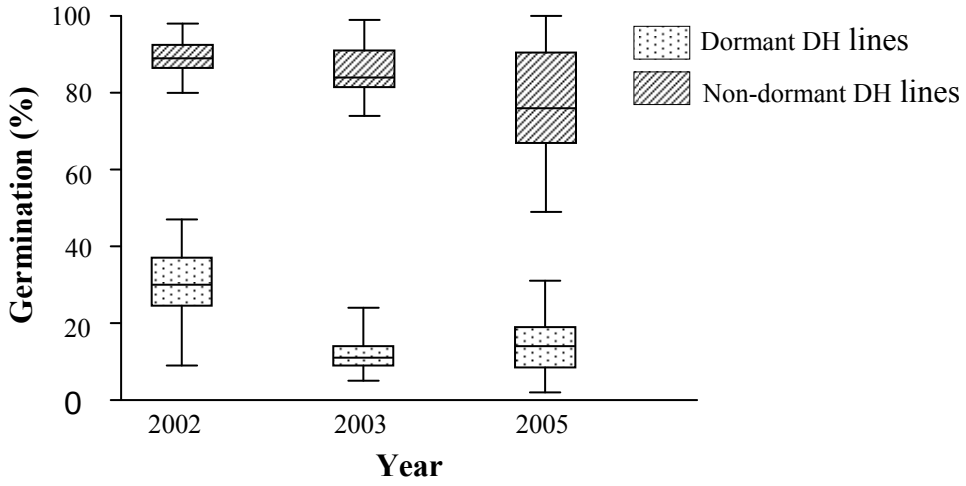


Figure 3.1. Box-and-Whisker plot of germination percentages of 20 'dormant' and 20 'non-dormant' DH lines in three years of field testing. The whiskers are the two lines out side the box that extend to the highest and lowest observations. The line inside the box represents the median of data set. The ends of boxes are upper and lower quartiles.

The sample types analyzed were dry seeds, seeds or spikes that had been pre-wetted for two days, and pre-wetted seeds or spikes that had been dried (Table 3.1). The pre-wetting treatment was done to simulate weather conditions that induce sprouting in the field. The α-amylase activity determined for 'W98616' samples varied from 3.32 to 5.6 μmoles of *p*-nitrophenol min^{-1} mg^{-1} protein (Table 3.1), thus the different treatments of the seeds or spikes had no effect on α-amylase activity in grain of the dormant cultivar. Dry seeds of the non-dormant 'Argent' showed a similar α-amylase activity level (5.46 μmoles of *p*-nitrophenol min^{-1} mg^{-1} protein) as dry seeds from the dormant cultivar. Thus, the level of α-amylase activity in the dry seeds at harvest could not be used to distinguish dormant lines from non-dormant lines. Pre-wetting of seeds or spikes increased α-amylase activity five- to seven-fold, respectively, but only for samples from the non-dormant 'Argent' (Table 3.1). No significant difference was seen between pre-wetted and pre-wetted / dried samples, but the latter sample type was preferred as it was easier to mill compared to the wet samples.

The α-amylase activity in the DH lines was determined on seeds or spikes pre-exposed to moisture then dried, and compared to α-amylase activities in dry seeds (Table 3.2). The mean enzyme activity in dry seeds of the 'dormant' and 'non-dormant' subgroup was 4.2±0.3 and 3.8±0.4 units, respectively (Table 3.2) and did not differ significantly from enzyme activities recorded for the parents (Table 3.1). Thus, it was not possible to separate the dormant and non-dormant lines based on α-amylase activity at harvest time. Similar findings were reported in a study by Hagemann and Ciha (1984). Pre-wetting of seeds or spikes raised the α-amylase activity in seeds of the 'non-dormant' subgroup, whereas α-amylase activity levels in the 'dormant' lines were affected only slightly (Table 3.2). The enzyme activities in pre-wetted samples of the DH lines showed a statistically significant correlation ($r = 0.60$) to germination frequencies (Table 3.3). Similar correlations between sprouting kernels in intact spikes and α-amylase activity ($r^2 = 0.30 – 0.79$) and germination of threshed kernels and of α-amylase activity ($r^2 = 0.07 – 0.81$) in different environments were reported by DePauw et al. (1989).

Table 3.1. Level of α-amylase activity in parental lines.

Treatment	α-amylase activity*	
	W98616 (dormant)	Argent (non-dormant)
Threshed Seeds		
Dry seeds at harvest	5.6 ± 0.3a	5.4 ± 0.4a
Pre-wetted seeds	3.3 ± 0.3a	27.3 ± 4.5b
Pre-wetted and dried	5.3 ± 0.3a	28.5 ± 5.2b
Spikes		
Pre-wetted	4.9 ± 1.4a	39.8 ± 1.2b
Pre-wetted spikes and dried	4.7 ± 0.2a	32.9 ± 9.5b

* Each data point represents three biological replications and three experimental assays in each biological replication. Activity values are expressed as μmoles of *p*-nitrophenol min^{-1} mg^{-1} protein. Values followed by same letter are not significantly different ($P<0.05$)

Table 3.2. Germination frequency, α-amylase activity and Falling Number in dry and imbibed seeds of white-grained wheat DH lines.

DH Lines	Germination Frequency (%)	α-amylase activity* (μmoles of *p*-nitrophenol $min^{-1}mg^{-1}$protein)		Falling Number (sec)
		Dry seeds	Pre-wetted, dried seeds	
Dormant	16±5.5	4.2±0.3	5.5±0.9	367±15.0
Non-dormant	74±7.7	3.8±0.4	16.2±4.6	374±14.4

* Each data point is presented as LS means ± standard error and represents four biological replications.

Table 3.3. Pearson Correlation coefficient between germination frequency, α-amylase activity and Falling Number in white-grained wheat.

	Germination frequency	**α-amylase activity**		**Falling Number**
		Dry seeds at harvest	Pre-wetted and dried seeds	Dry seeds at harvest
Germination frequency	-	-0.16*	0.60***	0.06^{NS}
α-amylase activity (Dry seeds at harvest)		-	0.05^{NS}	-0.83***
α-amylase activity (Pre-wetted and dried seeds)			-	-0.07^{NS}
Falling Number (dry seeds)				-

* significant at .05, *** significant at .0001, and ^{NS} non significant

3.4.3 Falling Number in dry seeds

Falling Number is a measure of starch hot paste viscosity rather than a direct measure of α-amylase activity. The mean Falling Number values of dormant and non-dormant DH lines at harvest were 367±15.0 and 374±14.4, respectively (Table 3.2). Similar to α-amylase activity at harvest, it was not possible to separate the dormant and non-dormant lines based on Falling Number values as no correlation to seed germination was noted (Table 3.3). Poor correlation between Falling Number and germination frequency was also found in a study by Reitan (1989). In a contrasting report, Hagemann and Ciha (1984) found a significant correlation between both germination index (GI) and percent germination (PG) with Falling Number values in un-germinated seeds. Hagemann and Ciha (1984) also concluded that Falling Number and enzymatic tests do not measure the seed's ability to sprout under optimum conditions. In a study by Humphreys and Noll (2002), a higher correlation was noted for in-spike sprouting score and Falling Number when samples were artificially weathered as compared to field weathering. In this study, some lines with high Falling Number were found to have high sprouting scores. Low repeatability of Falling Number due to variety x year interactions was found in a study where in-spike sprouting had a high repeatability (Hucl 1994). Barbeau et al. (2006) suggested that Falling Number should not be used as the sole criteria for determining degree of sprout damage because it does not quantify nor accurately reflect changes in protein composition and quality due to grain weathering.

The analysis of the parent lines and the DH lines for Falling Number revealed a negative correlation ($r = -0.83^{***}$) to α-amylase activity in dry seeds (Table 3.3). No correlation could be found between Falling Number in dry seeds and α-amylase activity in pre-wetted seeds or germination frequency (Table 3.3), and thus Falling Number was not a good predictor for PHS in this study. Similar to this study, the Falling Number and α-amylase activity in dry seeds were negatively correlated ($r = -0.91$) in recombinant inbred lines derived from a wheat x spelt cross (Zanetti et al. 2000), for which little variation in α-amylase activity was noted under dry harvest conditions relative to wet weather at harvest. Ringlund (1983) demonstrated that at low α-amylase activity levels, starch quality becomes the determining factor for Falling Number. Thus, Falling Number

reflects the endosperm quality at harvest time (Hagemann and Ciha 1984), but can fluctuate widely depending on the degree of ripening and the amount of rainfall prior to harvest (Mares 1993). Furthermore, Falling Number sample weight influences test precision, reproducibility and predictability of α-amylase activity (Finney 2001).

3.5 CONCLUSIONS

The primary reason for α-amylase accumulation in the grain is delayed harvest due to wet weather. As sprouting is strongly influenced by environment, the timepoint for sampling and method for evaluating the genotypic differences for PHS is critical. Germination percentage has shown high repeatability in predicting genetic differences in PHS resistance (Wu and Carver 1999), and showed high repeatability in our study based on three years of data. Although we found a strong correlation between FN and α-amylase activity in the dry harvested seeds, the data from these tests could not be correlated with germination percentage. However, a significant correlation was observed between α-amylase activity in pre-wetted seeds and germination percentage and this test could be used to predict PHS. However, artificial or field weathering and determination of α-amylase activity are tedious processes that require specialized equipment, which should be taken into consideration before choosing a method for evaluation of PHS. Given the simplicity of the germination test in combination with its good prediction of PHS resistance based on seed dormancy, this test provided a better proxy for seed dormancy in white grained wheat as compared to Falling Number and α-amylase activity. In addition to germination tests, α-amylase assays may be done to quantify sprout damage.

CHAPTER 4
IDENTIFICATION OF GENOMIC REGIONS ASSOCIATED WITH PHS RESISTANCE IN WHITE-GRAINED WHEAT

4.1 ABSTRACT

Pre-harvest sprouting (PHS) in bread wheat (*Triticum aestivu*m L.) is one of the constraints for consistent production of high quality wheat. White-grained wheat is generally more susceptible to PHS damage than red-grained wheat. The aim of this study was to identify molecular markers linked to quantitative trait loci (QTL) associated with PHS resistance in white-grained wheat. This trait was studied in a doubled haploid (DH) population derived from Argent (non-dormant, white-grained) x W98616 (dormant, white-grained). The DH population was mapped using 913 markers (356 SSR, 290 AFLP, 258 DArT and 9 EST) on different wheat chromosomes with a total genome coverage of 2,577 cM and an average marker density of 3.7 cM/marker. Five genomic regions associated with PHS resistance were identified by interval mapping on chromosomes 1A, 3A, 4A, 7A and 7D, and were all contributed by the dormant parent, W98616. The marker alleles associated with major QTL for grain dormancy on chromosome 4A in white-grained W98616 may be used for marker-assisted selection for introgression of these QTL in white-grained wheats to select PHS resistant genotypes.

4.2 INTRODUCTION

Pre-harvest sprouting (PHS) is defined as in-spike germination of mature grain before harvest due to high moisture conditions during maturation and before harvest. In Western Canada, cool and wet weather during harvest makes the crops susceptible to PHS. This condition is initiated by elevated α-amylase activities which catalyze hydrolysis of endosperm starch and thus provide energy for seed germination. PHS in wheat (*Triticum aestivum* L.) represents a major constraint for consistent production of high quality grain as it causes downgrading of grain, severely limits end-use applications

for wheat flour and results in substantial economic losses to farmers and food processors. Wheat flour from sprouted grains loses its thickening power and produces bread loaves with large holes, sticky crumb and dark-coloured crusts (Mares et al. 2004). The Canadian Grain Commission has set tolerance levels for sprouted grain for each wheat grade produced in Canada. Depending on the quantity of sprouted kernels present in a sample, pre-harvest sprouted wheat is reduced in grade and value, and often graded as low-value animal feed (Official Grain Grading Guide 2007). Producer revenue in Canada decreases by 8.5% for food use with a concomitant and an increase of 2.5% for feed wheat, which reflects the shift from food use to feed use for downgraded wheat (Wahl and O'Rourke 1994).

The development of wheat cultivars with harvest-time seed dormancy has been an important target for wheat improvement programs in many countries where moist harvest conditions frequently occur. Improving PHS resistance is challenging as PHS is expressed as a quantitatively inherited trait that is strongly affected by environmental factors (Anderson et al. 1993). The red grain colour has long been recognised as a genetic marker for PHS resistance in hexaploid wheat (Nilsson-Ehle 1914), but both red and white wheats vary in PHS susceptibility (Bassoi and Flintham 2005). Recent breeding efforts have been focused on developing new cultivars of hard white wheat suited to domestic and international markets, but the low level of PHS resistance is a major constraint for white wheat improvement. The potential resistance of wheat cultivars to PHS is based on seed dormancy (Mares 1987a).

The advent of molecular markers has revolutionized the genetic analysis of complex traits and identification of chromosomal regions associated with disease, insects, agronomic and grain quality traits in wheat (Huang et al. 2000; Liu et al. 2001; Huang et al. 2006). Several quantitative loci (QTL) or genomic regions affecting the PHS resistance or seed dormancy in wheat have been identified in different gene pools via linkage to molecular markers (Anderson et al. 1993; Roy et al. 1999; Zanetti et al. 2000, Kato et al. 2001; Mares and Mrva 2001, Flintham et al. 2002; Groos et al. 2002; Osa et al. 2003; Kulwal et al. 2005; Mares et al. 2005; Mori et al. 2005; Tan et al. 2006). The accumulated QTL mapping studies suggest that several genes are involved in determining

resistance and new loci/genes may be discovered as the number of mapping studies increases.

The aim of this study was to identify the genomic regions associated with PHS resistance in a doubled haploid (DH) mapping population of white-grained bread wheat and to develop molecular markers suitable for marker assisted selection in wheat breeding programs. A linkage map of 2,577 cM based on mapping data from a DH mapping population obtained from two white-grained spring wheat genotypes, W98616 (dormant) and Argent (non-dormant) was developed using SSR, AFLP, DArT and EST markers to identify the genomic regions associated with PHS resistance. DNA markers associated with the genomic regions represent a promising, environment-insensitive tool for selecting genotypes with increased PHS resistance.

4.3 MATERIALS AND METHODS

4.3.1 Mapping population

A mapping population of 151 DH lines was developed from a cross between two white-grained spring wheat (*Triticum aestivum* L.) genotypes, Argent and W98616 (Figure 4.1; Hucl et al. 2005) using the wheat x maize method (Knox et al. 2000). The parent W98616 is a white-grained, dormant line selected from the cross AUS1408/RL4137. Both the white-grained AUS 1408 and red-grained RL4137 are good sources of resistance for PHS. The parent Argent is a white-grained and non-dormant American cultivar.

4.3.2 Phenotyping for pre-harvest sprouting

A 155-entry (151 DH lines with four checks: Argent, W98616, RL4137 and AUS1408), two replicate trials using a RCBD experimental design was grown in 2002 and 2003 at the Seed Farm (SF) and Kernen Farm (KF), University of Saskatchewan, Canada. Individual plots consisted of single rows, 3.7 meter long and 0.3 meter apart at a seed rate of 250 seeds m^{-2}. Fertilizer was drilled in with the seed at a rate of 7 kg ha^{-1} of N and 29 kg ha^{-1} of P. At Zadoks' Growth Stage 92 (Zadoks et al. 1974), 40 spikes per plot were harvested from the upper canopies and air dried at room temperature (22 - 24˚C) for one week. The spikes were bulk threshed using a rubber belt thresher and seeds were stored in a freezer at -20˚C until needed. For seed dormancy determination, germination tests were

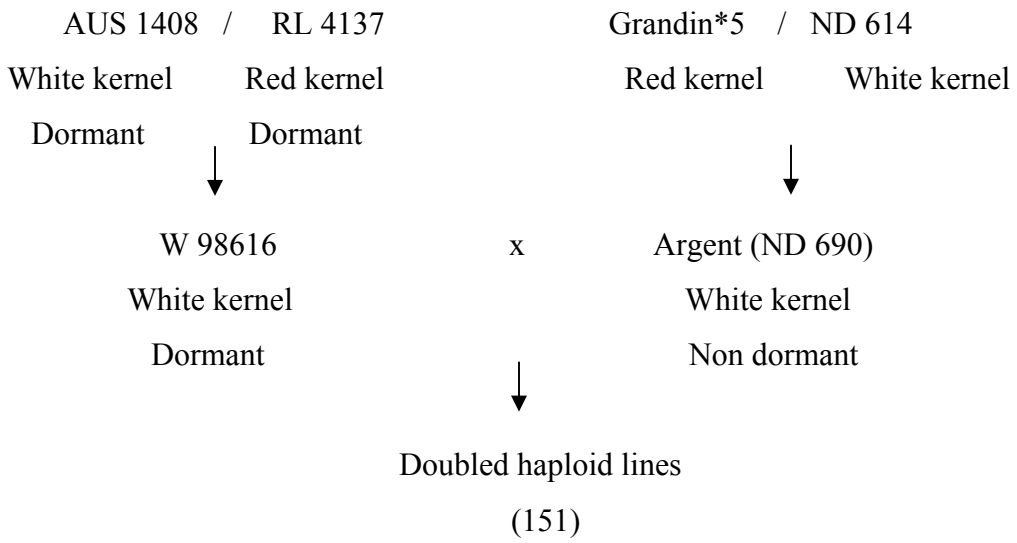


Figure 4.1. Pedigree of the DH mapping population used for identification of QTL for PHS tolerance.

conducted within two months of the harvest. Briefly, one hundred seeds were placed in Petri plates (8 cm diameter), containing a Whatman #1 filter paper soaked with 5.0 ml of distilled water. The Petri plates were incubated at a relative humidity level of >90 % and 20°C. The germination count was performed after seven days. Non germinated seeds were treated with 0.05% (w/v) gibberellic acid (GA_3) solution (1 ml/Petri plate) and incubated at 12°C. The final germination counts were performed after seven days to determine seed viability. Percent germination data were calculated as follows: (no. of germinated seeds/100 imbibed seeds) x 100. Statistical analysis was conducted using Minitab Version 13 (Minitab Inc, State College, PA).

4.3.3 Marker analysis

The parents and DH lines plants were grown under controlled conditions in a growth chamber under an 18 hr photoperiod (250 μmol m^{-2} s^{-1} light intensity, 23°C day and 18°C night temperature). The leaves were harvested after 20 days, freeze dried and genomic DNA was isolated using the CTAB method (Doyle and Doyle 1990). The DNA concentration was quantified using a DU® 800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA).

4.3.3.1 Microsatellite analysis

A total of 689 SSR markers (Table 4.1) were screened for polymorphism between the parental lines W98616 and Argent. The polymerase chain reaction (PCR) was assembled in a 25 μl volume containing 100 ng of genomic DNA, 2.5 μl of 10X PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer, 1.0 unit of REDTaq DNA polymerase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and incubated in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada). The thermocycling program consisted of an initial denaturation at 95°C for 4.15 min, followed by 30 cycles of 45 sec at 95°C, 20 sec at optimized annealing tempratures, 90 sec at 72°C and a final cycle of 10 min at 72°C. The PCR products were initially electrophoresed on 2% (w/v) agarose gel to detect amplification and polymorphism. PCR products were also scored on 6% (w/v) denaturing polyacrylamide gel electrophoresis (PAGE) to detect the polymorphism. A 4 μl aliquot of PCR product was mixed with 4 μl of 2X loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and

0.05% xyanol blue), denatured at 95°C for 2 min and immediately chilled on ice. Samples (3 µl) were loaded on 6% (w/v) denaturing polyacrylamide gels and electrophoresis was done (80 watts, 60 milli amperes and 3000 volts for 1 hr) using a Model S2 sequencing gel electrophoresis apparatus (Mandel Scientific Company Inc., Guelph, Ontario, Canada). The migration of fragments was visualized by silver staining as described by the Silver Sequence protocol (Promega, Madison, WI, USA).

