GENETICS AND LINKAGE ANALYSIS OF GRAIN DORMANCY AND TYROSINASE IN WHEAT

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
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DOCTOR OF PHILOSOPHY

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ABSTRACT

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Pre-harvest sprouting due to low levels of seed dormancy, and high levels of the enzyme tyrosinase adversely affect the quality of wheat flour. The present knowledge of the genetics of tyrosinase and dormancy is inconsistent and inconclusive. Knowledge of the genetics of resistance to dormancy and tyrosinase will aid selection in breeding programs and will also facilitate linkage analysis with molecular markers. The main objectives of this research were to determine the number of genes involved in seed dormancy and tyrosinase activity in wheat and to develop molecular markers for these genes. Seeds from recombinant inbred lines matured under controlled environmental conditions (16°C day/night) were evaluated for dormancy in germination tests performed at 15°C and 20°C. Two dormancy genes were identifed in the population (BiggarBSR/RL4555) and lines carrying either or both genes could be identified using the germination tests at both temperatures. A RAPD marker (primer UBC303) was identified as linked to one dormancy gene by 25.74 cM (+/- 5.2 cM) however the chromosome location of the dormancy gene was not determined.

A quantitative colorimetric microtiter plate assay was developed to measure levels of tyrosinase activity in individual wheat kernels. All currently registered Canadian common wheat cultivars had high levels of tyrosinase activity while all Canadian durum wheat cultivars had no tyrosinase activity. A synthetic hexaploid wheat (RL5710) with no tyrosinase activity was identified and used as a parent in crosses to produce segregating populations. A strong gene and a weaker gene which interact epistatically were identified in the segregating F_2 and F_3 populations. The genes were located on chromosomes 2DS and 2A as determined by the analysis of alien addition and substitution lines. The knowledge of the chromosome location allowed the developement of molecular markers linked to the tyrosinase loci from RFLP markers previously mapped to chromosome group 2. RFLP markers (B15C and ABG019) flanking the gene on 2DS were identified. Each marker was shown to be linked by approximately 15 cM on either side of the tyrosinase gene. This would result in a selection error rate of 8% when both markers were used together.

FORWARD

This thesis begins with a general abstract, introduction and a literature review. This is followed by the presentation of three chapters each comprised of specific research objectives. Chapters 3 and 5 are presented in the following format: Abstract, Introduction, Material and Methods, Results and Discussion while Chapter 4 presents the Results and Discussion together in one section. The thesis is written in the style of the Canadian Journal of Plant Science. The thesis concludes with a general discussion which brings all aspects of the research together and summarizes the main points of the research, and a list of references cited.

Chapter 5, entitled "Kernel tyrosinase in *Triticeae* spp. and the inheritance of genes controlling its expression in common wheat", has been published in part: **Bernier, A.M and Howes, N.K. 1994.** Quantification of Variation in Tyrosinase Activity Among Durum and Common Wheat Cultivars, Journal of Cereal Science 19:157-159. This chapter presents this published work as well as the subsequent research on the genetics and molecular marker analysis of the enzyme tyrosinase. Chapter 3 entitled "Inheritance of seed dormancy in wheat grown under controlled environmental conditions" describes the characterization of segregating populations for dormancy. A population characterized for dormancy was used for the analysis of RAPD markers linked to dormancy. Chapter 4, entitled "RFLP analysis of the group 2 chromosomes in wheat", presents a collection of data generated toward the objectives of mapping the group 2 chromosomes of wheat using previously mapped barley markers and the search for the potential involvement of the group 2 chromosomes in the expression of dormancy. The mapping of the group 2 chromosomes of wheat also led to the identification of a molecular marker linked to a smut resistance gene. This research was published in 1997 **(Procunier, J.D., Knox, R.E., Bernier, A-M., Gray, M.A., and Howes, N.K. 1997.** DNA markers linked to a T10 loose smut resistance gene in wheat (*Triticum aestivum* L.) Genome 40(2): 176-179) and this article is referred to in this chapter.