4.3.3.2 Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP analysis using *Eco*RI/*Mse*I primer/adaptor combination was performed as described by Vos et al. (1995). Genomic DNA (300 ng) in a 10 µl reaction volume containing 1 U *Eco*RI, 2 U *Mse*I, 50 pmol *Mse*I adaptor, 5 pmol *Eco*RI adaptor, 1U T4 DNA ligase and 1X digestion-ligation buffer (500 mM Tris Cl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 255 µg/ml BSA and 50 mM NaCl) was incubated overnight at room temperature. After ligation, the reaction mixture was diluted to 18 times and stored at -20°C. Pre-selective amplification was performed with two primers, one corresponding to the *Eco*RI-end with a pre-selective nucleotide A and another corresponding to the *Mse*I-end with a pre-selective nucleotide C. The PCR was performed in a 20 µl volume containing 4 µl of DNA (diluted DNA from ligation reaction), 2.0 µl of 10X PCR buffer, 200 µM of each dNTP, 58 pmol *Eco*RI primer and 57 pmol *Mse*I primer and 1.0 unit of REDTaq DNA polymerase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) in MyCycler™ thermal cycler (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada). The thermocycling program consisted of an initial cycle at 72°C for 3 min, followed by 20 cycles of 20 sec at 94°C, 30 sec at 56°C, 2 min at 72°C and a final step of 30 min at 60°C. The PCR product was diluted 20 times for selective amplification. Selective amplification was performed with different *Eco*RI/*Mse*I primer combinations having three selective nucleotides. The PCR was performed in a 20 µl volume containing 3 µl of DNA (diluted DNA from pre-selective PCR reaction), 2.0 µl of 10X PCR buffer, 200 µM of each dNTP, 6 ng *Eco*RI primer and 30 ng *Mse*I primer and 1.0 unit of REDTaq DNA polymerase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) in MyCycler™ thermal cycler (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada). The thermocycling program consisted of an initial

denaturation at 94°C for 2 min, followed by 20 sec denaturation step at 94°C, 30 sec annealing step (see below), 2 min extension step at 72°C. The annealing temperature in the first cycle was 66°C, which was subsequently reduced in each cycle by 1°C for the next 10 cycles and was continued at 56°C for the remaining 25 cycles. Sixty-four *Eco*RI and *Mse*I AFLP primer combinations were screened for polymorphism and 39 informative primer pairs were used to genotype the mapping population. The nomenclature for an AFLP marker is derived from the enzyme combination, the primer combinations, and the molecular weight (bp) of the product. The sizes of AFLP polymorphic fragments were determined from scanned images of gels by comparing the amplified products to that of standard DNA marker using Quantity One Software (Bio-Rad Lab, Hercules, CA, USA).

4.3.3.3 Diversity Array Technology (DArT®) markers

DArT markers were generated by Triticarte Pty. Ltd. Canberra, Australia (http://www.triticarte.com.au), which is a whole-genome profiling service laboratory, as described by Wenzl et al. (2004) and Akbari et al. (2006). DNA samples were prepared according to the company's specifications. 100 µl DNA of the parents in duplicate and 90 DH lines in a 96 well PCR plate were provided for genotyping. The locus designations used by Triticarte Pty. Ltd. were adopted in the linkage maps. DArT markers consisted of the prefix "wPt", followed by numbers corresponding to a particular clone in the genomic representation, where w stands for wheat, P for *Pst*I (primary restriction enzyme used) and t for *Taq*I (secondary restriction enzyme).

4.3.3.4 Expressed Sequence Tag (EST) analysis

The ESTs were selected from C-4AC-4AL12-0.43, C-4AS1-0.20 and 4AL13-0.59-0.66 deletion bins of chromosome 4A and corresponding regions in the rice genome (Table 3). The ESTs were selected based on possible candidate genes involved in germination, dormancy and ABA signaling genes. Two oligonucleotide primer pairs for each EST were designed using the software Primer3 (Rozen and Skaletsky 2000). Primers were designed to amplify a PCR product in the range of 300-500 bp. The PCR products that appeared monomorphic by agarose gel analysis were further analysed by single strand conformation polymorphism (SSCP) gels (Orita et al. 1989; Tondelli et al. 2005).

4.3.4 Genetic linkage analysis

JoinMap® 3.0 (van Ooijen and Voorrips 2001) was used for linkage analysis of mapping data. Markers were assembled into linkage groups at a LOD score of ≥3.0. A linkage group was assigned to a chromosome when it contained at least three SSR or DArT markers or both that had been assigned to a particular chromosome in previously published genetic maps (http://wheat.pw.usda.gov/ggpages/map_summary.html; Röder et al. 1998; Somers et al. 2004; Akbari et al. 2006; Semagn et al. 2006). Final mapping was done by combining two or more linkage groups that belong to the same chromosome. The final map was drawn using the MapChart program, v. 2.1 (Voorrips 2002). Recombination fractions were converted into genetic map distance in centiMorgans (cM) using the Kosambi mapping function (Kosambi et al. 1944). The segregation ratio at each locus and its deviation from the expected ratio were determined using the chi-square (χ^2) test.

4.3.5 QTL analysis

MapQTL® 5 (van Ooijen 2004) software was used for QTL analysis using two different analysis methods. Firstly, a Kruskal-Wallis rank sum test was performed to find the association between individual marker and germination percentage. From this test, putative QTL were identified for the markers with mean marker classes significantly different at $P<0.01$. Secondly, identification of major QTL on the genome was carried out by interval mapping (Lander and Botstein 1989). A log likelihood (LOD) score threshold of 3.0 was used to identify genomic regions containing putative QTL associated with PHS resistance.

4.4 RESULTS AND DISCUSSION

4.4.1 Analysis of germination frequency in doubled haploid population

There were clear differences in percent germination between Argent and W98616 and DH lines differed significantly in percent germination at 20ºC. The results shown in Figures 4.2 and 4.3 are based on the mean averaged over two years and two locations each year. W98616 (dormant) and Argent (non-dormant) parents showed germination percentage of 4% and 97%, respectively (Figure 4.3). Percent germination for the DH

lines differed significantly and ranged from 8 to 97% for DH lines. The DH lines showed a continuous distribution (Figure 4.2) of trait values which is in agreement with the distribution expected for a quantitatively inherited trait. The germination frequencies separated the dormant and non-dormant DH lines in both years, which indicated that the germination test is a reliable predictor for PHS resistance or seed dormancy in bread wheat (Chapter 3). Overall, a higher dormancy level was observed for DH lines in 2003 relative to 2002. Based on the germination percentage of DH population, 20 dormant lines (germination $\leq$ 28%) and 20 non-dormant lines (germination $\geq$ 83%) were used for mapping SSR and AFLP markers, whereas, 90 lines (40 from both extremes and 50 in the midrange) were used for mapping DArT markers (Figure 4.3).

4.4.2 Genetic linkage map of Argent X W98616

A total of 689 SSR markers (Table 4.1) were screened for polymorphism between the parent lines Argent and W98616. Between the two parents 316 (45.9%) of SSR markers showed polymorphism for one or more alleles. Fifty-two (16.5%) of polymorphic SSR primer pairs amplified more than one polymorphic allele between the two parents. Röder et al. (1998) also reported 20% of gwm primer pairs amplifying more than one locus. About 90% of the wheat genome consists of repetitive DNA, of which more than 50% are retro-elements (Li et al. 2004). If a SSR marker resides within these sequences, non-orthologous loci could be amplified (Röder et al. 1998).

Sixty-four *Eco*RI and *Mse*I AFLP primer combinations were screened for polymorphism and 39 informative primer pairs were used to genotype the mapping population (Table 4.2). Three hundred and six polymorphic markers were scored ranging from 2 to16 markers per *Eco*RI and *Mse*I AFLP primer combination. Twenty-five primer combinations resulted in either poor amplification or showed no polymorphism between parents, and thus, were not used for genotyping the mapping population.

The genotyping data of 295 DArT markers was generated by Triticarte Pty. Ltd. Canberra, Australia. Twenty-eight DArT markers were not included in linkage analysis because they were either monomorphic in the parents but polymorphic in the population or data on parents was missing. The overall call rate and P value of DArT markers was 94.69% and 84.97%, respectively.

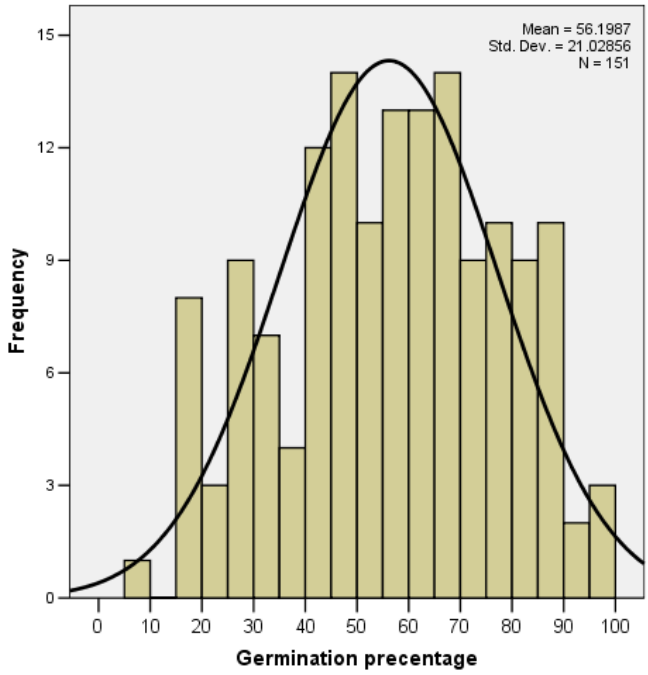


Figure 4.2. Frequency distribution of germination percentage at 20ºC for Argent x W98616 DH population. (Source: Matus-Cádiz and Hucl, Dept. of Plant Sciences, Univ. of Saskatchewan).

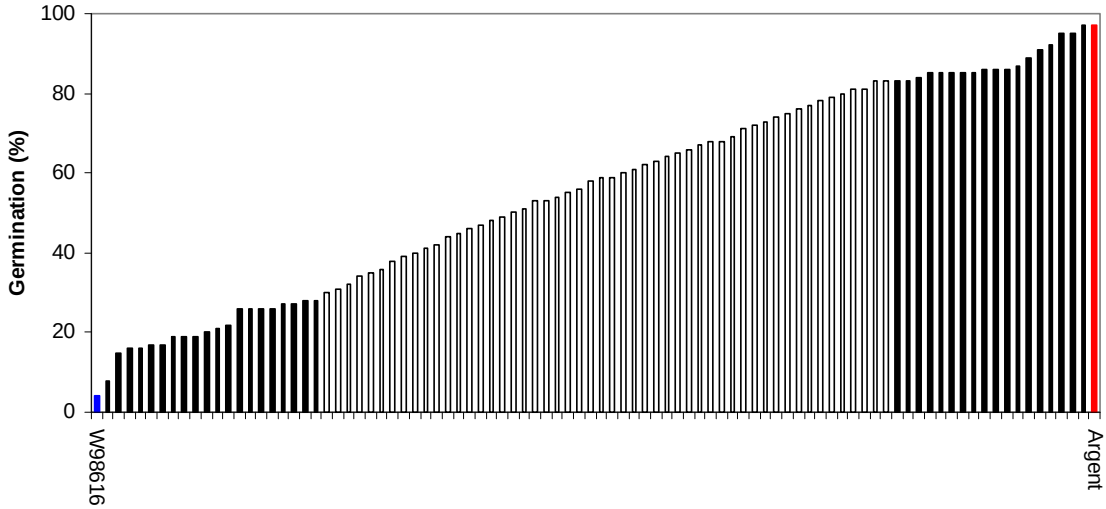


Figure 4.3. Germination percentage for 90 lines of Argent x W98616 DH population. DH lines represented by with black bars were used for mapping SSR and AFLP markers, and all 90 DH were used for mapping DArT markers.

A total of 972 polymorphic markers (390 SSR, 306 AFLP, 267 DArT and 9 EST) were obtained from the Argent / W98616 mapping population. Out of 972 markers, 913 markers (356 SSR, 290 AFLP, 258 DArT and 9 EST) were mapped on different wheat chromosomes (Table 4.3; 4.4), whereas 59 markers (34 SSR, 16 AFLP, 9 DArT) could not be mapped to any linkage group. Most of the unmapped SSR markers belonged to SSR primers which amplified more than one allele. The distribution of the markers among the 21 chromosomes was uneven, with the D genome showing lower polymorphism than the A and B genomes (Table 4.4). The number of markers on chromosomes ranged from 12 makers on 2D to 91 markers on 2B. The B genome had the highest number of markers (425), followed by A and D genomes with 312 and 176 markers, respectively. There was also variation in the number of markers on homoeologous groups. The homoeologous groups 1 and 7 had 152 markers, whereas, homoeologous group 3 had the lowest number of markers (99).

The genetic length of wheat chromosomes ranged from 26 cM on 2D to 183 cM on 5B. A, B and D genomes had genetic length of 962, 888 and 727 cM, respectively, with a total genome coverage of 2,577 cM. Similar sizes of maps were reported by Somers et al. (2004) covering 2,569 cM and a winter wheat map of 2,873 cM by Båga et al. (2007). However, genetic maps of 3,685 cM and 3,522 cM in length have been reported (Sourdille et al. 2003; Quarrie et al. 2005). The genetic length of maps derived from recombinant inbred lines (RILs) is generally longer compared to doubled haploid populations due to increased number of meiosis, and thus recombinations in RILs than DH lines (Somers et al. 2004).

The density of markers ranged from 2.0 cM/marker on 2B to 10.8 cM/marker on 4D, with an average of 3.7 cM/marker. There was variation in marker density on different homoeologous groups, ranging from 2.4 cM/marker for group 2 to 4.5 cM/ marker for group 3 chromosomes. The marker density in the B genome was the highest (2.7 cM/marker), followed by the A genome with 4.0 cM/marker. The D genome had the lowest marker density (5.4 cM/marker) compared to the A and B genomes. The low polymorphism in the D genome compared to the A and B genomes is well known and it is in agreement with the hypothesis of a monophyletic introduction of the D genome in

Table 4.1. Summary of SSR markers used in this study.

SSR code	SSR used in the study			Source
	Total	Polymorphic	Polymorphism %	
gwm	207	107	51.7	Röder et al. 1998
wmc	176	82	46.6	http://wheat.pw.usda.gov
barc	186	84	45.2	http://wheat.pw.usda.gov
cfa	20	5	25.0	http://wheat.pw.usda.gov
cfd	81	27	33.3	http://wheat.pw.usda.gov
gdm	11	8	72.7	Pestsova et al. 2000
DuPw	2	1	50	Eujayl et al. 2002
ksum	5	1	20	http://wheat.pw.usda.gov
gpw	1	1	100	Nicot et al. 2004
Total	689	316	45.9	

Table 4.2. AFLP primer pair combinations with selective bases and number of polymorphic bands.

*Mse*I primer		M47	M48	M49	M50	M59	M60	M61	M62
*Eco*RI primer	Selective bases	CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
E32	AAC	-*	6	8	-	-	-	-	-
E33	AAG	-	2	-	-	-	-	6	-
E35	ACA	-	3	7	-	-	3	9	7
E36	ACC	-	-	13	6	-	-	10	7
E37	ACG	13	11	12	3	-	6	4	12
E38	ACT	-	-	11	6	-	16	-	3
E40	AGC	-	11	12	5	9	6	6	11
E41	AGG	2	8	10	13	8	4	10	5

*- Poor amplification

Table 4.3. Expressed sequence tags (EST) mapped in this study.

EST	Putative function	Primer sequence (5' - 3')
BE406676	Oxalate oxidase GF-2.8 precursor (Germin)	Forward- GGTTCTGAAGGCTGAACTGC
		Reverse- CATGAACCGTGTGGACTTTG
BE426203	Translocation protein TolB precursor	Forward- AACGTCATCTTCTTCCACCG
		Reverse- TACTCGTAGCGACTGTGCGT
BE426646	Putative transport protein	Forward- AAAGTGGTGGCTCATCTGCT
		Reverse- CTGCCTAAAAAGCCATCTCG
BE442666	Lipoxygenase	Forward- CAACAAGCTGGAAGGCAACT
		Reverse- ATGGCAGGGTACTCGTTCAC
BQ170322	Signal transduction (Ca binding motif / Calmodulin)	Forward- CGCAAGATGAAGGACACTGA
		Reverse- CACCAGGGAGGACACAAGTT
CD920298	ABA induced plasma membrane protein PM19	Forward- TAACCATGCTGGTGGATACG
		Reverse- CATCCACATCCAGACACTGC
U80037	ABA induced plasma membrane protein PM19	Forward- CTGGTGCTGAACCTCATCAT
		Reverse- CATGGTTGCCAAACTGGTTC
P450 (Rice chromosome 3)	P450 monooxygenase	Forward- GTATGCTGTCCCGTGGTTCT
		Reverse- GACAGGAGGTCGTGCTTCTC

Table 4.4. The distribution of SSR, AFLP, DArT® and EST markers and the density of markers on the genetic linkage map of Argent x W98616.

Chromosome	No. of markers						No. of Linkage groups	Genetic length	Marker density (cM/marker)
	SSR	AFLP	DArT	EST	Total	Unique loci			
1A	18	21	14	0	53	38	1	154	4.1
1B	18	26	19	0	63	45	2	132	2.9
1D	17	7	12	0	36	28	1	80	2.9
2A	17	11	7	0	35	26	3	77	3.0
2B	23	39	28	1	91	65	3	132	2.0
2D	8	2	2	0	12	8	3	26	3.3
3A	21	11	11	0	43	37	2	172	4.6
3B	9	5	14	0	28	25	3	96	3.8
3D	13	8	7	0	28	18	2	90	5.0
4A	24	18	14	2	58	46	2	133	2.9
4B	14	8	8	3	33	28	2	74	2.6
4D	13	3	1	0	17	11	2	119	10.8
5A	16	4	3	0	23	18	3	137	7.6
5B	25	30	30	1	86	67	1	183	2.7
5D	22	3	6	1	32	28	2	175	6.3
6A	10	10	17	0	37	30	2	116	3.9
6B	16	25	20	0	61	46	2	150	3.3
6D	17	5	3	0	25	19	3	97	5.1
7A	23	30	9	1	63	44	2	173	3.9
7B	16	20	27	0	63	48	1	121	2.5
7D	16	4	6	0	26	23	2	140	6.1
A genome	129	105	75	3	312	239	15	962	4.0
B genome	121	153	146	5	425	324	14	888	2.7
D genome	106	32	37	1	176	135	15	727	5.4
Group1	53	54	45	0	152	111	4	366	3.3
Group2	48	52	37	1	138	99	9	235	2.4
Group3	43	24	32	0	99	80	7	358	4.5
Group4	51	29	23	5	108	85	6	326	3.8
Group5	63	36	39	2	141	113	6	495	4.4
Group6	43	40	40	0	123	95	7	363	3.8
Group7	55	54	42	1	152	115	5	434	3.8
Total	356	290	258	9	913	698	44	2577	3.7

bread wheat (Lagudah et al. 1991). Also, the D genome has a lower DNA content (4,024 Mb) than *A. speltoides*, the B-genome donor (4,886 Mb), and *T. monococcum*, the A-genome donor (5,751 Mb) (Arumuganathan and Earle 1991; Bennett and Leitch 1995). The high level of polymorphism in B genome of wheat may be that B genome has evolved more rapidly compared to the A and D genomes since the emergence of polyploidy (Sourdille et al. 2001). It may also be due to the outcrossing behavior of *Aegilops speltoides* or the B genome could have a polyphyletic origin as suggested by Vardi (1973).

A total of 913 loci could be arranged into 44 linkage groups (Figure 4.4). Each linkage group was assigned at a LOD score of $\geq$ 3.0. The linkage group on different chromosomes varied from one to three. Only four chromosomes viz. 1A, 1D, 5B, 7B, were represented by a single linkage group. The homoeologous group 2, and chromosomes, 3B, 4D and 6D had three linkage groups, whereas, the remaining chromosomes each had two linkage groups. The main reason for the occurance of multiple linkage groups was that only 40 DH lines were used for mapping SSR and AFLP markers. However, for DArT markers, 90 DH lines were used for genotyping. There could be some disagreement in the order of closely linked markers with the consensus map. In a smaller population the chances of informative recombinant progeny lines occurring in the mapping population to accurately position the markers is lower than in a larger population (Somers et al. 2004). The marker order is also conditioned by the population used and position of the cross-over along the chromosomes within the mapping population (Somers et al. 2004).

With a few exceptions, chromosomal location and marker order were similar to other published maps (Röder et al. 1998; Somers et al. 2004; Båga et al. 2007; http://wheat.pw.usda.gov/ggpages/map_summary.html; http://www.diversityarrays.com). A total of 22 (2.4%) loci showed different chromosomal locations (Table 4.5). These discrepancies between maps are likely due to mapping population size and genetic backgrounds, limitations of the software to resolve alternative markers, error in polymorphism scoring and different paralogous alleles scored in different mapping studies (Båga et al. 2007). Earlier studies also reported these discrepancies in chromosomal location of SSR and RFLP markers (Båga et al. 2007; Singh et al. 2007a).

Seventeen DArT markers were not mapped previously (Table 4.5) and the chromosomal location of these markers in this map may help in integrating these markers in future mapping studies.

4.4.2.1 Segregation distortion

The segregation ratios of all the markers were tested for fit to the expected 1:1 ratio by Chi-squared analysis. Of the markers used for mapping, 87 markers showed segregation distortion. Among the 87 distorted loci, 64 (7.02%) and 23 (2.52%) showed distortion at $p<0.05$ and $p\leq0.01$. Forty-one markers showed segregation distortion in favour of Argent and 46 showed segregation towards W98616. Distorted regions favoring the Argent allele were found on chromosomes 2B, 3B, 3D, 6A and 6D, whereas, regions on chromosomes 1B, 4A, 5B, 5D, 7A and 7D showed preference for W98616 alleles (Figure 4.4). The markers with distorted segregation were not randomly distributed and most of these markers were clustered in distorted regions on the chromosomes. Segregation distortion is a common phenomenon in all mapping populations (F_2, DH or RILs) and RILs have the highest probability of distortions due to continued selfing for five to six generations (Singh et al. 2007a). Segregation distortions have been reported in all the interspecific or intraspecific crosses used for generating linkage maps in diploid and hexaploid wheat species (Paillard et al. 2003; Liu et al. 2005 and Singh et al. 2007a). Segregation distortion in interspecific and intraspecific crosses mainly results from competition among male gametes in fertilization or from abortion of male gametes or zygotes (Lyttle 1991). Genetic differences among pollen may lead to gametophyte competition and selection, which result in non-random fertilization. Alternatively, hybrid sterility genes that cause the abortion of specific gamete or zygote genotypes can give rise to segregation distortion (Faris et al. 1998). Sears and Sears (1978) were unable to recover a telo-7DL plant in 'Chinese Spring'. It may be possible that the distorted segregation observed on 7D was caused by a gametophyte selection factor. A hybrid male sterlity (ms) gene is known to exists on 4B (Sears 1966) and it may be possible that a homoeoallele exists on 4A. The evidence of preferential male transmission factors present on 5DL could be a reason that ditelosomic 5AS, 5BS and 5DS are the few unavailable ditelosomic lines of Chinese Spring wheat (Faris et al. 1998). There are several other reasons for explaining non-Mendelian segregation within wheat × maize-

Table 4.5. Markers mapped on different chromosomes in the Argent x W98616 DH mapping population compared to published studies.

Markers	Chr.	Previously mapped	Markers	Chr.	Previously mapped
wPt-1179	7A	5B	wPt-8760	2B	
wPt-1695	6A	6D	wPt-8849	7B	
wPt-2410	2B		wPt-9205	5B	6A
wPt-2479	6B		wPt-9215	7B	3A
wPt-2587	6B		barc28	2A,6B	1A,5B
wPt-3773	4A		barc90	4B	2D,7B
wPt-4415	6B		barc287	1D	1A
wPt-5188	6B	2B/6A	cfa2141	5B	5A,5D,2D
wPt-5558	4B		gwm32	2B	3A
wPt-6199	2B		gwm44	5B	4A,7D
wPt-6239	3B		gwm107	1B	3B,4B,6B
wPt-6376	3B		gwm132	7B	2B,6A,6B,6D
wPt-6673	7B		gwm304	1D	2A,5A
wPt-6878	6B		gwm340	3A	3B
wPt-7320	2B		gwm410	4D	2B,5A
wPt-7629	2A		gwm448	6A	2A,2D,4B
wPt-7642	7D	6B	gwm558	1A	2A
wPt-7894	7B		gwm642	6A	1D
wPt-7906	6A		wmc762	7A	3B

Refer to the following links to find the previously mapped position of markers:
http://wheat.pw.usda.gov/ggpages/map_summary.html;
http://www.diversityarrays.com

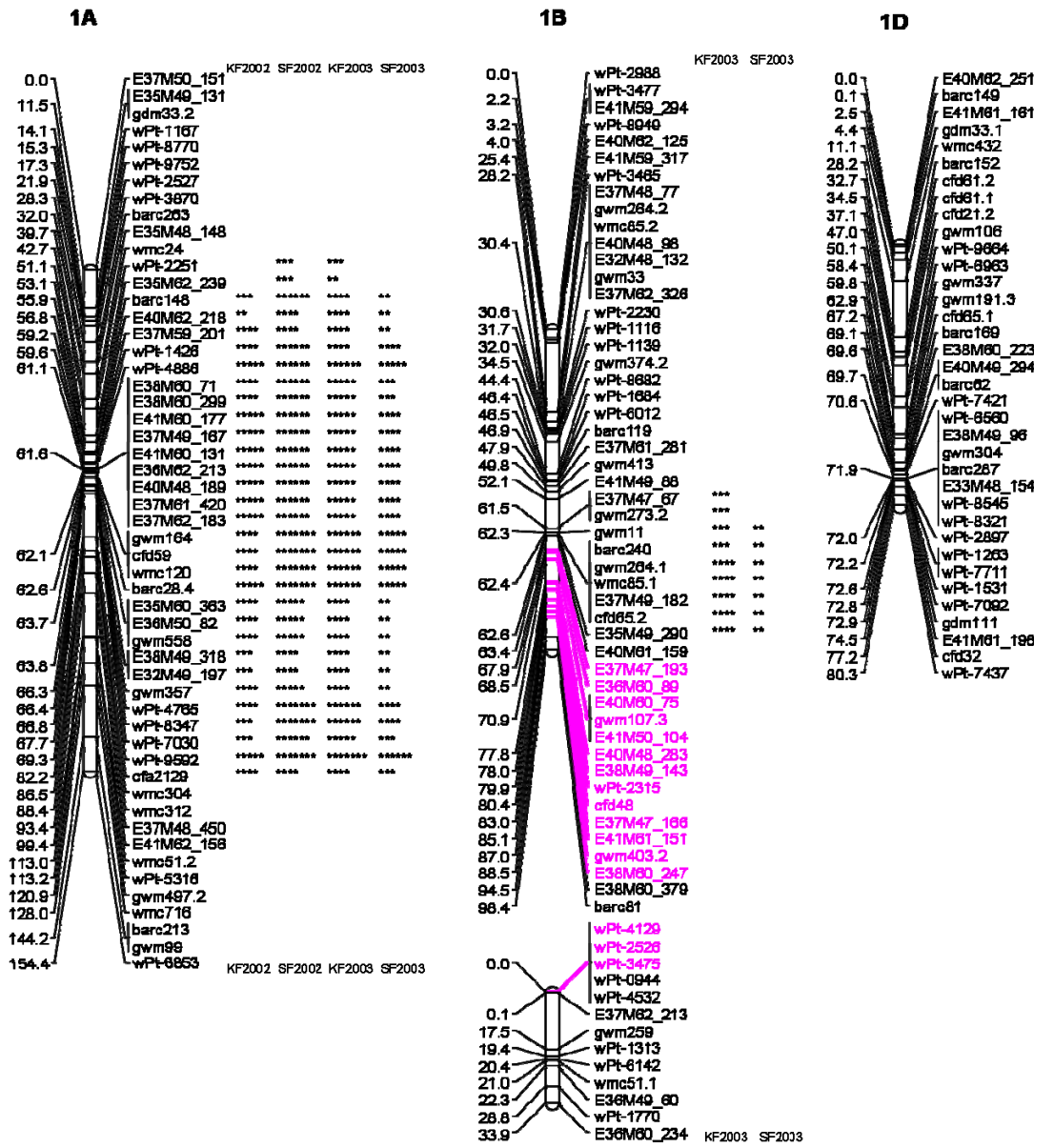
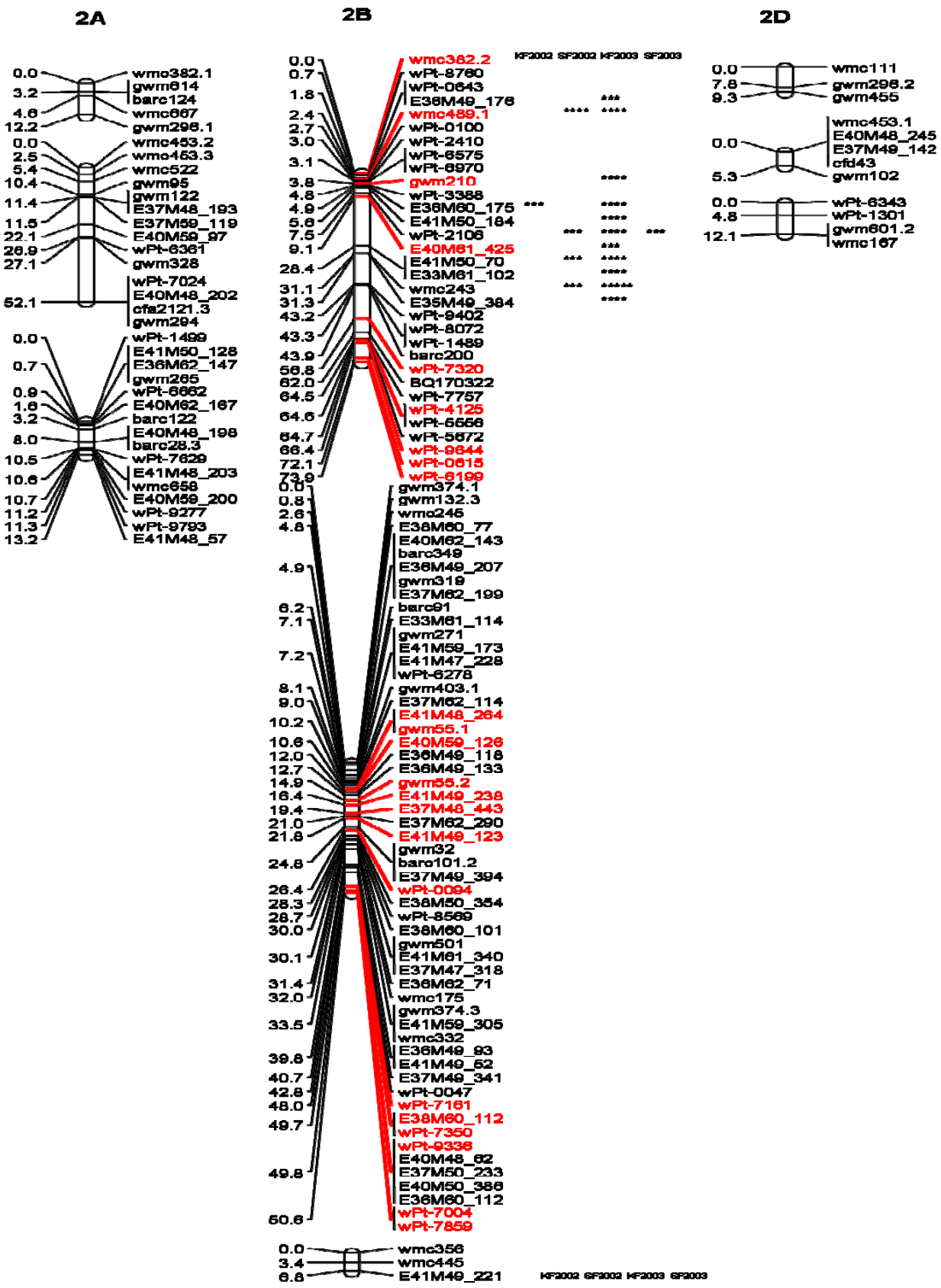


Figure 4.4. Genetic linkage map of Argent x W98616 DH population and location of markers associated with PHS resistance. Loci that cosegregated are grouped beside a vertical line *on the right side of chromosome bars*. Marker positions are given in Kosambi cM *on the left side of chromosome bars*. Microsatellites amplifying more than one locus have the suffix ".1", ".2" or ".3". Single markers associated with PHS resistance based on Kruskal-Wallis test are indicated by ** (p<0.05), *** (p<0.01), **** (p<0.005), ***** (p<0.001), ****** (p<0.0005), ******* (p<0.0001) in four trials Kernen farm (KF) and Seed farm (SF) in 2002 and 2003. Markers in magenta and red colours are distorted in favor of W98616 and Argent alleles, respectively.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2B
2D
KF2002 SF2002 KF2003 SF2003
wmc382.2
wPt-8760
wPt-0643
E38M49_176
wmc489.1
wPt-0100
wPt-2410
wPt-6575
wPt-8970
gwm210
wPt-3388
E38M60_175
E41M50_184
wPt-2106
E40M61_425
E41M50_70
E33M61_102
wmc243
E35M49_384
wPt-9402
wPt-8072
wPt-1489
barc200
wPt-7320
BQ170322
wPt-7757
wPt-4125
wPt-6566
wPt-5672
wPt-9644
wPt-0615
wPt-8199
gwm374.1
gwm132.3
wmc245
E38M60_77
E40M62_143
barc349
E38M49_207
gwm319
E37M62_199
barc91
E33M61_114
gwm271
E41M59_173
E41M47_228
wPt-6278
gwm403.1
E37M62_114
E41M48_264
gwm55.1
E40M59_126
E38M49_118
E38M49_133
gwm55.2
E41M49_238
E37M48_443
E37M62_290
E41M49_123
gwm32
barc101.2
E37M49_394
wPt-0094
E38M50_354
wPt-8569
E38M60_101
gwm501
E41M61_340
E37M47_318
E38M62_71
wmc175
gwm374.3
E41M59_305
wmc332
E38M49_93
E41M49_62
E37M49_341
wPt-0047
wPt-7161
E38M60_112
wPt-7350
wPt-9336
E40M48_62
E37M50_233
E40M50_386
E36M60_112
wPt-7004
wPt-7859
wmc356
wmc445
E41M49_221
KF2002 SF2002 KF2003 SF2003
wmc382.1
gwm614
barc124
wmc667
gwm296.1
wmc453.2
wmc453.3
wmc522
gwm95
gwm122
E37M48_193
E37M59_119
E40M59_97
wPt-6361
gwm328
wPt-7024
E40M48_202
cfa2121.3
gwm294
wPt-1499
E41M50_128
E38M62_147
gwm265
wPt-6662
E40M62_167
barc122
E40M48_198
barc28.3
wPt-7629
E41M48_203
wmc658
E40M59_200
wPt-9277
wPt-9793
E41M48_57
wmc111
gwm296.2
gwm455
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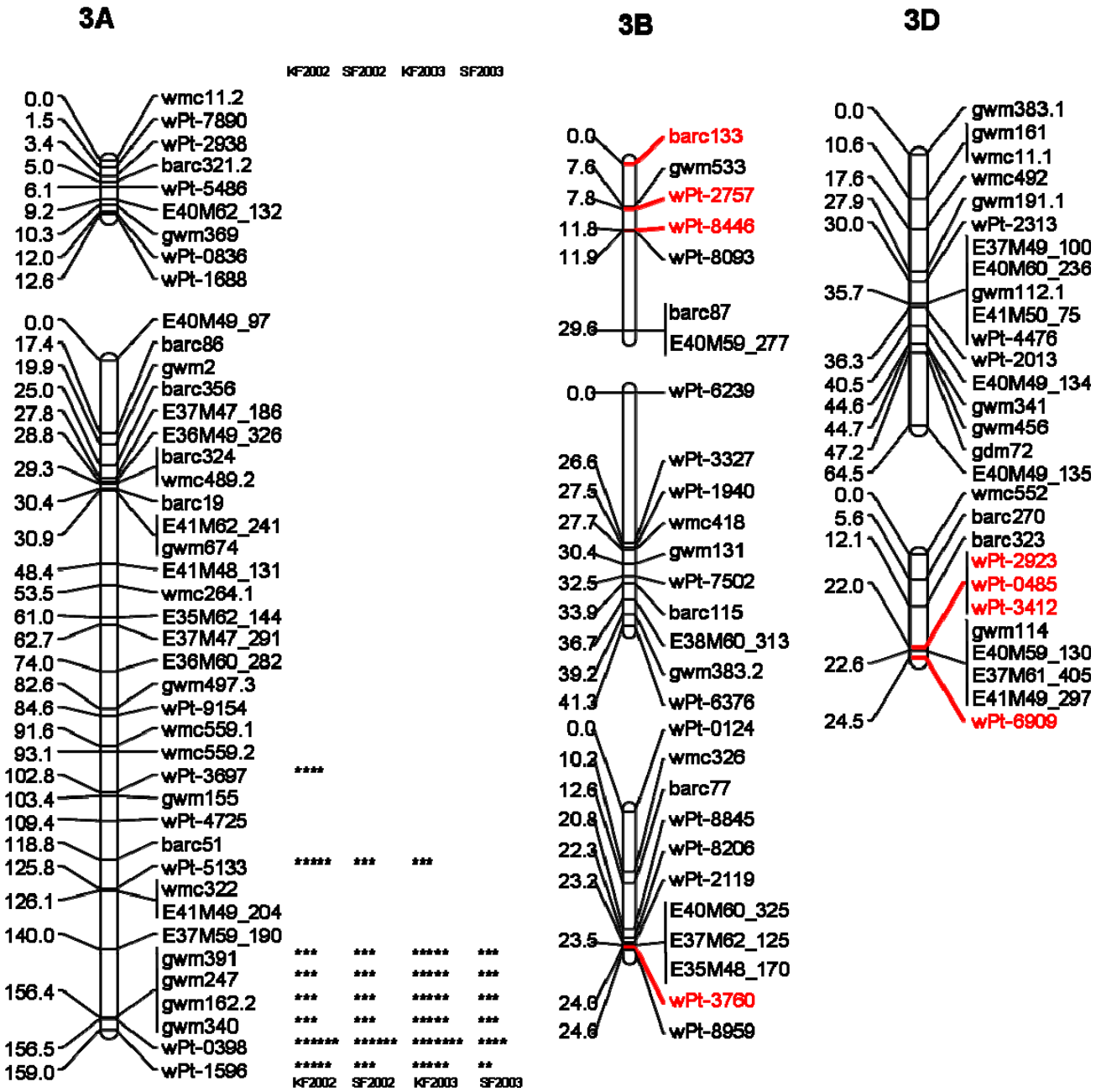
E37M49_142
cfd43
gwm102
wPt-6343
wPt-1301
gwm601.2
wmc167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3B
3D
KF2002 SF2002 KF2003 SF2003
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3.4 wPt-2938
5.0 barc321.2
6.1 wPt-5486
9.2 E40M62_132
10.3 gwm369
12.0 wPt-0836
12.6 wPt-1688
0.0 E40M49_97
17.4 barc86
19.9 gwm2
25.0 barc356
27.8 E37M47_186
28.8 E36M49_326
29.3 barc324
wmc489.2
30.4 barc19
30.9 E41M62_241
gwm674
48.4 E41M48_131
53.5 wmc264.1
61.0 E35M62_144
62.7 E37M47_291
74.0 E36M60_282
82.6 gwm497.3
84.6 wPt-9154
91.6 wmc559.1
93.1 wmc559.2
102.8 wPt-3697
103.4 gwm155
109.4 wPt-4725
118.8 barc51
125.8 wPt-5133
126.1 wmc322
E41M49_204
140.0 E37M59_190
156.4 gwm391
gwm247
gwm162.2
gwm340
156.5 wPt-0398
159.0 wPt-1596
0.0 barc133
7.6 gwm533
7.8 wPt-2757
11.8 wPt-8446
11.9 wPt-8093
29.6 barc87
E40M59_277
0.0 wPt-6239
26.6 wPt-3327
27.5 wPt-1940
27.7 wmc418
30.4 gwm131
32.5 wPt-7502
33.9 barc115
36.7 E38M60_313
39.2 gwm383.2
41.3 wPt-6376
0.0 wPt-0124
10.2 wmc326
12.6 barc77
20.8 wPt-8845
22.3 wPt-8206
23.2 wPt-2119
23.5 E40M60_325
E37M62_125
E35M48_170
24.6 wPt-3760
24.6 wPt-8959
0.0 gwm383.1
10.6 gwm161
17.6 wmc11.1
27.9 wmc492
30.0 gwm191.1
35.7 wPt-2313
E37M49_100
E40M60_236
gwm112.1
E41M50_75
36.3 wPt-4476
40.5 wPt-2013
44.6 E40M49_134
44.7 gwm341
47.2 gwm456
64.5 gdm72
E40M49_135
0.0 wmc552
5.6 barc270
12.1 barc323
22.0 wPt-2923
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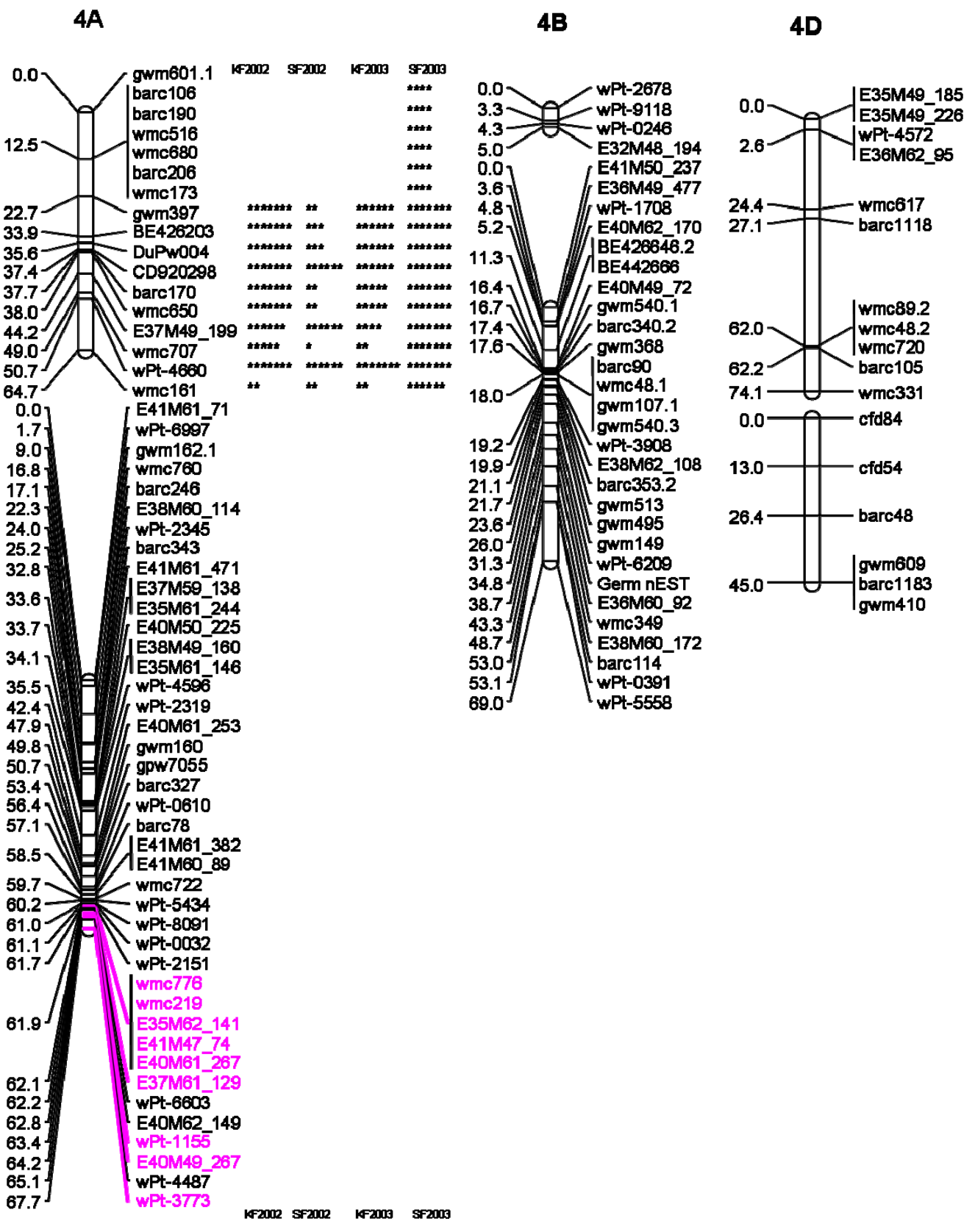
wPt-3412
22.6 gwm114
E40M59_130
E37M61_405
E41M49_297
24.5 wPt-6909