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CHAPTER 1
Introduction

Wheat cultivars without adequate dormancy can succumb to preharvest sprouting if cool and moist environmental conditions prevail prior to harvest. Preharvest sprouting in the spike causes yield losses, reduced test weight and reduced end-use quality due to high levels of α-amylase. These high levels of α-amylase can have severe consequences for the processing of wheat, causing a sticky crumb in bread and the collapse of loaves. Chinese and Japanese noodles made from such flour are also discoloured and lack elasticity (Derera, 1988).

The enzyme tyrosinase is found in the pericarp of the kernels of most cereals. Although the physiological function of this enzyme in the cereal kernel remains unknown, its effects on the end-use quality of wheat are well documented (Matheis and Whitaker, 1984, Kobrehel *et al.* 1974, Kruger *et al.* 1994b, Baik *et al.* 1994). The primary effect of high levels of tyrosinase is the discoloration of products such as oriental noodles, chapattis and flat breads.

Medium-hard white wheats with medium strength dough suitable for the production of alkali noodles in Asia have been identified as a growing market for Canada. Existing Canadian Prairie Spring wheat cultivars exhibit relatively low levels of seed dormancy, are mostly red kernelled and have high levels of tyrosinase. The development of wheat cultivars with increased seed dormancy is a breeding objective in many countries where preharvest sprouting is a concern. White kernelled germplasm with higher levels of dormancy have been identified and can therefore be used as sources of dormancy. A clearer understanding of the genetics of dormancy in these lines will provide a more efficient means of selection. The incorporation of this trait into new cultivars could also be facilitated by the development of molecular markers for dormancy genes.

Germplasm having low or zero levels of tyrosinase needs to be identified and incorporated into white cultivars. A survey of the genetic variation in tyrosinase activity

amongst durum and common wheat cultivars may enable the reduction of the browning of wheat products by identifying and selecting germplasm having lower levels of the enzyme. A thorough understanding of the genetics of this trait should improve selection aimed at reducing or eliminating tyrosinase. The development of molecular markers for tyrosinase genes providse potential to further accelerate the selection process.

The primary objectives of this research project were to determine the number of genes involved in seed dormancy and tyrosinase activity in wheat and to develop molecular markers linked to genes for these two traits. The strategy used in the first case was to identify dormancy genes that were expressed at different germination temperatures in segregating populations grown under controlled environmental conditions. Genes identified in this way were then targetted by random amplified polymorphic DNA analysis. A second approach to the identification of markers linked to dormancy genes was a chromosome specific search involving the group 2 chromosomes and restriction fragment length polymorphism DNA markers. The strategy for the genetic study of tyrosinase involved the use of cytogenetic stocks such as alien substitution and addition lines to identify the chromosome location of the gene(s). Once the chromosome location was identified, chromosome specific molecular markers could be used to target the tyrosinase loci.

CHAPTER 2
Literature review

1. INTRODUCTION

Many factors are involved in the determination of the quality of wheat products such as bread, noodles and pasta. Reduction in end use quality may be caused by factors such as sprouted grain and high levels of the enzyme tyrosinase. Sprouted wheat contains germination specific enzymes such as alpha-amylase and proteases which can affect the loaf volume and crumb characteristics of wheat, and the quality of noodle products. The germination of seeds in the heads before harvest is called preharvest sprouting (PHS) and is due to a low level of dormancy in the grain. To better understand the complexities of dormancy this review will summarize the research published to date on the physiology, testing methodologies and the genetics of dormancy.

Tyrosinase is present in the bran of most common wheat cultivars and is responsible for the undesirable browning of many wheat end-products. A complete review of tyrosinase in cereals will be presented with sections on the physiology, assay methodologies, proposed roles of the enzyme in the plant and the impact of the enzyme on end-product quality. Theories pertaining to the involvement of tyrosinase in dormancy will be presented.

An integral part of the research presented in this thesis involved the use of random amplified polymorphic DNA markers (RAPD) and restriction fragment length polymorphism (RFLP) markers to locate genes to specific chromosomes. A separate section will be presented here on the use and importance of these molecular markers in plant breeding and genetic studies, including a brief discussion of the advantages and disadvantages of these types of markers and a review of their successful applications to tagging and mapping specific genes in cereals and other crop plants.