4A
4B
4D
KF2002
SF2002
KF2003
SF2003
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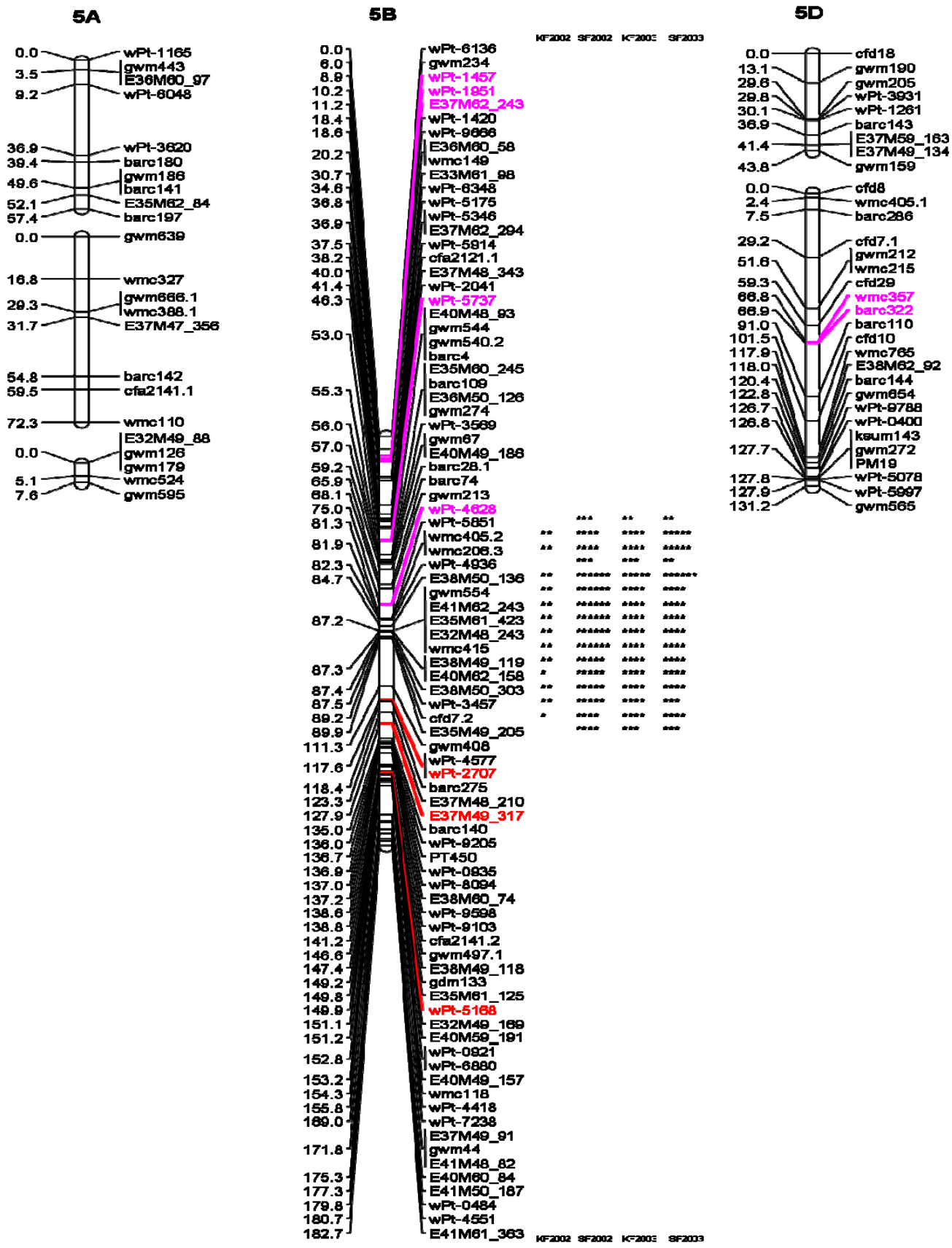
barc106
barc190
wmc516
12.5 wmc680
barc206
wmc173
22.7 gwm397
33.9 BE426203
35.6 DuPw004
37.4 CD920298
37.7 barc170
38.0 wmc650
44.2 E37M49_199
49.0 wmc707
50.7 wPt-4660
64.7 wmc161
0.0 E41M61_71
1.7 wPt-6997
9.0 gwm162.1
16.8 wmc760
17.1 barc246
22.3 E38M60_114
24.0 wPt-2345
25.2 barc343
32.8 E41M61_471
33.6 E37M59_138
E35M61_244
33.7 E40M50_225
34.1 E38M49_160
E35M61_146
35.5 wPt-4596
42.4 wPt-2319
47.9 E40M61_253
49.8 gwm160
50.7 gpw7055
53.4 barc327
56.4 wPt-0610
57.1 barc78
58.5 E41M61_382
E41M60_89
59.7 wmc722
60.2 wPt-5434
61.0 wPt-8091
61.1 wPt-0032
61.7 wPt-2151
61.9 wmc776
wmc219
E35M62_141
E41M47_74
E40M61_267
62.1 E37M61_129
62.2 wPt-6603
62.8 E40M62_149
63.4 wPt-1155
64.2 E40M49_267
65.1 wPt-4487
67.7 wPt-3773
0.0 wPt-2678
3.3 wPt-9118
4.3 wPt-0246
5.0 E32M48_194
0.0 E41M50_237
3.6 E36M49_477
4.8 wPt-1708
5.2 E40M62_170
BE426646.2
11.3 BE442666
16.4 E40M49_72
16.7 gwm540.1
17.4 barc340.2
17.6 gwm368
barc90
18.0 wmc48.1
gwm107.1
gwm540.3
19.2 wPt-3908
19.9 E38M62_108
21.1 barc353.2
21.7 gwm513
23.6 gwm495
26.0 gwm149
31.3 wPt-6209
34.8 Germ nEST
38.7 E36M60_92
43.3 wmc349
48.7 E38M60_172
53.0 barc114
53.1 wPt-0391
69.0 wPt-5558
0.0 E35M49_185
E35M49_226
2.6 wPt-4572
E36M62_95
24.4 wmc617
27.1 barc1118
wmc89.2
62.0 wmc48.2
wmc720
62.2 barc105
74.1 wmc331
0.0 cfd84
13.0 cfd54
26.4 barc48
gwm609
45.0 barc1183
gwm410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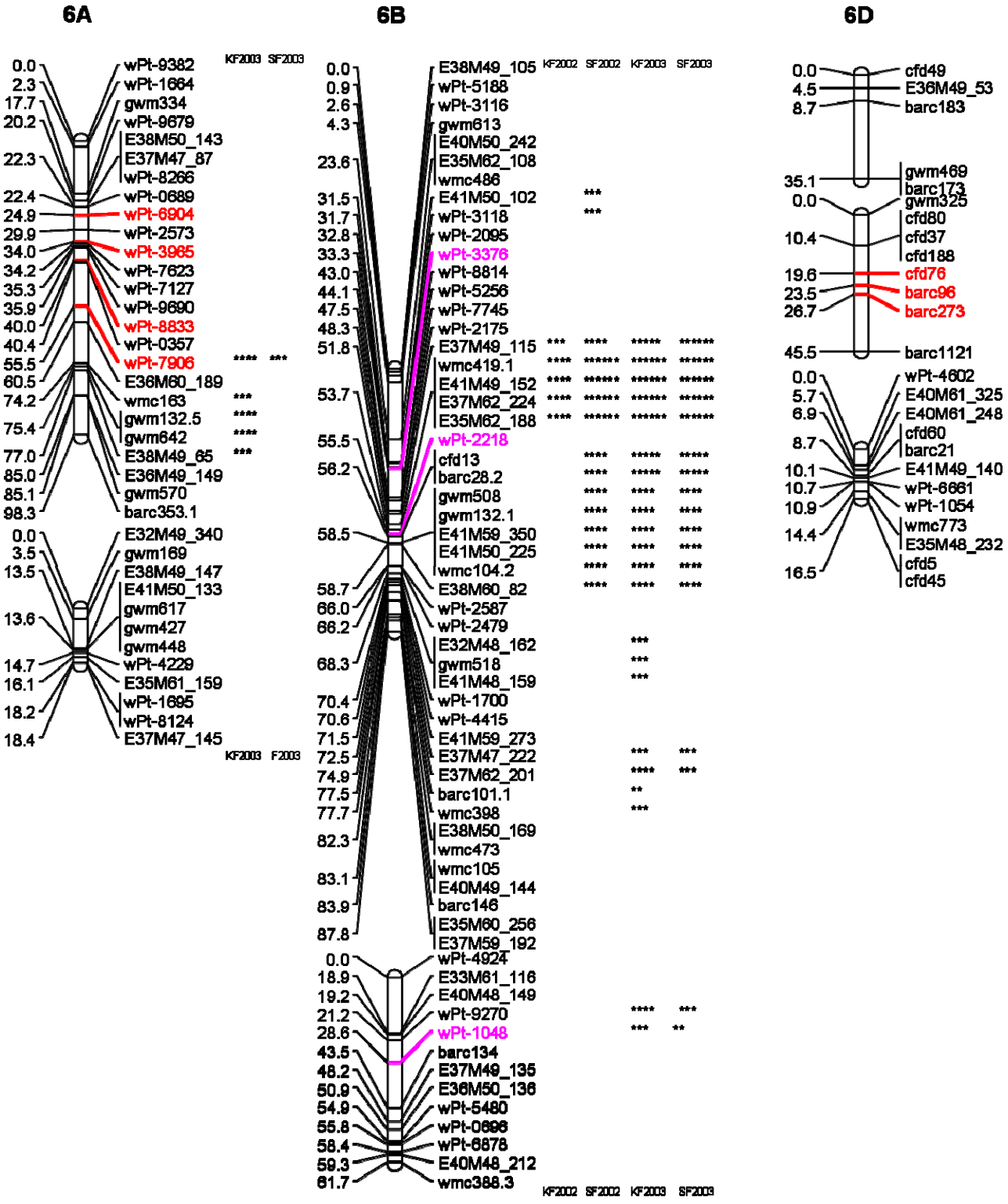
3.5 gwm443
E36M60_97
9.2 wPt-6048
36.9 wPt-3620
39.4 barc180
49.6 gwm186
barc141
52.1 E35M62_84
57.4 barc197
0.0 gwm639
16.8 wmc327
29.3 gwm666.1
wmc388.1
31.7 E37M47_356
54.8 barc142
59.5 cfa2141.1
72.3 wmc110
0.0 E32M49_88
gwm126
gwm179
5.1 wmc524
7.6 gwm595
5B
KF2002 SF2002 KF2003 SF2003
0.0 wPt-6136
6.0 gwm234
8.9 wPt-1457
10.2 wPt-1851
11.2 E37M62_243
18.4 wPt-1420
18.6 wPt-9666
E36M60_58
20.2 wmc149
30.7 E33M61_98
34.6 wPt-6348
36.8 wPt-5175
36.9 wPt-5346
E37M62_294
37.5 wPt-5814
38.2 cfa2121.1
40.0 E37M48_343
41.4 wPt-2041
46.3 wPt-5737
E40M48_93
53.0 gwm544
gwm540.2
barc4
E35M60_245
55.3 barc109
E36M50_126
gwm274
56.0 wPt-3569
57.0 gwm67
E40M49_188
59.2 barc28.1
65.9 barc74
68.1 gwm213
75.0 wPt-4628
81.3 wPt-5851
81.9 wmc405.2
wmc208.3
82.3 wPt-4936
84.7 E38M50_136
gwm554
E41M62_243
87.2 E35M61_423
E32M48_243
wmc415
87.3 E38M49_119
E40M62_158
87.4 E38M50_303
87.5 wPt-3457
89.2 cfd7.2
89.9 E35M49_205
111.3 gwm408
117.6 wPt-4577
wPt-2707
118.4 barc275
123.3 E37M48_210
127.9 E37M49_317
135.0 barc140
136.0 wPt-9205
136.7 PT450
136.9 wPt-0835
137.0 wPt-8094
137.2 E38M60_74
138.6 wPt-9598
138.8 wPt-9103
141.2 cfa2141.2
146.6 gwm497.1
147.4 E38M49_118
149.2 gdm133
149.8 E35M61_125
149.9 wPt-5168
151.1 E32M49_169
151.2 E40M59_191
152.8 wPt-0821
wPt-6880
153.2 E40M49_157
154.3 wmc118
155.8 wPt-4418
169.0 wPt-7238
171.8 E37M49_91
gwm44
E41M48_82
175.3 E40M60_84
177.3 E41M50_187
179.8 wPt-0484
180.7 wPt-4551
182.7 E41M61_363
KF2002 SF2002 KF2003 SF2003
5D
0.0 cfd18
13.1 gwm190
29.6 gwm205
29.8 wPt-3931
30.1 wPt-1261
36.9 barc143
41.4 E37M59_163
E37M49_134
43.8 gwm159
0.0 cfd8
2.4 wmc405.1
7.5 barc286
29.2 cfd7.1
51.6 gwm212
wmc215
59.3 cfd29
66.8 wmc357
66.9 barc322
91.0 barc110
101.5 cfd10
117.9 wmc765
118.0 E38M62_92
120.4 barc144
122.8 gwm654
126.7 wPt-9788
126.8 wPt-0400
127.7 ksum143
gwm272
PM19
127.8 wPt-5078
127.9 wPt-5997
131.2 gwm565