2. PREHARVEST SPROUTING IN WHEAT

2.1 Introduction

At the turn of the century seed scientists noticed that morphologically mature seeds did not always germinate even under favourable conditions (as cited in Derera *et al.* 1977). Such seeds exhibit a phenomenon called dormancy. Dormancy is an important characteristic for the survival of many wild plant species. Some seeds stay dormant for several years to bypass unfavourable growing seasons. In the cereal industry, dormancy is also an important characteristic. Under moist environmental conditions, cereal seeds with little or no dormancy can sprout directly in the head before harvest can occur. The damage caused by this pre-harvest sprouting (PHS) of the kernels includes harvest losses, reduced test weights, loss of seed viability and reduced end-use quality. Preharvest sprout damage is relatively common in the major wheat producing areas, occurring in 3 to 4 out of every 10 years (Derera 1989). The incidence and severity of sprout damage is highly variable, reflecting the variability in the weather at harvest and differences in susceptibility of the cultivars grown to pre-harvest sprouting .

2.2 Physiology of dormancy in wheat

The exact mechanism by which a seed can resist germination under favourable conditions remains unknown , however, over the years many factors which may contribute to dormancy have been investigated. Some of the factors that have been proposed as involved in increased levels of dormancy in wheat include reduced levels of alpha-amylase (Gale and Marshall 1973, 1975), alpha-amylase inhibitors (Weselake *et al.* 1983 a,b), slow water uptake (King 1984), and interference with gas exchange by the embryo by the enzyme polyphenol oxidase (Côme *et al.* 1984). All these factors are briefly discussed here in relation to the their proposed involvement in dormancy. Red seed coat colour in wheat has been associated with high levels of preharvest sprouting resistance. A more detailed review of the involvement of red seed coat colour in dormancy is presented as it was a factor investigated in the present study.

2.2.1 GA effects

The main detrimental effect associated with PHS is the increase in α-amylase activity in germinated kernels. The enzyme is detrimental in flour because it has negative effects on bread and noodle quality (Morad and Rubenthaler 1983, Edwards *et al.* 1989, Sorrells *et al.* 1989, Bushuk and Lukow, 1987). Alpha-amylase is produced *de novo* in germinating seeds in response to gibberellic acid (GA) (Paleg 1960a , 1960b). GA is produced in the embryo and is secreted to the aleurone cells of the kernel which in turn produce α-amylase. The amylase is secreted in to the endosperm where it degrades the starch into simple sugars which are used by the embryo during germination.

Gale and Marshall (1973) discovered that the dwarf wheat cultivar Tom Thumb did not respond to GA with the expected release of α-amylase compared to normal tall genotypes. They suggest that this attribute could be used to increase PHS resistance in wheat. Tom Thumb carries the dwarfing gene *Rht3* which is pleiotropic or very tightly linked to the gibberellin insensitivity gene *Gai3*. The *Rht3/Gai3* gene is located on chromosome 4A (Gale and Marshall 1975) and is referred to as one locus on the genetic map of wheat (McIntosh 1988).

Rht3 was consistently associated with a 77% reduction in kernel α-amylase in wheat grown under field conditions conducive to sprout damage (Flintham and Gale 1982) . Flintham and Gale (1982) also found that *Rht3* does not affect α-amylase isozyme distribution but rather decreases enzyme activity in general. Although α-amylase activity is reduced, *Rht3* does not prevent the emergence of the coleoptile or radicle. *Rht3* does prevent accumulation of α-amylase messenger RNA more effectively at high temperature (25°C) than at low temperatures (Lenton and Appleford 1992).

Bhatt *et al.* (1977) examined the potential use of *Rht3* in breeding for PHS resistance in F_2 plants from the cultivar Tordo (*Rht3*) and a semi-dwarf cultivar. Resistance to sprouting was found to be strongly linked with the severe dwarf plant habit. They suggested that the severe dwarfism associated with the *Rht3* gene will be problematic in a plant breeding program which aims at exploiting the GA insensitivity of the *Rht3* genotypes. Other sources of dwarfism exist (eg. Japanese cultivar Norin 10) but

these do not possess the aleurone insensitivity to GA. These carry the genes *Rht1* and *Rht2* which have only small effects on the reduction of α-amylase during sprouting (Flintham 1989, Mares *et al.* 1983)

2.2.2 Alpha-amylase inhibitor

A proteinaceous inhibitor of α-amylase has been isolated from barley (Weselake *et al.* 1983 a,b), wheat (Mundy *et al.* 1984) and rye (Hejgaard *et al.* 1984). This protein specifically inhibits germination specific α-amylase isozymes. The level of inhibitor in barley varies widely among cultivars (Weselake *et al.* 1985, Munck *et al.* 1985) and is found throughout the mature endosperm and in aleurone and embryo tissues. The α–amylase inhibitor in barley and rye is encoded by a single structural gene on chromosome 2H in barley and on chromosome 2R in rye (Hejgaard *et al.* 1984). The locus was assigned the symbol *Isa-1* (McIntosh 1988). Three inhibitor loci identified in wheat were found on the long arms of chromosomes 2A, 2B and 2D (Masojc *et al.* 1993) using isoelectric focusing, cytogenetic stocks and a monoclonal antibody developed by Zawistowski *et al.* (1992).

Zawistowska *et al.* (1988) showed that the addition of pure barley α-amylase inhibitor to sprout damaged wheat flour improved its baking quality. This however does not provide direct evidence of a role in the regulation of α-amylase activity during pre-harvest sprouting. Synthesis of the inhibitor occurs during grain development and reaches a constant level 4 to 5 weeks post anthesis followed by a decrease (Robertson and Hill 1989). It is unlikely that the inhibitor plays a direct role in dormancy since its action on α-amylase would have to occur during germination. The inhibitor may act more like a germination attenuator. The inhibitor in the mature seed could have some action on the initial α-amylase produced in germination: however this would be only temporary as the large increase in α-amylase produced during germination would rapidly bind all the available inhibitor (Robertson and Hill 1989). Alpha-amylase synthesis is stimulated during germination by GA in aleurone tissues while synthesis of the α-amylase inhibitor is inhibited by GA (Mundy *et al.* 1986). The inhibitor suppresses a germination specific

cereal α-amylase and a bacterial protease (Mundy *et al.* 1983), indicating that it may have a role in slowing down seedling metabolism or in restricting infection by microorganisms.

Munck *et al.* (1985) studied the levels of the inhibitor in barley cultivars having varying degrees of sprouting resistance. No correlation between germination and α-amylase inhibitor levels was found. Abdul-Hussein and Paulsen (1989) found that the inhibitors interact with calcium but do not have primary roles in PHS.

2.2.3 Water uptake

Wheat ear characteristics are potential factors which could influence the level of dormancy by altering water uptake by the enclosed grain. A water uptake study of 50 cultivars by Kings and Richard (1984) showed that awns and their associated structures account for some of the cultivar differences. Cultivars and near isogenic lines with awns absorbed up to 30% more water than cultivars and near isogenic lines without awns (King and Richards 1984). Sprouting in the ear was less severe in the awnless lines while glaucousness or pubescence did not affect the level of sprouting. Kings and Richards (1984) suggested that selection for awnlessness could reduce sprouting. King (1984) also studied water uptake in relation to grain characteristics such as grain coat colour, pericarp or testa thickness, grain hardness or grain protein content. Cultivars used in the study varied in water uptake, however this was not correlated to any of the grain characteristics studied.