6A
6B
6D
KF2003 SF2003
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wPt-1664
gwm334
wPt-9679
E38M50_143
E37M47_87
wPt-8266
wPt-0689
wPt-6904
wPt-2573
wPt-3985
wPt-7623
wPt-7127
wPt-9690
wPt-8833
wPt-0357
wPt-7906
E36M60_189
wmc163
gwm132.5
gwm642
E38M49_65
E36M49_149
gwm570
barc353.1
E32M49_340
gwm169
E38M49_147
E41M50_133
gwm617
gwm427
gwm448
wPt-4229
E35M61_159
wPt-1695
wPt-8124
E37M47_145
KF2003 F2003
KF2002 SF2002 KF2003 SF2003
E38M49_105
wPt-5188
wPt-3116
gwm613
E40M50_242
E35M62_108
wmc486
E41M50_102
wPt-3118
wPt-2095
wPt-3376
wPt-8814
wPt-5256
wPt-7745
wPt-2175
E37M49_115
wmc419.1
E41M49_152
E37M62_224
E35M62_188
wPt-2218
cfd13
barc28.2
gwm508
gwm132.1
E41M59_350
E41M50_225
wmc104.2
E38M60_82
wPt-2587
wPt-2479
E32M48_162
gwm518
E41M48_159
wPt-1700
wPt-4415
E41M59_273
E37M47_222
E37M62_201
barc101.1
wmc398
E38M50_169
wmc473
wmc105
E40M49_144
barc146
E35M60_256
E37M59_192
wPt-4924
E33M61_116
E40M48_149
wPt-9270
wPt-1048
barc134
E37M49_135
E36M50_136
wPt-5480
wPt-0696
wPt-6878
E40M48_212
wmc388.3
KF2002 SF2002 KF2003 SF2003
cfd49
E36M49_53
barc183
gwm469
barc173
gwm325
cfd80
cfd37
cfd188
cfd76
barc96
barc273
barc1121
wPt-4602
E40M61_325
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barc21
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wPt-1054
wmc773
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cfd5
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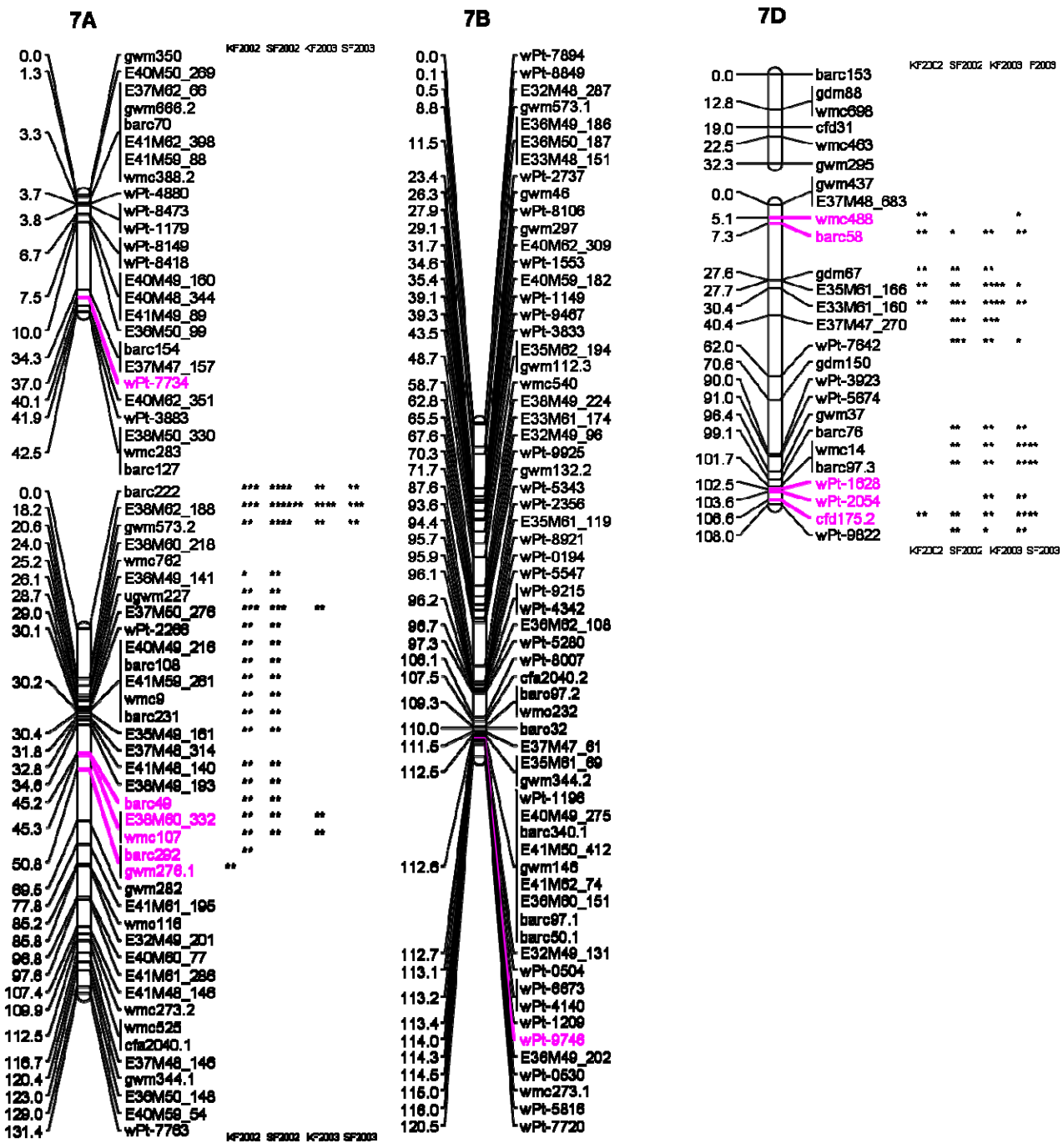
7A
7B
7D
KF2002 SF2002 KF2003 SF2003
0.0 gwm350
1.3 E40M50_269
E37M62_66
gwm666.2
barc70
3.3 E41M62_398
E41M59_88
wmc388.2
3.7 wPt-4880
3.8 wPt-8473
wPt-1179
6.7 wPt-8149
wPt-8418
7.5 E40M49_160
E40M48_344
E41M49_89
10.0 E36M50_99
34.3 barc154
E37M47_157
37.0 wPt-7734
40.1 E40M62_351
41.9 wPt-3883
E38M50_330
42.5 wmc283
barc127
0.0 barc222
18.2 E38M62_188
20.6 gwm573.2
24.0 E38M60_218
25.2 wmc762
26.1 E36M49_141
28.7 ugwm227
29.0 E37M60_276
30.1 wPt-2266
E40M49_216
barc108
30.2 E41M59_261
wmc9
barc231
30.4 E35M49_161
31.8 E37M48_314
32.8 E41M48_140
34.6 E36M49_193
45.2 barc49
45.3 E36M60_332
wmc107
barc282
50.8 gwm276.1
69.5 gwm282
77.8 E41M61_195
85.2 wmc116
85.8 E32M49_201
96.8 E40M60_77
97.6 E41M61_286
107.4 E41M48_146
109.9 wmc273.2
112.5 wmc525
cfa2040.1
116.7 E37M48_146
120.4 gwm344.1
123.0 E36M50_148
129.0 E40M59_54
131.4 wPt-7763
0.0 wPt-7894
0.1 wPt-8849
0.5 E32M48_287
8.8 gwm573.1
E36M49_186
11.5 E36M50_187
E33M48_151
23.4 wPt-2737
26.3 gwm46
27.9 wPt-8106
29.1 gwm297
31.7 E40M62_309
34.6 wPt-1553
35.4 E40M59_182
39.1 wPt-1149
39.3 wPt-9467
43.5 wPt-3833
48.7 E35M62_194
gwm112.3
58.7 wmc540
62.8 E36M49_224
65.5 E33M61_174
67.6 E32M49_96
70.3 wPt-9925
71.7 gwm132.2
87.6 wPt-5343
93.6 wPt-2356
94.4 E35M61_119
95.7 wPt-8921
95.9 wPt-0194
96.1 wPt-5547
96.2 wPt-9215
wPt-4342
96.7 E36M62_108
97.3 wPt-5280
106.1 wPt-8007
107.5 cfa2040.2
109.3 barc97.2
wmc232
110.0 barc32
111.5 E37M47_61
112.5 E35M61_89
gwm344.2
wPt-1196
E40M49_275
barc340.1
E41M50_412
112.6 gwm148
E41M62_74
E36M60_151
barc97.1
barc50.1
112.7 E32M49_131
113.1 wPt-0504
113.2 wPt-6673
wPt-4140
113.4 wPt-1209
114.0 wPt-9748
114.3 E36M49_202
114.5 wPt-0530
115.0 wmc273.1
116.0 wPt-5818
120.5 wPt-7720
0.0 barc153
12.8 gdm88
wmc698
19.0 cfd31
22.5 wmc463
32.3 gwm295
0.0 gwm437
E37M48_683
5.1 wmc488
7.3 barc58
27.6 gdm67
27.7 E35M61_166
30.4 E33M61_160
40.4 E37M47_270
62.0 wPt-7642
70.6 gdm150
90.0 wPt-3923
91.0 wPt-5674
96.4 gwm37
99.1 barc76
wmc14
101.7 barc97.3
102.5 wPt-1628
103.6 wPt-2054
106.6 cfd175.2
108.0 wPt-9822
KF2002 SF2002 KF2003 F2003
KF2002 SF2002 KF2003 SF2003

derived doubled haploid populations. These include heterogeneity within the parents, selection associated with the doubled haploid production process, outcrossing and admixture of seed during increase for trials (Kammholz et al. 2001). Segregation distortion of particular loci could cause serious problems in plant breeding if they are closely linked to agronomically important genes.

4.4.3 Chromosomal regions associated with PHS

Single marker analysis of the phenotypic and genotypic data revealed ten chromosomes; 1A, 1B, 2B, 3A, 4A, 5B, 6A, 6B, 7A and 7D involved in PHS resistance with the threshold significance value set to $P \leq 0.01$ (Figure 4.4). The putative QTL on chromosome 1A, 3A, 4A, 5B, 6B, 7A and 7D were represented by multiple markers and were significant in all four trials. The QTL on chromosomes 1B and 6A, and 2A were significant in two and three trials, respectively. In all the putative QTL revealed from single marker analysis, the increase in dormancy or decrease in percent germination was contributed by the alleles from the dormant parent (Table 4.6). Two putative QTL on chromosome 4A were suggested based on *P*-value, where markers DuPw004 (36cM) and wPT-4660 (51cM) showed peak values (Table 4.6).

Interval mapping using MapQTL5 (van Ooijen, 2004) was performed to identify genomic regions containing the putative QTL. The significant threshold LOD score (*P=0.05*) for detection of QTL on the whole genome was determined on 1,000 permutation tests (Churchill and Doerge 1994). In this study, the permutation tests estimated significant LOD scores of 3.7, 3.7, 3.5 and 4.5 in KF2002, SF2002, KF2003 and SF2003 trials, respectively. Five genomic regions on chromosomes 1A, 3A, 4A, 7A and 7D were detected by interval mapping (Figure 4.5). These QTL accounted for 14-15% (2 trails), 15-16% (2 trials), 11-30% (3 trials), 19-23% (2 trials) and 27-31% (2 trials) of phenotypic variance, respectively, and were all contributed by the dormant parent, W98616.

In bread wheat, all chromosomes except chromosome 1D have been reported to carry QTL for PHS resistance or dormancy (Flintham et al. 2002). The QTL on chromosome 1A was located between 60-70 cM and likely resides near the centromeric

Table 4.6. Chromosomal regions associated with PHS resistance in the Argent x W98616 mapping population.

Chromosome	Position[a] (cM)	Peak Marker(s)[b]	Significance (*p*-value)[c] Trial site and year				LOD	Mean[d]	
			KF2002	SF2002	KF2003	SF2003		Argent	W98616
1A	62.1	gwm164	****	*******	******	*****	4.26	64	40
	69.3	wPt9592	******	*******	*******	******	4.44	65	38
3A	156.4	gwm340	***	***	*****	***	3.77	66	27
	156.5	wPt0398	******	******	*******	****	3.77	58	33
4A	22.7	gwm397	*******	**	******	*******	4.97	60	41
	37.4	CD920298	*******	******	******	*******	5.77	80	22
	50.7	wPt4660	*******	******	*******	*******	9.58	71	33
7A	18.2	E38M62_188	***	******	****	***	3.85	77	36
7D	30.4	E33M61_160	**	***	****	**	3.32	72	37

[a]Chromosomal position in centiMorgans (cM)

[b]Markers with highest *p*-value

[c] Significance level: ** ($p<0.05$), *** ($p<0.01$), **** ($p<0.005$), ***** ($p<0.001$), ****** ($p<0.0005$), ******* ($p<0.0001$).

[d] Phenotypic mean value of germination percentage from the trial with underlined *p*-value.

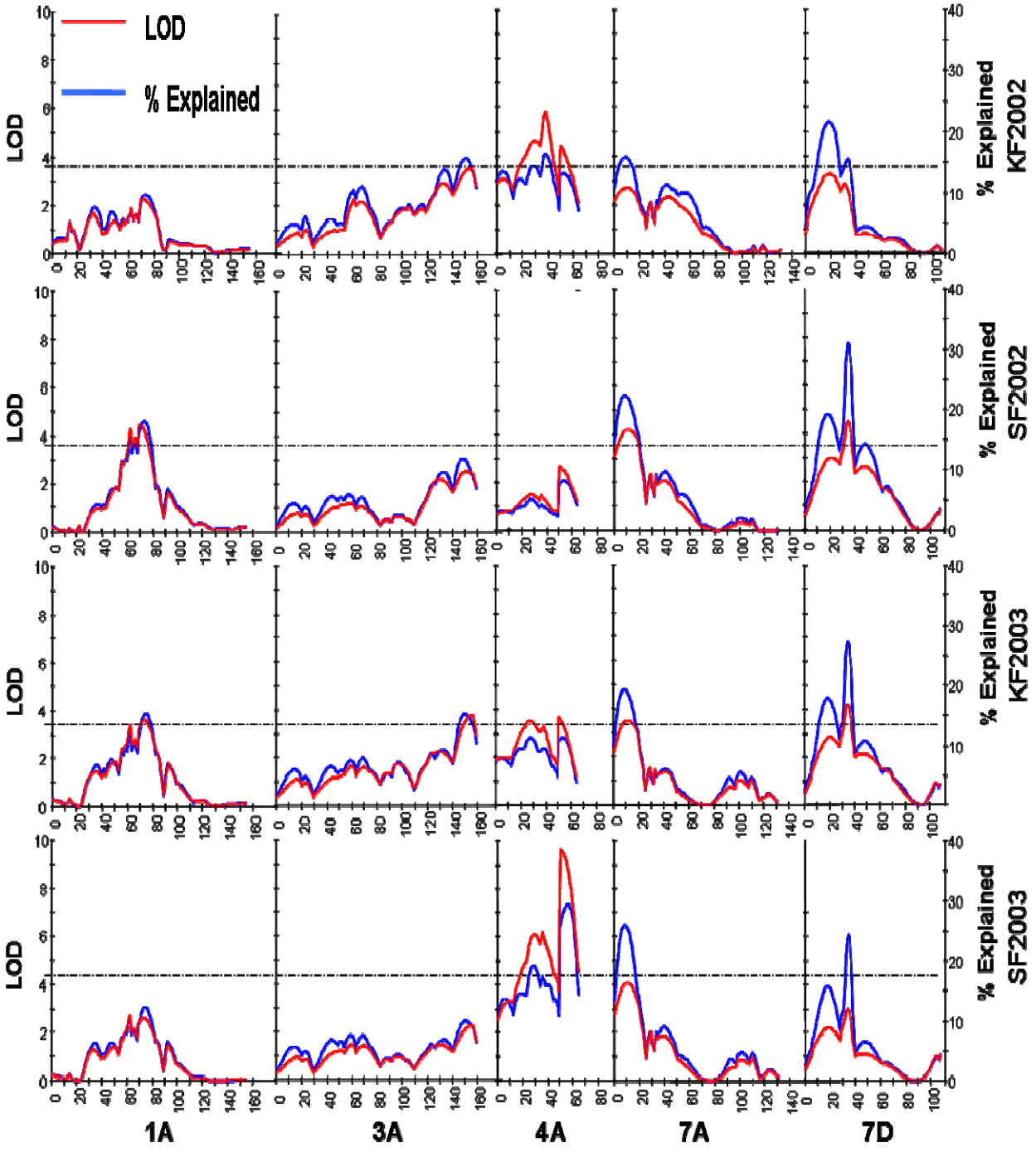

Figure 4.5. Interval maps of QTL for PHS resistance on chromosomes 1A, 3A, 4A, 7A and 7D. *Horizontal dashed line* (- - - -) indicates the genome-wide threshold LOD score determined in each trial. KF2002, SF2002, KF2003, SF2003 indicates four trials at Kernen Farm (KF) and Seed Farm (SF) in 2002 and 2003.

region. Zanetti et al. (2000) reported a QTL for Falling Number in a wheat x spelt cross at 60 cM and a QTL for α-amylase activity at 64 cM. The QTL in our study may corresponds to the QTL for Falling Number and α-amylase activity because the position of *Xpsr1327* reported by Zanetti et al. (2000) is close to the *gwm164* and *gwm357* markers. There is an inverse curvilinear relationship between α-amylase activity and Falling Number (Mares 1987b) but Falling Number values fluctuate widely depending on the degree of ripening and the amount of rainfall prior to harvest (Mares 1993). Also Falling Number and enzymatic tests do not measure the ungerminated seed's ability to sprout under optimum conditions (Hagemann and Ciha 1984). The QTL on chromosome 1A may likely be associated with α-amylase activity or Falling Number and may not be associated with grain dormancy *per se*. Two QTL were also reported on the short arm of chromosome 1A based on RFLP analysis (Anderson et al. 1993).

The QTL on chromosome 3A was identified at the end of long arm between *gwm340* and *wPt-1596*. The QTL on 3AL in our study corresponds to the QTL identified by Groos et al. (2002) and Kulwal et al. (2005). Osa et al. (2003) identified PHS resistance QTL on both arms of 3A, a major QTL on 3AS (*QPhs.ocs-1*) and a minor QTL on 3AL (*QPhs.ocs-2*) located between the centromere and *taVp1* locus. The wheat *taVp1* locus was mapped 30 cM from the centromere and about 30 cM proximal to the red grain (*R*) locus that controls seed colour and coat imposed dormancy (Bailey et al. 1999). The expression levels of the *Vp1* correlates with the level of seed dormancy in dormant and non-dormant cultivars (Nakamura and Toyama 2001). However, McKibbin et al. (1999) reported that the abundance of *taVp1* transcript was similar in developing embryos of dormant and non-dormant genotypes and might not be associated with the level of dormancy of the wheat grain. Later studies showed that mis-splicing of *VP1* gene contributes to the susceptibility to PHS in hexaploid wheats (McKibbin et al. 2002). However, in this study there was no LOD peak in the *TaVP1* region and thus grain dormancy in this population is not expected due to the direct effect of *TaVP1*.

Interval mapping revealed two peaks on chromosome 4A in marker intervals *gwm397/wmc650* and *wmc707/wmc161*. Mapping of more markers may resolve whether the two peaks correspond to two different QTL or one. Very few markers have been reported in the genomic region between *gwm397* and *wmc161* in most of the genetic

maps. The QTL on 4AL was likely located in bin 4AL13-0.59-0.66 based on available markers. In our study we were able to map one EST (CD920298) (Table 4.3) to that region. This EST was described as ABA induced plasma membrane protein PM19 (http://www.ncbi.nlm.nih.gov/). Tan et al. (2006) also reported a QTL between marker interval *gwm397* and *wPt-2788*. A highly significant QTL on chromosome 4A associated with grain dormancy was identified in three bread wheat genotypes, two white-grained wheats (AUS 1408 and SW95-50213) and one red-grained wheat (AUS 1490) (Mares et al. 2005). Based on the comparisons between dormant red and white genotypes, and a white mutant derived from the red genotype, they postulated that the 4A locus confers an intermediate dormancy on its own and a dormant phenotype when combined with the red pigmentation genes in red wheat and unidentified gene(s) in white wheats. Noda et al. (2002) suggested that wheat embryo sensitivity to germination inhibition by abscisic acid (ABA) and dormancy is controlled by major gene(s) located on the long arm of chromosome 4A. In cereal crops, embryo sensitivity to ABA has been shown to be a key factor in the mechanism of seed dormancy (Walker-Simmons 1987; Kawakami et al. 1997). ABA signaling genes were also mapped between *gwm397* and *gwm637* in a population of diploid wheat RILs derived from a cross between *Triticum monococcum* and *T. boeoticum* for identification of QTL for dormancy (Nakamura et al. 2007). One major QTL on the long arm of $5A^m$, two minor QTL on the long arm of $3A^m$ and one minor QTL on the long arm of $4A^m$ were detected. They concluded that the minor QTL on $4A^m$ is orthologous to the QTL identified in previous studies (Mares et al. 2005; Torada et al. 2005). Three major haplotypes were observed on chromosome 4AL, designated RL4137-type allele, AUS1408-type allele and synthetic-hexaploid-type allele in a study of haplotype diversity of PHS QTL in wheat (Ogbonnaya et al. 2007). These results indicated that RL4137-type allele was prevalent in Canadian cultivars and RL4137 is one of the grand-parents in our mapping population (Figure 4.1).

The QTL on short arm of chromosome 7A spans a 21 cM marker interval between *barc222/gwm573*. This region corresponds to the locus *Xpsp3050* associated with PHS resistance reported in a mapping population of RL4137 x Timgalen (Flintham et al. 2002). This locus may be another PHS resistance allele contributed by RL4137 in both our population and Flintham et al. (2002) population.

The QTL on chromosome 7D showed a peak in marker interval gdm67/wPt7642, but the marker E33M61_160 in this region has a highest LOD score of 3.3. This genomic region corresponds to the location of the *α-Amy-D2* locus on the long arm of chromosome 7D. During germination, the products of the two gene families *α-Amy-1* and *α-Amy-2* are present (Gale and Ainsworth 1984). Han et al. (1997) demonstrated that marker-assisted selection for a QTL associated with a marker *Amy2* was highly effective in improving α-amylase activity and other malting characteristics. Varshney et al. (2001) also reported a QTL for PHS resistance on 7DL.