2.2.4 Tyrosinase

Tyrosinase (Phenol oxidase E.C. 1.14.18.1) is found in most plant tissues. In most cereals seeds such as wheat and barley it is located in the pericarp. Dormancy may be broken by facilitating transport of oxygen to the embryo by the removal of the pericarp or by damaging the pericarp (Mares and Ellison 1990). Interference with gas exchange by the embryo may occur by the enzymatic consumption of oxygen by the testa, glumes and pericarp, thereby depriving the embryo of oxygen and inhibiting germination. Pericarp phenol oxidases are thought to be involved in this enzymatic oxygen consumption

(Kruger 1976, Gordon 1980, Gordon 1979). A more complete summary of phenol oxidases in wheat is presented elsewhere in this review as a genetic and chromosome mapping study of the enzyme was an integral part of this thesis research.

A French group of scientists has been involved in the elucidation of the role of polyphenol oxidase (PPO) in dormancy. They have found that in barley, the hulls interfere with germination by consuming oxygen (Côme et al. 1984, Corbineau and Côme, 1980). Hulls contain phenolic compounds which can be readily oxidised by PPO thus imparting a state of hypoxia in the embryo. At higher temperatures more oxygen was used by the hulls in spikes containing both dormant and non-dormant seeds, with the dormant seeds fixing three times more oxygen than the non-dormant seeds (Côme *et al.* 1984). The pericarp itself could be involved in oxygen consumption (Côme et al. 1984, Corbineau *et al.* 1980). Pericarp induced dormancy is generally less intense than hull induced dormancy in barley, and is more readily eliminated through after ripening of the seeds.

Lenoir *et al.* (1986) found, however, that the breaking of dormancy during afterripening was not due to a qualitative or quantitative modification of phenols or PPO in barley. They observed that the hulls of dormant seeds take up O_2 at the beginning of imbibition while the hulls of non-dormant seeds start to take up oxygen only after about 10 hours of imbibition. This delay in oxygen consumption could allow germination. Gfeller and Svejda (1960) also found that the level of PPO was not related to dormancy in a study comparing levels of tyrosinase in dormant and non-dormant cultivars.

Although there is no direct evidence for a causal relationship between PPO and dormancy every physical or biochemical treatment which reduces the oxidation reaction by PPO and facilitates oxygen passage to the embryo leads to better germination (Côme *et al.* 1984).

2.3 Red seed coat colour and dormancy

The development of wheat cultivars with increased seed dormancy is a plant breeding objective in many countries where the threat of moist harvest conditions exists. Among all classes of wheat, those with a white seed coat colour are most susceptible to preharvest sprouting while the red seed coat colour has long been associated with higher levels of PHS. Gordon (1979) suggested that a link existed between chemical precursors of the red pigment and resistance to PHS. Freed *et al.* (1976) report that the most dormant cultivars tested have three genes for seed colour while those with less dormancy have two genes. Thus, they concluded that different colour genes contribute different levels of PHS resistance. Gene dosage levels do not always account for observed variations in sprouting resistance within red seeded wheat. McEwan (1980) found that single red gene stocks of the cultivar Hilgendorf-61 have as much dormancy as their three red gene donor parent. He concluded that high levels of sprouting resistance can be conferred by single red gene factors. However, Flintham (1993) reports that the sprouting scores for red grained lines of an inbred population increased significantly with increases in red gene dosage. This did not, however hold true in a survey of 83 cultivars and lines conducted in the same study. The red gene dosage effect on resistance was not always observed, as cultivars with three genes varied from very resistant to sprouting to very susceptible. Gordon (1983) detected only a moderate relationship between seed coat colour and resistance to sprouting in 97 *Triticum* sp. genotypes. The results from all these studies show that there is genetic variation for dormancy within the red classes of wheat which is not always linked to the red genes.

A series of investigations were undertaken to try to recombine dormancy and white seed coat colour using red seeded sources of dormancy. Gfeller and Svejda (1960) hybridized a red seeded dormant cultivar and a non-dormant white seeded cultivar and failed to find a dormant white seeded recombinant. Freed *et al.* (1976) also reported the absence of recombination between seed colour and seed dormancy in a number of crosses and backcrosses involving red dormant and white nondormant cultivars. Soper *et al.*

(1989) concluded that the relationship between red colour and dormancy is either due to very tight linkage or pleiotropy in durum wheats.

Numerous other studies were performed in an attempt to recombine dormancy and white seed colour. Reitan (1980) detected 2 mechanisms controlling dormancy in an 8x8 diallele cross. One mechanism was associated with red seed coat colour, the other was independent. Depauw and McCaig (1983) also recombined white seed colour with resistance to sprouting. In a cross between the red seeded line RL4137 and a white seeded wheat they recovered white seeded F_3 lines which were as resistant as some of the red seeded dormant controls. These white seeded progenies did not however exhibit a level of dormancy equal to RL4137. These results suggest that RL4137 has a genetic mechanism for dormancy associated with red seed colour, and one or more mechanisms not associated with seed colour. The line RL4137 has been used extensively in genetic studies because of its very high level of dormancy. The cultivar Columbus was the first red spring wheat cultivar developed possessing dormancy from RL4137 (Noll and Czarnecki 1980). The germplasm Losprout, a white seeded wheat developed by Depauw *et al.* (1985), also has sprouting resistance derived from RL4137 .

Clarke *et al.* (1984) studied weathering damage on 14 cultivars as measured by falling number values. Falling number is an assay which measures the levels of α-amylase activity in a flour sample. A high falling number value reflects a low level of amylase activity. The mean falling number value for sprouting (weathering) resistant white kernelled wheat exceeded that of sprouting susceptible red kernelled wheats in several years of studies. Hence, a range of genotypic expression of weathering resistance exists in both red and white wheat.

2.4 Environmental effects and testing methodologies

The manifestation of dormancy is a cultivar specific characteristic but is also highly influenced by the temperature and moisture conditions that occur during seed development and maturation as well as the post harvest temperatures. It is therefore important to distinguish the effects of temperature at harvest maturity and temperature

effects during grain development when looking at environmental effects on dormancy. Screening in plant breeding programs requires methods to determine the genetic contribution to dormancy.

Wilson (1928) grew three cultivars of wheat over five environments over two years. Germination tests on harvest ripe material were performed at 10, 15, 20, 25 and 30°C to evaluate dormancy. The percent germination decreased at temperatures above 15° with the lowest levels of germination obtained at 30°C. A temperature of 15° was found to be optimal for germinating the three varieties. In a study using mature grain of three wheat cultivars tested at several temperatures during grain filling, George (1967) found that the level of dormancy in the three cultivars was similar at 10°C while at 20°C the dormancy period varied from 20 to 60 days depending on the cultivar. At 30°C all cultivars had a deep persistent dormancy. Hagemann and Ciha (1987) also found that high temperatures were not conducive to sprouting in mature seeds. Rain accompanied by cool temperatures just before harvest has been shown to increase sprouting in wheat (Belderok 1968). The developing grain can be affected by temperature only during a specific critical period before harvest coinciding with the dough stage. Hot weather at this stage of development shortens the dormancy period while cool weather prolongs this period (Belderok 1968, Briggle 1980).

In a similar study Mares (1984) concluded that for PHS damage to occur in the more dormant lines, lower temperatures or longer periods of unfavourable weather would be required. The more dormant lines in the study (Kenya 321 and RL4137) required a temperature of ten degrees for 100% of the seeds to germinate, while the non-dormant cultivar (Timgalen) reached 100% germination at twenty degrees. Germination increased during post-harvest storage or after-ripening (AR). For after-ripened grain 30°C was the optimal temperature for complete germination. It was also shown that a cold (5° to 15°C) pre-treatment of imbibed seeds with a moisture content of less that 25% stimulated germination of grain.

Strand (1990) supported these findings in research on the effects of climactic factors on dormancy. Stronger seed dormancy was obtained at 20°C compared to 10°C,

for wheat harvested at two different dates. Temperature and global radiation were positively correlated to dormancy in wheat whereas rain fall and humidity were not. All these factors were however correlated to dormancy in oats and barley. It was suggested that seed germination at 20°C provided better differentiation between cultivars than 10°C germination temperatures. Mares (1993) failed to find temperature effects on dormancy in a similar study using Australian wheats but found a significant effect of rainfall in a short time period prior to harvest. To test for dormancy an index of sprouting tolerance in relation to total rainfall in the 20 days prior to harvest could be used to predict levels of PHS resistance in breeding programs (Mares 1993).