4.5 CONCLUSIONS

Insufficient seed dormancy at harvest can contribute to pre-harvest sprouting in bread wheat. This undesirable trait negatively impacts grain producers and the processing industry in terms of economic losses and functional quality of the wheat flour. White-grained wheat genotypes are more prone to PHS as compared to red-grained wheat genotypes, although some white-grained wheats have relatively high levels of PHS resistance. Seed dormancy is an important trait for white-grained wheat breeding programs in order to prevent PHS. Seed dormancy and tolerance to PHS in wheat are complex traits, expressed as quantitatively inherited traits. The improvement of PHS resistance based on phenotypic selection is a difficult process because environments favorable for PHS are not always available and the controlled environments may not prove to be suitable to screen large numbers of breeding lines. Marker-assisted selection could provide a tool to overcome these difficulties. In this study, a detailed genetic map covering 2,577 cM of the wheat genome was developed on a mapping population derived from two parents differing widely in PHS. Five genomic regions on chromosomes 1A, 3A, 4A, 7A and 7D were found to be associated with PHS resistance and all five QTL were contributed by the white-grained and dormant parent, W98616. As these QTL are not related to red-grain colour, they should be of particular interest for breeding white-grained wheat genotypes with increased PHS resistance. Synteny between wheat, Brachypodium and rice genomes may be one of the strategies to saturate the genomic regions of PHS resistance with more markers. This strategy is not straight forward as micro-syntenic rearrangements may complicate the transfer of genomic information from

rice to wheat (Li et al. 2004). Another strategy may be the use of Target region amplified polymorphism (TRAP) markers (Hu and Vick 2003) in which one fixed primer is designed from a known EST, while the other primer is arbitrary with an AT- or GC-rich core to anneal with an intron or exon. This genetic map may also be helpful in detecting QTL for other quality or agronomic traits if the parents differ in those traits.

CHAPTER 5
VALIDATION OF MOLECULAR MARKERS FOR PHS RESISTANCE IN BREAD WHEAT

5.1 ABSTRACT

The selection for pre-harvest sprouting (PHS) resistance in bread wheat (*Triticum aestivum* L.) in early generations during breeding process is difficult because it is expressed as a quantitatively inherited trait and subject to environmental effects. The objectives of this study were to validate a major quantitative trait locus (QTL) for pre-harvest sprouting (PHS) resistance on chromosome 4A in bread wheat and to isolate near-isogenic lines for this QTL using marker-assisted selection (MAS). A total of 60 Canadian wheat cultivars and experimental lines were screened with three SSR markers in the QTL region for PHS resistance. The SSR markers DuPw004, barc170 and wmc650 explained 67%, 75% and 60% of total variation in germination (%), respectively, among different wheat genotypes. Marker assisted back crossing with DuPw 004 reduced the population size in BC_1F_1 and BC_2F_1 generation by 41% and 59%, respectively. A survey of pedigrees of different genotypes revealed that RL4137 is a major source of increased PHS resistance in a number of Western Canadian wheat cultivars. These microsatellite markers (DuPw004, barc170 and wmc650) will be useful for the plant breeders to pyramid this QTL with QTL from other PHS resistance sources.

5.2 INTRODUCTION

Pre-harvest sprouting (PHS) is the in-spike germination of physiologically mature grain in response to relatively high humidity due to untimely rains prior to harvest. PHS in bread wheat (*Triticum aestivum* L.) results in substantial economic loss, as it causes yield loss due to a reduction in grain weight and decreases the functional quality of the wheat flour. In Western Canada, the estimated economic losses due to PHS were up to $400 million for the period 1978-1988 (Derera 1990). The flour from sprouted wheat

leads to sticky dough, and breads baked from this have a sticky crumb, dark-coloured crust and large holes in the loaves that are difficult to slice (Mares et al. 2004). Noodles made from sprouted wheat show a five-fold greater number of spots as compared with alkaline noodles made from sound flour (Hatcher and Symons 2000). Breeding for PHS tolerance in wheat is challenging on a phenotypic basis because PHS is inherited quantitatively and it is highly influenced by environmental conditions (Anderson et al. 1993). Seed dormancy confers PHS resistance (Mares 1987a), but little is known about the genetic factors regulating seed dormancy in cereals. Red grain colour is a traditional marker for resistance to sprouting in wheat improvement programs. White-grained wheat is generally more susceptible to PHS than red-grained wheat, although both groups vary in PHS (Bassoi and Flintham 2005). Molecular markers linked to the PHS resistance trait represent a more reliable tool for selecting PHS resistant genotypes at early stages in wheat breeding programs. SSR (simple sequence repeat) or microsatellite markers have become a DNA marker system of choice in wheat. Several SSR markers have been identified which are linked to disease, insect, agronomic and grain quality traits in wheat (Huang et al. 2000; Liu et al. 2001; Huang et al. 2006).

Once a target trait has been identified and tagged, breeders can use the molecular markers to efficiently and effectively accelerate the crop improvement programs by tracing the favorable alleles in the genomic background of genotype to be improved and ensuring the presence of elite alleles at the selected loci through repeated cycle of selection (Dreher et al. 2003). Marker-assisted selection (MAS) is based on genetic information retrieved through the application of molecular markers. MAS involves using the presence/absence of a marker as a substitute for/or, to assist in phenotypic selection, in a way which may make it more efficient, effective, reliable and cost-effective compared to conventional plant breeding methodology. Marker-assisted selection has several advantages; selection of genotypes at an early growth stage, elimination of environmental effects, selection for traits with low heritability and gene pyramiding (Collard et al. 2005). Backcross breeding is a well-known procedure for the introgression of a target gene from a donor genotype into the genomic background of an elite recipient genotype. The objective is to increase the recipient genome content of the progeny, by repeated backcrosses to the recipient line.

The aim of this study was to test if any of the microsatellite markers associated with a major QTL on chromosome 4A for PHS resistance could be used across different genetic backgrounds to identify PHS resistant lines and to incorporate this allele in elite genotypes through marker-assisted backcrossing. These markers may be suitable for marker assisted selection of increased PHS tolerance.

5.3 MATERIALS AND METHODS

5.3.1 Plant materials

Sixty wheat lines and cultivars (Table 5.1; Table 5.2) were obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada for the validation of markers. A 42-entry (Table 5.2), four replicate trials in RCBD experimental design was grown at Seed Farm, University of Saskatchewan, Saskatoon, Canada in 2006. At Zadoks' Growth Stage 92 (Zadoks et al. 1974), 50 spikes per plot were harvested. The spikes were held at room temperature for seven days, bulk threshed using a rubber belt thresher and seeds were stored in a freezer at -20˚C. The seed samples were removed from a freezer (-20˚C) and stored at room temperature for three weeks before dormancy testing. Thirty advanced lines (F_7), 113 BC_1F_1 plants and 102 BC_2F_1 from different crosses were also used in this study (Table 5.3; Table 5.4).

5.3.2 Germination assay

Fifty seeds were placed in Petri plates (8 cm diameter), containing a Whatman #1 filter paper soaked with 3.0 ml of distilled water. The Petri plates were placed in a large plastic container containing water saturated paper towels and incubated at 20°C. The germination count was done after seven days. Non germinated seeds were treated with 0.05% (w/v) gibberellic acid (GA_3) solution (1 ml/Petri plate) and incubated at 12°C. The final germination count was done after seven days to determine the seed viability.

5.3.3 DNA extraction and molecular marker analysis

Leaves were harvested from 2-3 weeks old seedlings grown in a growth chamber under an 18 hr photoperiod (250 μmol m^{-2} s^{-1} light intensity, 23°C day and 18°C night temperature). The leaves were freeze dried and an aliquout of 50 mg was ground to a fine powder in a 2 ml centrifuge tube containing two glass beads (5 mm) using a grinding apparatus at 50 MHz for 10 min. Total genomic DNA was isolated from the pulverized

leaves using the CTAB method (Doyle and Doyle 1990). For molecular marker analysis, a polymerase chain reaction (PCR) was performed in a 25 µl volume containing 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer (Fermentas), 200 µM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$ and 1.0 unit of *Taq* DNA polymerase (Fermentas) in MyCycler^{TM} thermal cycler (Bio-Rad). The thermocycling program consisted of an initial denaturation at 95°C for 4.15 min, followed by 30 cycles of 45 sec at 95°C, 20 sec at annealing temprature, 90 sec at 72°C and a final cycle of 10 min at 72°C. The following annealing temperatures were used: barc170 (60°C), wmc650 (60°C) and DuPw004 (65°C). The DuPw004 products were separated by 2% (w/v) agarose gel electrophoresis and gel was stained with ethidium bromide. PCR products for barc170 and wmc650 were separated by 6% denaturing polyacrylamide gel electrophoresis and gel was stained with silver nitrate.

5.3.4 Data analysis

Single-marker linear regression analysis was used to determine the association between molecular marker and germination (%). The marker allele *nd* (non-dormant) was coded 0 and the allele *d* (dormant) was coded 1 for conducting regression analysis. The magnitude of the marker-associated phenotypic effect was described by the coefficient of determination (R^2) which is a fraction of the total variance accounted by the marker genotype.

5.4 RESULTS AND DISCUSSION

5.4.1 Marker validation

A major QTL for PHS resistance on Chromosome 4A has been mapped in a doubled haploid mapping population of 'Argent' (white-grained, non-dormant) x 'W98616' (white-grained, dormant) (Chapter 4). Three SSR marker alleles, DuPw004 (200 bp), barc170 (158 bp) and wmc650 (109 bp), inherited from the dormant parent ('W98616'), whereas non-dormant parent ('Argent') carried the DuPw004 (300 bp), barc170 (177 bp) and wmc650 (90 bp) alleles. To test these three markers in different genetic backgrounds, initially we selected genotypes (Table 5.1) for which information about seed dormancy were available in previous studies (Osanai and Amano 1993; Hucl and Matus-Cádiz 2002a; Hucl and Matus-Cádiz 2002b; Hucl, personal communication).

A PCR product of 200 bp was amplified from all the PHS resistant wheat lines (Figure 5.1). The same marker produced a 300 bp allele from PHS susceptible wheat lines. Similarly, dormant parent amplified a PCR product of 158bp and 109bp with barc170 and wmc650 SSR markers, respectively (Table 5.1). It should be noted that the allele size in 'W98616' was 158bp, whereas, the allele sizes in RL4137 and AUS1408 were 158bp and 197bp, respectively. 'W98616' has 'AUS1408' and 'RL4137' in the pedigree. So this allele is likely contributed by 'RL4137'.

The cvs. 'Columbus' and 'AC Domain' have 'RL4137' in their parentage as the source of PHS resistance. The cv. 'Columbus' is in the pedigree of cv. 'AC Majestic', 'McKenzie', 'Prodigy', 'AC Barrie', '5600HR', 'HR5500', '5601HR', 'Journey', 'Lovitt' and 'Snowstar'(Table 5.1; Table 5.2; Figure 5.2), whereas, cv. 'AC Domain' is in the pedigree of cvs. 'Harvest', 'AC Superb', 'PT434', 'PT435', 'PT559', 'BW384' and 'KANE'. The line 'OS72-36' had 'Zenkoujikomugi' ('Zen') as one of the parent, which is a Japanese red-spring wheat with an extremely high level of seed dormancy and tolerance to PHS (Osanai and Amano 1993). Similarly the source of PHS resistance in cv. 'Snowbird' and 'W98616' is 'RL4137'. The genotypes 'CDC EMDR9' and 'W98616' have 'AUS1408' in their parentage. 'Losprout' is the source of dormancy in 'AC Vista'. These results indicate 'RL4137' as a major source of increased PHS resistance in number of Western Canadian wheat cultivars. 'AC Eatonia' has 'Leader' as source of PHS resistance (DePauw et al. 1994). 'RL4137' and 'Leader' have 'Frontana' in their pedigree, a possible source of seed dormancy (Hucl, personal communication). Andreoli et al. (2006) also suggested that 'Frontana' as most likely source of seed dormancy in Canadian wheat cultivars.

Red grain colour traditionally has been associated with seed dormancy, but now it is clear that red colour is not sufficient to guarantee dormancy (Flintham et al. 1999; Bassoi and Flintham 2005). This is also evident in this study, as a large number of red grained wheat genotypes are non-dormant and also four out of nine white wheat genotypes are dormant. The genes responsible for red grain colour (*R*) are located on the long arms of the homeologous group 3 chromosomes in wheat (Flintham and Gale 1996).

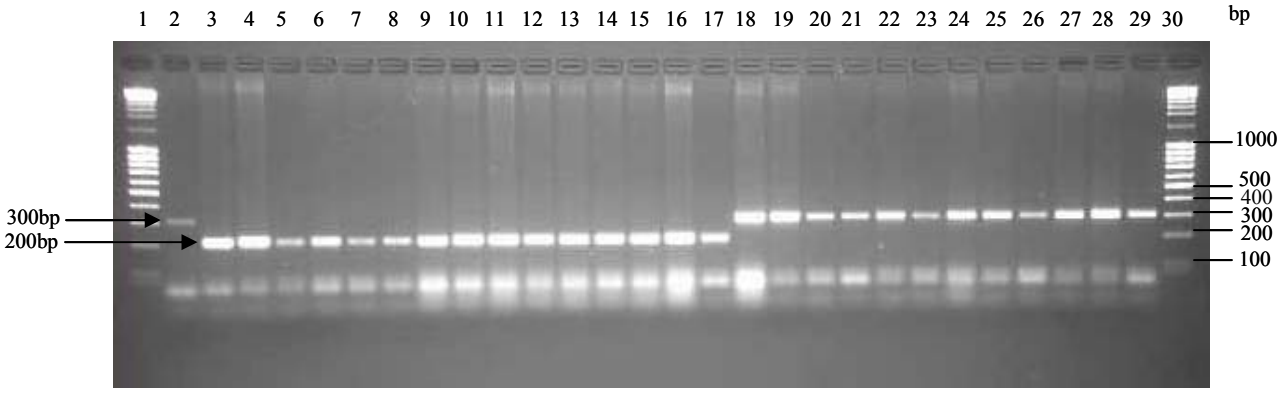

Figure 5.1. Screening of wheat cultivars and genotypes with a PHS resistance marker (DuPw004). Analysis of DuPw004 PCR amplification products by 2% (w/v) agarose gel electrophoresis. Migration of DNA size standard in lanes 1 and 30. The following lines were analyzed: 2- Argent, 3- W98616, 4- AUS1408, 5- RL4137, 6- Columbus, 7- CDC EMDR9, 8- OS72-36, 9- Zen, 10- PT434, 11- PT435, 12- Snowstar, 13- BW384, 14- KANE, 15- AC Majestic, 16- AC Domain, 17- AC Eatonia, 18- Tordo, 19- AC Crystal, 20- AC Cadillac, 21- CDC Merlin, 22- AC Intrepid, 23- Lillian, 24- CDC Go, 25- Katepwa, 26- CDC Alsask, 27- Roblin, 28- AC Reed, 29- CDC Teal.

Table 5.1. Validation of microsatellite markers associated with PHS resistance in indifferent genetic backgrounds.

Genotype	Pedigree	Class	DuPw004 allele/bp	barc170 allele/bp	wmc650 allele/bp
Argent	Grandin*5/ND 614	White Spring	300	177	90
W98616	AUS 1408/RL 4137	White Spring	200	158	109
AUS1408	Australian Winter Cereal Collection accession number 1408	White Spring	200	197	109
RL4137	Frontana/4/McMurachy//Exchange/3/2 *Redman /5/Thatcher*6/Kenya Farmer	Red Spring	200	158	109
Columbus	Neepawa*6/RL4137	CWRS[1]	200	158	109
CDC EMDR9	AUS1408/Park	Red Spring	200	197	109
OS72-36	Tordo/ Zenkoujikomugi	Red Spring	200	158	109
AC Reed	PT303/Dikwin//Kenya321/Fieldwin	CWSWS[2]	300	177	90
Zenkoujikomugi (Zen)	Derived from Igachikugo-Oregon by γ-ray radiation breeding	Red Spring	200	151	103
PT434	AC Domain*6/Lr22a	Red Spring	200	158	109
PT435	AC Domain*6/Lr22a	Red Spring	200	158	109
Snowstar	94B46*G22/McKenzie	CWHWS[3]	200	158	109
BW384	BW150*2//Tp/Tm/3/2*BW252/4/ 98A190/5/BW252	Red Spring	200	158	109
KANE	AC Domain/McKenzie	CWRS[1]	200	158	109
AC Majestic	Columbus *2// Saric 70/Neepawa /3/Columbus *5//Saric 70/Neepawa	CWRS[1]	200	158	109
AC Domain	ND499/RL4137//ND585	CWRS[1]	200	158	109
Ptarmigan	Yorkstar/Norstar	Soft White Winter	300	169	102
Tordo	Nainari-60*2//Tom-Thumb/Sonora-64/3/Lerma-Rojo-64/Sonora-64	White Spring	300	177	90

[1]CWRS - Canada Western Red Spring, [2]CWSWS - Canada Western Soft White Spring, [3]CWHWS - Canada Western Hard White Spring.

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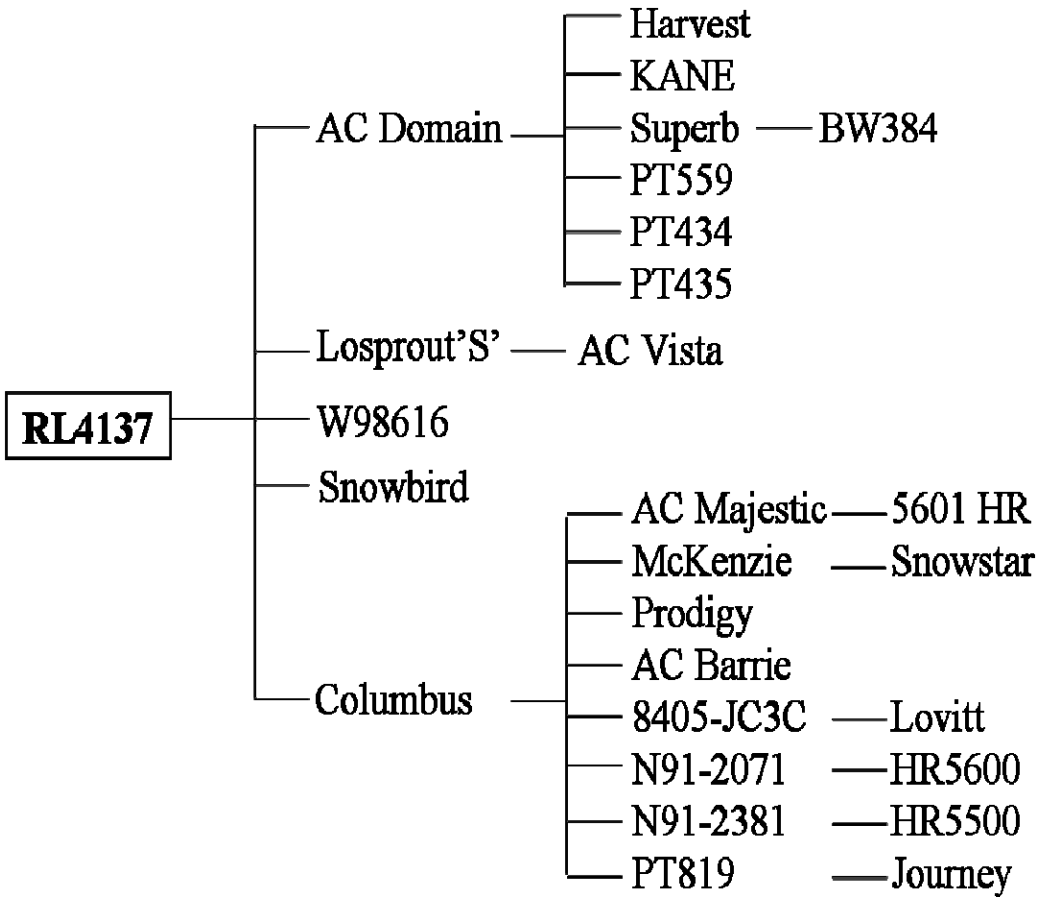

Figure 5.2. Dormant wheat genotypes with RL4137 in their pedigree.

Table 5.2. Evaluation of microsatellite marker associated with PHS resistance in Canadian wheat cultivars.