Nielson *et al.* (1984) undertook a series of experiments to determine differences in susceptibility among genotypes and to identify weather variables affecting PHS. Alpha-amylase enzyme activity was determined in grain from mature heads of wheat before and after simulated rain fall. They analyzed the effects of accumulated field temperatures, daily temperature fluctuations and accumulated precipitation on α -amylase. The daily temperature fluctuations before physiological maturity and precipitation after physiological maturity significantly affected α-amylase activity in grain not treated with rain. This confirmed prior studies results which stated that weather factors before as well as after physiological maturity had effects on PHS (Belderok 1968, LaCroix *et al.* 1976, Ollson and Mattson 1976).

Seed moisture content is also an important factor in the evaluation of seeds for dormancy. Premature seed desiccation and subsequent low temperatures increase the rate of germination (Wellington 1956). Water loss from the pericarp which precedes the loss of water from the whole seed, is sufficient to induce germination (Mitchell *et al.* 1980). Skinnes and Sorrells (1990) studied the effects of seed moisture content on dormancy using germination tests. High seed moisture content caused an increase in the level of seed dormancy in the red wheat varieties tested, whereas in the white variety tested it caused a reduction. High levels of moisture may make the seeds less permeable to oxygen by affecting the passage of oxygen through the pericarp, as was observed by Côme et al. (1984) in other species.

Environmental conditions during after-ripening (AR) can also influence the length of the dormancy period. Hagemann and Ciha (1987) studied the influence of field environmental conditions during grain filling on dormancy as well as the effects of various AR temperatures in winter wheats. The level of seed dormancy was evaluated by using a germination index and percent germination values. Seed dormancy at physiological maturity was a function of the genotype and the environment during grain maturation. The loss of seed dormancy was influenced by genotype, the environment during grain filling and the after-ripening temperature. High temperatures during the AR period accelerated the loss of dormancy. The length of the AR period is certainly of importance in the testing of plant material. If grain is stored too long before testing, dormancy may be lost and as such, inaccurately scored for these lines. A method of preserving dormancy from freshly harvested wheat grain developed by Noll and Czarnecki (1980) involved storing the grain at -15°C. This methods takes into consideration the effects of temperature during the AR period thereby extending the period of screening for several months. Mares (1983) confirmed these findings, however grain moisture content had to be less than 12%. Paterson *et al.* (1989) found that storage of grain at -20° for 3 months was effective in maintaining dormancy. Significant losses of dormancy were observed however, after 6 months of storage. These studies were all performed on plant material which had been grown under field conditions. Reddy *et al.* (1985) ripened five winter wheat cultivars at 15° and 26°C in growth cabinets. They used a weighted germination percentage which takes into account both the rate and number of sprouted seeds over time. They found that the greatest degree of dormancy was observed in seeds developed at 15°C. The maximum potential of a cultivar could be discerned by growing plants at 15° and performing gemination tests at three temperatures, 15°, 20° and 26°C. If a seed does not germinate at 15° the cultivar has high genetic potential for dormancy. If a seed germinates at 15° but fails to germinate at 20° it has intermediate dormancy potential, while if it germinates at 15° and 20° but not at 26°C it has very low genetic potential for dormancy. Plett and Larter (1986) conducted a similar experiment to provide information on the influence of the temperature (20°, 25°, 30°) during kernel

maturation on sprouting tolerance in both wheat and triticale. The results from this research indicate that a 25°C germination temperature ranked the four genotypes consistently in terms of sprouting tolerance over a range of maturation temperatures. A 17°C germination temperature ranked the cultivars differently depending upon the maturation temperature.