Genotypes	Pedigree	Class *	Germination (%)	DuPw004 allele/bp	barc170 allele/bp	wmc650 allele/bp
AC Eatonia	Leader/Lancer	CWRS	8	200	158	109
Harvest	AC Domain*2/ND640	CWRS	14	200	158	109
AC Foremost	HY320*5/BW553//HY320*6/ 7424-BW5B4	CPSR	26	200	158	109
AC Superb	Grandin*2/AC Domain	CWRS	39	200	158	109
5601HR	N93-2410/AC Majestic	CWRS	45	200	158	109
5500HR	N91-2381/AC Minto	CWRS	46	200	158	109
Lovitt	8405-JC3C*2/BW152	CWRS	47	200	158	109
Prodigy	SWP2242/Stoa (SWP = Columbus/BW85)	CWRS	51	200	158	109
Snowbird	RL4137*6//Tc/Poso48/3/AC Domain	CWHWS	53	200	158	109
AC Barrie	Neepawa/Columbus//BW90	CWRS	56	200	158	109
McKenzie	Columbus/Amidon	CWRS	58	200	158	109
PT559	SD3055/AC Domain	CWRS	61	200	158	109
5600HR	N91-2071/AC Minto	CWRS	63	200	158	109
Journey	CDC Teal//Grandin/PT819	CWRS	66	200	158	109
CDC Rama	McNeal/Glenlea	CWES	70	200	205	90
AC Vista	HY344/Losprout'S'//HY358* 3/BW553	CPSW	79	200	158	109
CDC Walrus	Glenlea*3/McNeal	CWES	80	300	177	90
Infinity	Kulm/8405-JC3C//AC Elsa	CWRS	80	300	177	90
AC Elsa	BW90/Laura	CWRS	85	300	177	90
Lillian	BW621*3/90B07-AU2B	CWRS	86	300	177	90
5700PR	N91-3051/AC Foremost	CPSR	87	300	177	90
CDC Imagine	CDC Teal*4/FS2 (FS=Fidel)	CWRS	90	300	177	90
AC Crystal	HY377/L8474-D1	CPSR	92	300	177	90
Marquis	Hard Red Calcutta/Red fife	Red Spring	93	300	177	94

CDC Go	Grandin/SD3055	CWRS	94	300	177	90
Glenlea	Pemdina*2/Bage//CB100	CWES	94	300	177	90
AC Abbey	BW608/93464//BW591	CWRS	95	300	146	109
AC Taber	HY320*2/BW553	CPSR	95	300	177	90
CDC Osler	AC Cora/PT534	CWRS	95	300	177	90
AC Splendor	Laura/RL4596//Roblin/BW107	CWRS	96	300	177	90
CDC Alsask	AC Elsa/AC Cora	CWRS	96	300	177	90
Katepwa	Nep*6/RL2938/3/Nep*6//CI8154/2*Fcr	CWRS	97	300	177	90
5701PR	N89-3003/N87-446//Oslo	CPSR	98	300	177	90
AC Andrew	Dirkwin/SC8021V2//Treasure/Blanca	CWSWS	98	200	195	109
AC Cadillac	BW90*3/BW553	CWRS	98	300	177	90
CDC Merlin	RL4386//BW525/BW37	CWRS	99	300	177	90
CDC Zorba	RL5407/Common Winter Spelt	Spring Spelt	100	300	186	105
AC Intrepid	Laura/RL4596//CDC Teal	CWRS	100	300	177	90
CDC Bounty	Katepwa/W82624//Kenyon	CWRS	100	300	177	90
CDC Teal	BW514/Benito//BW38	CWRS	100	300	177	90
Red Fife	Reselection from a Polish Introduction in Canada	Red Spring	100	300	177	90
Roblin	RL4302/RL4356//RL4359/RL4353	CWRS	100	300	177	90
$LSD_{0.05}$			16			

*CWRS - Canada Western Red Spring, CWES - Canada Western Extra Strong, CPSR - Canada Prairie Spring Red, CPSW - Canada Prairie Spring White, CWSWS - Canada Western Soft White Spring, CWHWS - Canada Western Hard White Spring.

However, in this study we used the molecular markers associated with PHS resistance on chromosome 4A. A highly significant QTL associated with grain dormancy on 4A has been identified in two white grained wheat genotypes; 'AUS1408' and 'SW95-50213' and a Japanese red-grained wheat (Mares et al. 2005; Tan et al. 2006). Noda et al. (2002) also suggested that chromosome 4A has the major gene(s) for embryo sensitivity to abscisic acid and dormancy. Flintham et al. (2002) reported the *Phs* locus on the long arm of chromosome 4A, in the region of an ancestral translocation/inversion point between chromosomes 4AS and 5AL.

Effectiveness of molecular markers should be validated by determining the target phenotype in independent populations and different genetic backgrounds which is referred to as marker validation (Sharp et al. 2001; Collins et al. 2003). There is no guarantee that molecular markers identified in one population will be useful in other populations, when the populations originate from distantly related germplasm (Yu et al. 2000). Parker et al. (1998) identified a marker for wheat flour colour on chromosome 7A based on the cross Schomburgk/Yaralinka and later confirmed it's usefulness in the Cranbrook/Halberd and Sunco/Tasman crosses (Mares and Campbell 2001). But the same marker was not applicable to yellow colour characteristics of lines such as Cunningham and Janz, but was applicable to material with Schomburgk-type yellow flour colour (Sharp et al. 2001). Sharp et al. (2001) also identified a marker linked to stem rust *Sr2* gene in Chinese Spring x Chinese Spring (Hope3B) on chromosome 3B, but the polymorphism was not diagnostic when assayed in a wide range of CIMMYT, Australian and other cultivars of known *Sr2* genotypes. In our study, the ability of the markers to detect the appropriate allele in different backgrounds supports its usefulness for selecting PHS resistant genotypes in wheat improvement programs.

5.4.2 Molecular marker vs. germination (%)

The marker data on different genotypes at the *XDuPw004*, *Xbarc170* and *Xwmc650* loci and percent germination of individual genotypes were used for QTL analysis through the single-marker linear regression approach. The regression of germination (%) on the DuPw004 marker was significant, indicating an association between the molecular marker and germination (%). The R^2-value of 0.67 suggested that the quantitative trait locus linked with SSR marker DuPw004 contributed 67% of total

variation in germination (%) among different genotypes (Figure 5.3). Similarly, single-marker linear regression analysis was used to determine the association between molecular markers, barc170 and wmc650, and germination (%). The marker alleles of 148bp (barc170) and 109bp (wmc650) were considered as dormant (*d*) and all other alleles were considered as non-dormant (*nd*) for conducting the regression analysis. The magnitude of the marker-associated phenotypic effect (R^2) was 0.75 (y = -45.793x = 93.259) and 0.60 (y = -39.765x + 93) for barc170 and wmc650, respectively.

5.4.3 Screening of F_7 lines with PHS resistance marker

Thirty advanced wheat breeding lines (F_7) from different crosses were screened for the presence of PHS resistance marker (DuPw004). These lines were developed to incorporate the PHS resistance from 'AC Majestic' ('BW173') into 'CDC Teal' which is a PHS susceptible cultivar. 13 lines were homozygous for the presence of PHS resistance marker, whereas, 16 lines were negative for the marker (Table 5.3). During the first two backcrosses, progeny were selected on the basis of germination tests and in homozygous generations on the basis of falling number (Hucl, personal communication). As presented in Chapter 3, we did not observe any correlation between Falling Number and germination tests upon screening a DH population of Argent x W98616 cross (Singh et al. 2007b). Thus, as Falling Number is not an indicator of germination frequency, there is a possibility of losing the desired allele during the selfing process. One of the lines was found to be heterozygous on the basis DuPw004 marker. There are two possible reasons; first, the selected line had not reached homozygosity at this locus and second, DNA was extracted from 3-4 plants and not from a single plant.

5.4.4 Screening of BC_1F_1 and BC_2F_1 with PHS resistance marker

As PHS is inherited quantitatively and highly influenced by environmental conditions, selecting PHS resistant genotypes on a phenotypic basis at early stages of a breeding process is difficult. DNA-based markers have gained wider recognition as tools for increasing the selection efficiency for plants of a desirable genotype. The presence of molecular markers linked to an economically important trait allows selection of plants with the desirable allele and discarding of those with the undesirable allele in each generation. A total of 113 BC_1F_1 plants from four different backcrosses were screened with DuPw004 marker associated with PHS resistance (Table 5.4, Figure 5.4). Nineteen

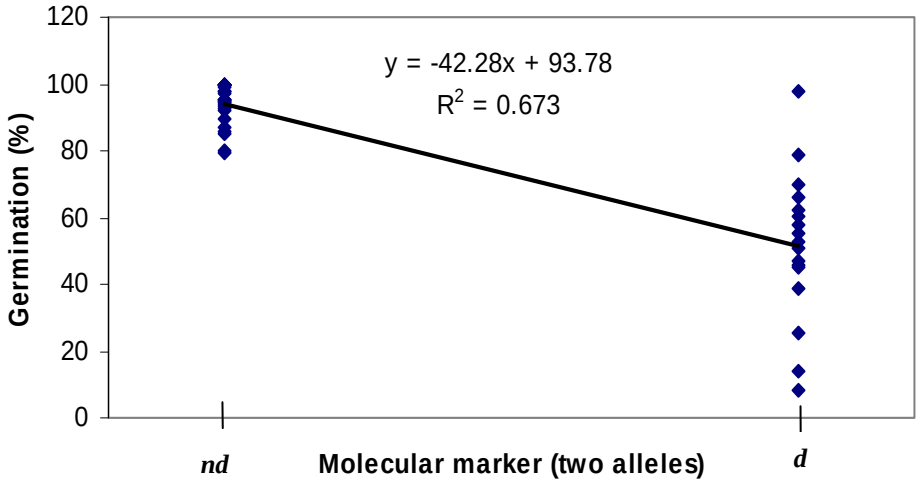

Figure 5.3. Regression of germination percentage (Y) on PHS resistance molecular marker DuPw004, drawn using single marker linear regression analysis. *nd* – non-dormant allele, *d* – dormant allele.

Table 5.3. Screening of advanced F_7 lines from different backcrosses with a PHS resistance marker (DuPw004).

Cross	Generation	Total no. of lines	Positive (200 bp)	Negative (300 bp)	Heterozygous (200 and 300 bp)
Teal/AC Majestic (F_3)//Teal	BC_1F_7	11	8	3	0
Teal*3/ AC Majestic	BC_2F_7	9	3	6	0
Teal*4/ AC Majestic	BC_3F_7	10	2	7	1

BC_1F_1 plants from the cross 'BW384'// 'CDC Go'/ 'PT434' were homozygous for the presence of the 200 bp allele while 11 plants were heterozygous. As expected, no plant was homozygous for the absence of the marker because 'BW384' and 'PT434' were dormant and 'CDC Go' was non-dormant. Ten plants each from 'CDC Go'*2 / 'PT434' and 'CDC Go'*2 / 'PT435' were heterozygous for the marker, whereas, 19 and 14 plants were negative for the marker in the two crosses, respectively. Similarly, 17 BC_1F_1 plants from the cross 'Lillian' // 'CDC Go' / 'PT434' were heterozygous for the marker and 13 plants were negative for the marker in this cross. Similarly, 102 BC_2F_1 plants were screened with PHS resistance markers. Fifty-two BC_2F_1 plants showed the PHS resistance markers in heterozygous condition, whereas, 50 plants were negative for the desired marker. Screening of plants from different backcrosses did not differ significantly from a 1:1 ratio (Table 5.4).

In the context of backcross breeding, DNA based markers can be used to control the target gene (foreground selection) and/or to hasten the return to the recipient genotype on chromosomal regions outside the target gene (background selection). The efficiency of such marker-assisted introgression programs has been analyzed in a series of theoretical works (Visscher et al. 1996). The results indicate that marker-assisted introgression is expected to be economically important, because it permits a gain of time of about two backcross generations, compared to conventional backcross programs, which is economically important. The quantitative trait loci (QTL) with larger effects are very useful for corresponding trait improvement through MAS. Enabling favourable allele frequency to be increased in early generation through molecular markers would deliver substantial efficiency gains in a crop improvement program (Koebner and Summers 2003).

5.5 CONCLUSIONS

The primary objective of this study was to validate SSR markers associated with a major QTL for PHS resistance on chromosome 4A in different genetic backgrounds. A major QTL was identified in a DH mapping population of 'Argent' x 'W98616' in this study. Both the parents have white kernels but 'Argent' is a non-dormant genotype, whereas, 'W98616' is a dormant genotype. The main reason to use this population for

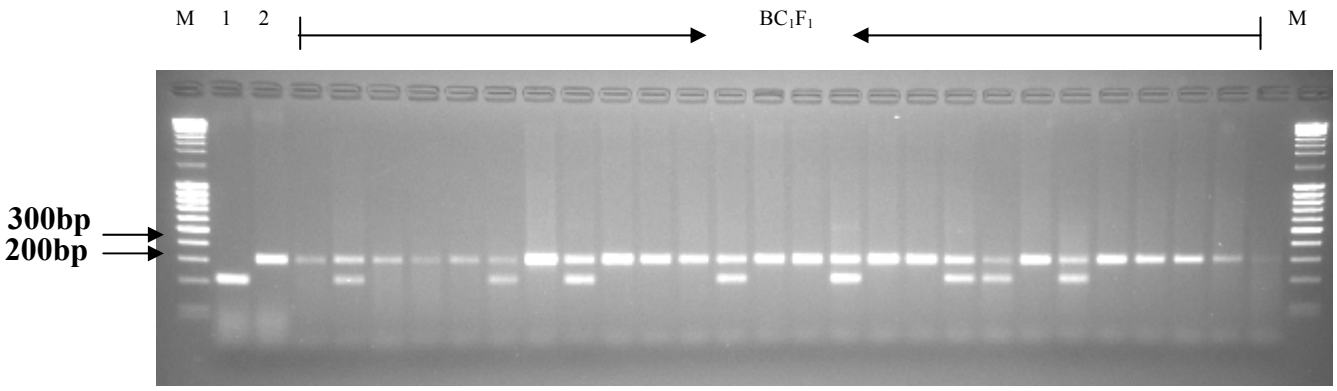

Figure 5.4. Genotyping of BC_1F_1 progeny from CDC Go*2/PT434 cross. The PCR products for DuPw004 were analyzed on 2% (w/v) agarose gel stained with ethidium bromide. M- MassRuler, 1- PT434, 2- CDC Go.

Table 5.4. Screening of backcross F_1 progeny with a PHS resistance marker (DuPw004).

Cross	Generation	Total No. of plants	Homozygous		Hetero-zygous	$\chi 2$	*P*
			200bp	300bp			
CDC Go*2/PT434	BC_1F_1	29	0	19	10	2.8	0.09
BW384//CDC Go/PT434	BC_1F_1	30	19	0	11	2.1	0.14
Lillian// CDC Go /PT434	BC_1F_1	30	0	13	17	0.5	0.47
CDC Go *2/PT435	BC_1F_1	24	0	14	10	0.7	0.41
CDC Go *3/PT434	BC_2F_1	44	0	20	24	0.4	0.55
CDC Go /3/Lillian// CDC Go /PT434	BC_2F_1	16	0	7	9	0.3	0.62
CDC Go *3/PT435	BC_2F_1	42	0	23	19	0.4	0.54

Genotype	Allele size (bp)	Phenotype
CDC Go	300	Non-dormant
Lillian	300	Non-dormant
PT434	200	Dormant
PT435	200	Dormant
BW384	200	Dormant

marker detection was to exclude the effect of grain colour on dormancy for which genes have been reported on homeologous group 3 chromosomes of wheat (Flintham et al. 1996). The QTL detected on 4A chromosome may help in the selection for PHS resistance excluding effect of grain colour. The codominant nature of these markers is one of the major advantages in selection of homozygous and heterozygous plants. Marker assisted back-crossing reduced the population size in the BC_1F_1 and BC_2F_1 generation by 41% and 59%, respectively. This reduction in population size will reduce the labour for analysis of advanced lines for other grain quality traits. The marker DuPw004 assay is PCR and agarose gel based; it can be implemented routinely to transfer this desired allele for PHS resistance in wheat breeding programs. The identification of markers associated with PHS resistance will allow breeders to select for the trait at the DNA level rather than relying on phenotypic expression. Near-isogenic lines for the PHS resistance QTL on 4A will be developed to investigate the effect of this QTL. These lines will be valuable parents for developing new mapping populations for high resolution mapping and map-based cloning of this major QTL.

CHAPTER 6
GENERAL DISCUSSION

Pre-harvest sprouting (PHS) in rain-affected wheat at harvest time represents a limitation to the reliable production of high-quality grain for export and domestic use. In wheat-growing areas that are prone to unfavorable weather conditions during the harvest period, tolerance to PHS is, therefore, a highly desirable trait. Improving PHS tolerance is difficult on a phenotypic basis since PHS is a quantitatively inherited trait and strongly affected by environmental factors. Moreover, screening for PHS resistance is hampered by the existence of genotype x environment interactions. DNA markers linked to genes involved in PHS represent a promising, environment-insensitive tool for selecting genotypes that are more resistant to PHS. The present study focused on the identification of genomic regions associated with PHS resistance in white-grained wheats with the following objectives:

(1) Identification of a suitable method for PHS phenotyping.

(2) Identification of genomic regions associated with PHS resistance.

(3) Validation of DNA markers linked to QTL associated with PHS resistance.

The level of grain dormancy is a major component of genetic variation in resistance to PHS in wheat (Mares 1987a; Mares 1993). Two basic approaches have evolved to identify genotypes with improved PHS resistance based on grain dormancy. PHS can be measured by sprouting tests of spikes subjected to artificial or natural wetting treatments and germination tests on threshed seeds (Mares 1989). The α-amylase activity and Falling Number are also used as indicators of starch degradation due to PHS. In this study, different methods of PHS phenotyping e.g. germination test, Falling Number and α-amylase activity in dry and pre-wetted seeds were performed on 40 doubled-haploid lines (20 dormant and 20 non-dormant) derived from a cross between two white-grained spring wheat genotypes ‘Argent’ (non-dormant) and ‘W98616’ (dormant). The

germination percentage segregated the dormant and non-dormant DH lines in the three years of testing, indicating that the germination test is a repeatable and reliable predictor of PHS resistance or dormancy. Falling Number and α-amylase activity at harvest time could not be correlated with dormant and non-dormant DH lines. However, the α-amylase activity in pre-wetted and dried seeds showed a positive correlation (r = 0.60) with germination percentage. There is an inverse curvilinear relationship between Falling Number and α-amylase activity (Mares 1987b). Falling Number provides a measure of endosperm quality at the harvest time (Hagemann and Ciha 1984), but it fluctuates widely depending on the degree of ripening and the amount of rainfall prior to harvest time (Mares 1993). At low α-amylase activity levels, starch quality affects the Falling Number values (Ringlund 1983). Also, α-amylase may accumulate in the grain during seed development independent of PHS (Lunn et al. 2001b; Mares and Mrva 2007).

There are three basic requirements for the genetic mapping of quantitatively inherited traits such as PHS; mapping population, marker system and linkage map of the population under consideration. In this study a doubled haploid mapping population derived from Argent (non-dormant, white-grained) x W98616 (dormant, white-grained) was used to identify the genomic regions associated with PHS resistance in white-grained bread wheat. During the last two decades, a number of DNA-based molecular marker systems have been developed for construction of linkage maps in different crop plants (Vos et al. 1995; Röder et al. 1998; Gupta et al. 1999; Jaccoud et al. 2001; Gupta and Rustgi 2004; Varshney et al. 2005). Microsatellite or SSR markers are most commonly used for development of linkage map, as they are highly polymorphic, can be exchanged between laboratories and highly transferable between populations (Röder et al. 1998; Somers et al. 2004). Amplified Fragment Length Polymorphism (AFLP), a technique based on selective PCR amplification of genomic restriction fragments, can detect a large number of dominant polymorphic markers by using a limited set of generic primers and without any prior knowledge of genomic sequence (Vos et al. 1995). Diversity Arrays Technology (DArT), a microarray hybridization-based marker technique, has the potential for increasing marker density within a short time and at low cost (Jaccoud et al. 2001; Wenzl et al. 2004). In this study, these three different types of DNA-based marker systems (SSR, AFLP and DArT) were used to construct a linkage map of Argent x

W98616. We were also able to incorporate a few expressed sequence tags (ESTs) in the linkage maps. Out of 972 polymorphic markers, 913 markers (356 SSR, 290 AFLP, 258 DArT and 9 EST) could be arranged into 44 linkage groups at a LOD score of $\geq$ 3.0 on different wheat chromosomes with total genome coverage of 2,577 cM. There was variation in marker density on different chromosomes. The marker density in the B genome was highest (2.7 cM/marker) while the D genome had the lowest marker density (5.4 cM/marker). The high level of polymorphism in the B genome may be due to the outcrossing behavior of *Aegilops speltoides* (B genome donor), polyphyletic origin of B genome (Vardi 1973) or the B genome has evolved more rapidly (Sourdille et al. 2001). Out of 913 mapped markers, 87 (9.5%) markers showed segregation distortion. Segregation distortion is a common phenomenon in different mapping populations (Singh et al. 2007a). The reasons for non-Mendelian segregation in wheat x maize-derived doubled haploid populations may be due to heterogeneity within parents, selection associated with DH production process or outcrossing (Kammholz et al. 2001).

Single marker analysis of the phenotypic and genotypic data identified ten chromosomes involved in PHS resistance. However, only five genomic regions on chromosomes 1A, 3A, 4A, 7A and 7D were detected by interval mapping at a genome-wide threshold LOD score (Figure 4.5, Figure 6.1). The QTL on chromosome 1A was located at a linkage interval of 60-70 cM and likely resides near the centromeric region. This QTL, in our study, most likely corresponds to the QTL for Falling Number and α-amylase activity because the position of the *Xpsr1327* marker associated with Falling Number and α-amylase reported by Zanetti et al. (2000), is close to the *gwm164* and *gwm357* markers. The QTL on chromosome 3A was identified at the end of the long arm between *gwm340* and *wPt-1596*. The QTL on 3AL in our study corresponds to the QTL identified by Groos et al. (2002) and Kulwal et al. (2005). The *vivipary* loci (*taVp1*) that contribute to PHS susceptibility and the red grain loci (*R*) that control seed colour and coat imposed dormancy have been mapped to homoeologous group 3 chromosomes (Bailey et al. 1999). However, in this study there was no LOD peak in the *TaVP1* region and thus grain dormancy was not due to the direct effect of *TaVP1*.

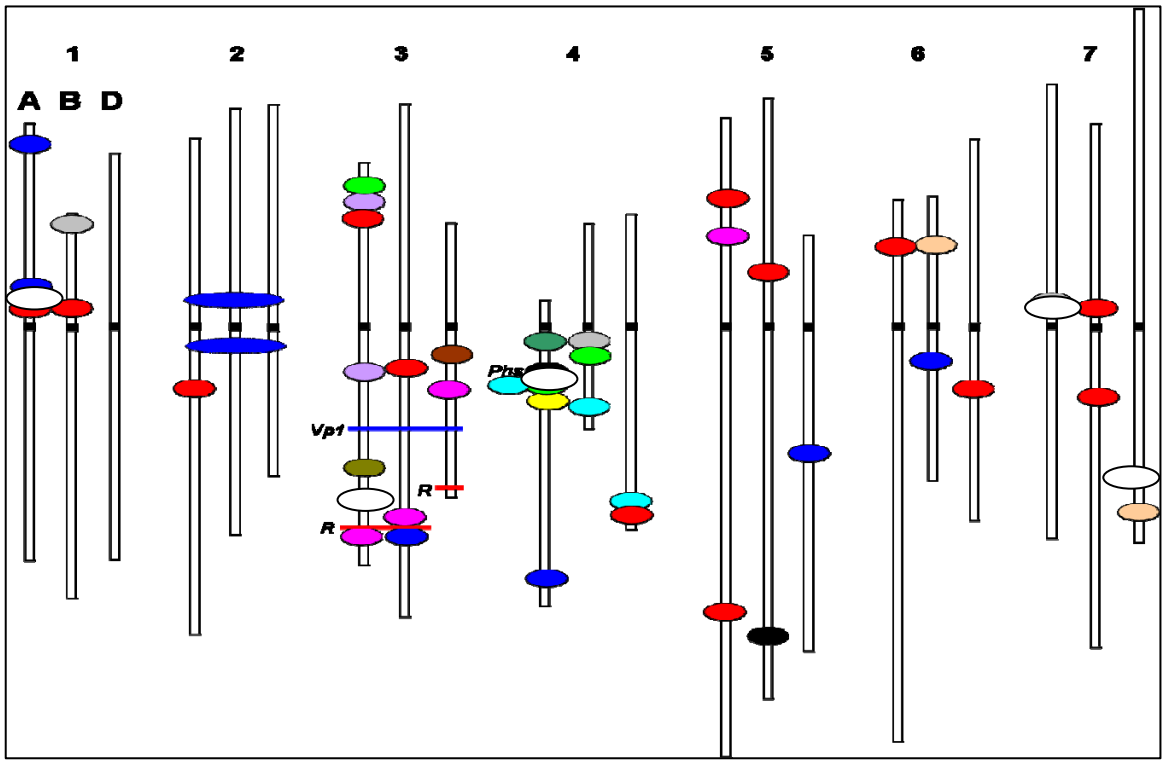

Figure 6.1. Summary of genomic locations of QTL associated with PHS resistance in present and previous studies. ***White oval structures represent the QTL identified in this study.*** Approximate locations of QTL in other studies: – Anderson et al. 1993; – Roy et al. 1999; – Zanetti et al. 2000; - Mares and Mrva 2001; - Kato et al. 2001; – Flintham et al. 2002; - Groos et al. 2002; – Mares et al. 2002; - Osa et al. 2003; - Kulwal et al. 2005; – Mares et al. 2005; – Mori et al. 2005; – Tan et al. 2006. The chromosome length and centromere positions are based on a wheat consensus map (Somers et al. 2004).

Interval mapping revealed two peaks on chromosome 4A in marker intervals *gwm397/wmc650* and *wmc707/wmc161*. Mapping of more markers may resolve whether the two peaks observed correspond to two different QTL or one. In this study, in the marker interval *gwm397/wmc650*, we were able to map one EST (CD920298) described as ABA induced plasma membrane protein PM19 (http://www.ncbi.nlm.nih.gov/). Noda et al. (2002) suggested that wheat embryo sensitivity to germination inhibition by abscisic acid (ABA) is controlled by major gene(s) located on the long arm of chromosome 4A. The QTL on the short arm of chromosome 7A at the interval between *barc222/gwm573* corresponds to the locus *Xpsp3050* associated with PHS resistance reported in a mapping population of RL4137 x Timgalen (Flintham et al. 2002) and may be a putative locus contributed by RL4137 as the mapping population in this study also has RL4137 as a grand-parent.The QTL on chromosome 7D corresponds to the *α-Amy-D2* locus on the long arm of chromosome 7D. The QTL on chromosome 4A were significant in three out of four trials, whereas, QTL on chromosome 1A, 3A, 7A and 7D were significant in two trials at a genome-wide threshold LOD score.

Before using molecular markers in actual plant breeding programs, it is necessary to undertake studies on marker validation, a process of examining the behavior of markers and associated polymorphism in different genetic backgrounds. During this investigation, a study was also carried out to validate molecular markers in QTL on chromosome 4A for PHS resistance in different genetic backgrounds and to incorporate the superior alleles in different elite genotypes through backcrossing. The marker classification of different wheat genotypes at the *XDuPw004*, *Xbarc170* and *Xwmc650* loci and percent germination were used for QTL analysis via the single-marker linear regression approach. DuPw 004, barc170 and wmc650 explained 67%, 75% and 60% of total variation in germination (%), respectively. A survey of pedigrees of different genotypes revealed that RL4137 is a major source of increased PHS resistance in a number of Western Canadian wheat cultivars. Red grain colour has traditionally been associated with seed dormancy, however, it is now clear that red colour alone is not adequate to guarantee dormancy (Bassoi and Flintham 2005; Flintham et al. 1999). In this study, a large portion of the red-grained wheat genotypes are non-dormant while four out of nine white-grained wheat genotypes carried dormancy QTL. The microsatellite

markers (DuPw004, barc170 and wmc650) may be useful for the plant breeders to pyramid this QTL with QTL from other PHS resistance sources.

6.1 Future research directions

Excess α-amylase activity impairs wheat grain quality since enzymatic hydrolysis of starch leads to processing problems and unsatisfactory end-products. The major cause of excess α-amylase activity is PHS, when ripe grains germinate in the field in wet weather due to lack of dormancy. However, excess α-amylase activity also accumulates early during wheat grain development and not due to PHS (Lunn et al. 2001a; 2001b). This includes late maturity α-amylase (LMA) (Mares and Mrva 2007), also known as pre-maturity α-amylase and retained pericarp α-amylase (Lunn et al. 2001b). Unlike sprout damage, LMA may be triggered by low temperature shock (12-18°C) during the second half of grain filling period at 25-35 days after flowering (Mrva and Mares 2001). Rarely, α-amylase activity also accumulates due to germination in the early development stage when grains are still at high moisture content, normally before 35%, and is known as pre-maturity sprouting (Lunn et al. 2001b). Therefore, these routes of α-amylase accumulation should be taken into consideration while breeding for PHS resistance/dormancy because LMA is completely independent of PHS and can be expressed in sprouting tolerant or dormant genotypes (Nakatsu et al. 1996).

Thirty advanced lines (F_7) from three crosses (Table 5.3) developed to introgress PHS resistance from 'AC Majestic' into the PHS susceptible cultivar 'CDC Teal', were selected on the basis of germination tests during the first two backcrosses and in homozygous generations on the basis of Falling Number as PHS resistant (Hucl, personal communication). Thirteen lines were positive for the presence of PHS resistance marker (DuPw004) based on the germination test. It will be interesting to phenotype and correlate these genotypes based on germination percentage. Markers in QTL on 1A and 7D can be used on these genotypes as these QTL were also reported to be associated with Falling Number and α-amylase activity. Similarly, the backcross line (Table 5.4) screened for the DuPW004 marker should be phenotyped with the germination test to correlate with the genotypic data.

6.2 Conclusions

- Germination testing of seeds was the most reliable method for measuring PHS resistance in white-grained wheat.
- The level of α-amylase activity in pre-wetted and dried seeds can predict the level of PHS. The enzyme activities in pre-wetted samples of the DH lines showed a statistically significant correlation ($r = 0.60$) to germination percentage.
- Falling Number showed a strong correlation with α-amylase activity in dry seeds at harvest time, but was not correlated with percent germination.
- Five genomic regions associated with PHS resistance identified by interval mapping on chromosomes 1A, 3A, 4A, 7A and 7D were all contributed by the dormant parent, W98616.
- The SSR markers DuPw 004, barc170 and wmc650 explained 67%, 75% and 60% of the total variation in germination (%), respectively, among different wheat genotypes.
- Marker analysis on the chromosome 4A QTL and a survey of pedigrees of different genotypes revealed that RL4137 is a major source of increased PHS resistance in a number of Western Canadian wheat cultivars.
- Marker assisted back-crossing with DuPw 004 reduced the population size in the BC_1F_1 and BC_2F_1 generations by 41% and 59%, respectively.

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APPENDIX I

PLANT DNA EXTRACTION

The plants were grown under controlled conditions in the growth chamber under an 18 h photoperiod (250 µmol m^{-2} s^{-1} light intensity, 23°C day and 18°C night temperature) or green house. The leaves were harvested after 20 days of seeding and freeze dried. The genomic DNA was isolated from freeze dried leaves using the CTAB method (Doyle and Doyle 1990). Approximately 20 mg of leaves were ground to fine powder in 2 ml centrifuge tube containing 2 glass balls (5 mm) in the grinding apparatus at 50 MHz for 10 min. After addition of 800 µl of DNA extraction buffer, the tubes were vortexed briefly and incubated at 60ºC for 10 min. The tubes were inverted 3-4 times during the incubation period to keep the samples well mixed. Then 800 µl of chloroform: isoamyl alcohol (24:1) was added to the tubes and mixed by inverting the tubes several times to obtain a homogeneous solution. The tubes were centrifuged at 13,000 rpm for 5 min at room temperature and 750 µl of the upper phase was transferred to new centrifuge tubes. Again, 750 µl of chloroform: isoamyl alcohol (24:1) was added to the tubes, mixed well and centrifuged at 13,000 rpm for 5 min at room temperature. Then, 700 µl of the upper phase was transferred to new centrifuge tubes and 700 µl of DNA precipitation buffer was added, mixed immediately and kept at room temperature for 20 min. The samples were centrifuged at 13,000 rpm for 10 min. The supernatant was carefully poured off and the last droplets of supernatant were removed by placing the tubes upside-down on a paper tissue (Kimwipe). The pellets were resuspended in 500 µl of 1M NaCl and the samples were kept at 60ºC for 5 min to ensure that all the pellets become dissolved. Then, 1 ml of absolute ethanol was added, mixed well and incubated at -20ºC overnight. The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 6 min at room temperature. The supernatant was discarded and 1.5 ml of 80% (v/v) ethanol was added to the pellet and centrifuged at 13,000 rpm for 6 min at room temperature. The supernatant was carefully poured off. The tubes were again centrifuged to collect the remaining liquid to the bottom of the tube and all the liquid from the tubes was removed by pipette. The pellets were air dried and dissolved in 150 µl of Tris-EDTA buffer (1X).

The DNA samples were treated with RNase to remove residual RNA. 0.5 µl of RNase A (10 mg RNase A/ ml) was added to each DNA sample and incubated at 37ºC for 1 hr. Then 150 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each sample and mixed by inverting the tubes. The tubes were centrifuged at 13,000 rpm for 10 min at room temperature and 140 µl of upper phase was transferred to new centrifuge tube. Then 140 µl of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 13,000 rpm for 5 min at room temperature. 140 µl of the upper phase was transferred to a new centrifuge tube and, 1/10 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol was added and mixed. Samples were incubated at -80ºC for 10 min to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 15 min at room temperature. The pellets were washed by adding 1ml of 80% (v/v) ethanol and centrifuged at 13,000 rpm for 5 min. The 80% ethanol washing step was repeated again. The pellets were air dried and dissolved in 100 µl of Tris-EDTA buffer (1X). The concenteration of DNA was determined using a DU® 800 spectrophotometer (Beckman Coulter). The spectrophotometer was calibrated with Tris-EDTA buffer (1X). DNA was diluted to 60 times with Tris-EDTA buffer (1X) and quantified.

AGAROSE GEL ELECTROPHORESIS

PCR products were analysed by 2% (w/v) agarose gel electrophoresis. Agarose powder was mixed with 0.5X TAE electrophoresis buffer to the desired concentration and heated in a microwave oven until completely melted. Ethidium bromide was added to the gel (final concentration 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis. The solution was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After solidification of the gel, the comb was removed and the gel in the plastic tray was placed in the electrophoresis chamber. The electrophoresis chamber was filled with 0.5X TAE buffer so that it covered the gel. DNA samples mixed with loading buffer were then pipeted into the sample wells. Electrophoresis was carried out at constant 100 volts for 1 hr. The gels were then visualized with an ultraviolet transilluminator (BioRad Gel Documentation system).

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Gel preparation

The small and large glass plates were thoroughly cleaned on one side twice with 95% ethanol and Kimwipes® tissue papers. Repel Silane (3 ml) was applied on the cleaned side of the large plate and spread over the entire surface with a Kimwipes® tissue paper. After 5 min, the excess of Repel Silane was removed with Kimwipe® tissue paper saturated with deionized water and plate was allowed to dry. The cleaned small plate was treated with 3 µl of Bind Silane in 1 ml of 0.5% acetic acid in 95% ethanol. The Bind Silane was spread over the entire surface with Kimwipe® tissue paper. After 5 min, the small plate was wiped with 95% ethanol and Kimwipe® tissue paper 3-4 times to remove the excess of binding solution and allowed to dry. The glass plates were assembled by placing 0.4 mm side spacer between the plates and plates were fitted in casting boot (Life Technologies^{TM}). Care was taken not to allow the treated surfaces of the glass plates to touch each other.

50 µl of TEMED and 500 µl of 10% (w/v) ammonium persulfate was added to 6% gel mix in a beaker. The solution was carefully poured between the glass plates using a 60 ml syringe. A constant flow of solution was maintained at one side of the assembled plates to prevent the formation of bubbles in the gel. The straight side of the sharktooth comb was inserted between the plates up to a depth of 6 mm. The comb was secured with 4 clamps. The gel was allowed to polymerize for 1 hr.

Sample preparation

The samples were prepared by mixing 4 µl of PCR product with 4 µl of STR 2X loading dye. The samples were denatured by heating at 95°C for 2 min and immediately chilled on ice.

Gel electrophoresis

After polymerization of the gel, the clamps were removed and excess polyacrylamide was shaved from the comb with a surgical blade. The comb was then removed from the plates. The plates were removed from the casting boot and fixed in the Model S2 sequencing gel electrophoresis apparatus (Mandel Scientific Company Inc., Guelph, Ontario, Canada). with the longer plate facing out and the well side on top. 0.5X TBE buffer was added in both upper and lower buffer chambers of the electrophoresis

apparatus. The air bubbles were removed from the top of the gel with a syringe filled with the buffer. The gel was allowed to run at 80 watts, 60 milli amperes and 3000 volts to achieve a gel surface temperature of approximately 50°C. After the prerun, the urea was flushed from the well area with a syringe filled with buffer. The comb teeth of the sharktooth comb were inserted into the gel approximately 1-2mm. The combs were kept inserted in the gel during both gel loading and electrophoresis. 3µl of each sample was loaded in the respective wells and also a 50 bp DNA ladder was loaded in the three wells. Electrophoresis was conducted 80 watts, 60 milli amperes and 3000 volts for 1 hr.

Gel staining

After electrophoresis, the glass plates were removed from the apparatus. The comb and side spacers were removed from the plates, and the plates were separated from each other. The gel remained affixed to the small glass plate and placed in a shallow plastic tray. The gel was stained with silver stain using following steps:

Step	Solution	Time
1	fix / stop solution	20 min
2	deionized water	2 min
3	repeat step 2, twice	2 x 2 min
4	staining solution	30 min
5	deionized water	10 sec
6	developer solution (4-10°C)	up to 5 min (until alleles and ladder became visible)
7	fix / stop solution*	5 min
8	deionized water	2 min

*Added directly to the developer solution to stop the reaction

The gel (small plate) was positioned upright and allowed to dry overnight. The gel was scanned with an Espon Expression 1680 scanner.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

Gel preparation

The small and large glass plates were thoroughly cleaned with 95% ethanol and Kimwipes® tissue papers. The glass plates were assembled by placing 1.5mm side spacer between the plates and the plates were fitted in gel casting assembly. 34 µl of TEMED and 165 µl of 10% (w/v) ammonium persulfate were added to 50 ml of 10% gel mix in a beaker. The solution was carefully poured between the glass plates. A constant flow of solution was maintained on one side of the assembled plates to prevent the formation of bubbles in the gel and the comb was inserted between the plates. The gel was allowed to polymerize for one hour.

Sample preparation

PCR product (5 µl) was mixed with 9 µl of formamide loading dye (99.6% formamide, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cynol). The samples were denatured by heating at 95°C for 5 min and kept on ice for 10 min.

Gel electrophoresis

After polymerization of the gel, the comb was removed from the plates and excess of polyacrylamide was shaved from the top of the plates with a surgical blade. The plates were removed from the casting assembly and fixed in the gel electrophoresis apparatus with the longer plate facing out and the well side on top. 0.6X TBE buffer was added in both the upper and lower buffer chambers of the electrophoresis apparatus. The gel was allowed to run at 3 watts, 50 milli amperes and 200 volts for 1 hr. The temperature of the gel was maintained at 18°C with the gel cooling system. After a prerun of the gel, the wells were cleaned with a syringe filled with buffer. Ten µl of each sample was loaded in the respective wells. The electrophoresis was conducted at 3 watts, 50 milli amperes and 250 volts for 14-16 hr at 18°C.

Gel staining

After electrophoresis, the glass plates were removed from the electrophoresis apparatus. The side spacers were removed from the plates, and the plates were separated from each other. The gel was separated from the plates and placed in a shallow glass tray on a shaker. The gel was stained with silver stain in using the following steps:

Step	Solution	Time
1	fix solution	3 min
2	staining solution	5 min
3	deionized water	10 sec
4	developer solution	up to 10 min (until bands become visible)
5	deionized water	2 min