Seed dormancy is induced at low temperatures during seed development. Unfortunately the low temperature that favours the induction of dormancy also is effective at breaking dormancy during germination (Belderok 1968). Therefore, even if a cultivar does have the genetic capacity to develop dormancy in a cool growing season, it may not remain dormant in cool rainy weather at the end of the season. This perhaps exemplifies the complexity of dormancy as an agronomic trait. The complete nature of the interaction between genotype and environment is still poorly understood.

Various procedures have been developed for the assessment of PHS tolerance in either field grown or cabinet grown plant material. These tests can essentially be classified into the three following categories: Germination test on hand threshed seeds reported as percent germination or as a germination index over time, germination tests on intact spikes subjected to either high moisture or simulated rain, and α-amylase enzyme activity assays (Hageman and Ciha 1984, Paterson *et al.* 1989, Mares 1988).

Each of these types of tests have advantages and disadvantages pertaining to ease of application and reproducibility. Some of these tests were evaluated side-by-side by Hagemann and Ciha (1984) to determine their effectiveness in indicating susceptibility to sprouting. They also evaluated these tests for their use in breeding programs. Three winter wheat cultivars varying in sprouting susceptibility to PHS were field grown under 6 environments. Germination tests on threshed seeds were performed at 15°, 20°, 25°, and 30°C. PHS tolerance was assayed in intact spikes incubated in a humidity chamber (McMaster and Derera 1976), spikes buried in moist sand, and spikes rolled in moist paper towels. Alpha-amylase activity was assayed in flour from ungerminated and germinated seeds using the falling number test (FN), enzyme diffusion test, and a photometric method using dye releasing tablets and an α-amylase analyzer. The

germination tests using intact spikes rolled in paper towels and threshed seeds were the more accurate tests as these identified significant differences between the cultivars and environments. The use of intact spikes was advantageous because it allowed the measurement of sprouting under natural conditions where the spike characteristics could influence PHS response. The enzymatic tests measured the quality of the endosperm but not necessarily the sprouting potential of a cultivar. These would be more applicable to the measurement of actual sprout damage. Under the conditions used in this study, threshed seed germinated in petri dishes was the most satisfactory test because the temperature could be easily manipulated, it was less time consuming, and the coefficient of variation (CV) values were low enough to suggest that the seeds were all being exposed to the same microclimate during the test.

Plett and Larter (1986) found that with plants grown under controlled conditions, results of rain simulation on intact heads closely paralleled those from germination tests on threshed seeds. McCaig and DePauw (1992) compared artificial rain, germination tests and field weathering tests. Once again artificial rain and germination tests differentiated and ranked the cultivars according to PHS tolerance. Field weathering was found unsatisfactory, this may however, have been due to a lack of rainfall during the testing period. Paterson *et al.* (1989) conducted similar studies. For spike wetting tests, genotype was the largest source of variation in the analysis. Environment and interaction effects were generally smaller for spike wetting than for germination tests. Spike wetting more closely duplicates the field conditions under which PHS occurs. Germination tests are a more direct assessment of seed dormancy, however, this test is more sensitive to non-genetic factors (environmental interactions) and therefore may require testing of material grown over different environments.

2.5 Genetics and heritability of dormancy

Although the literature has provided evidence to suggest that there may be relatively tight linkage between kernel colour and dormancy, genetic variation exists within colour classes of spring and winter hexaploid wheat (Derera *et al.* 1977, DePauw

and McCaig 1983, Clarke *et al.* 1984, Mares 1987, McCaig and DePauw 1992 and McCrate *et al.* 1981). Breeding for resistance to sprouting in white wheats has been aided by the development and identification of several genetic sources of resistance such as that possessed by the cultivars Ford, Kenya 321 sib (Derera *et al.* 1977), Bihar 124 (Mares 1987) Clark's Cream and Brevor (McCrate *et al.* 1981). The inheritance of dormancy in these and other genetic sources remains relatively unknown.

The inheritance of dormancy was studied by Gfeller and Svejda (1960) in F_7 lines from the cross of a dormant red cultivar (Renown) and a white non-dormant cultivar (Cascade). They estimated heritability at 73% for seed dormancy. This dormancy was correlated with red seed coat colour. Since then various results have been reported as to the heritability of dormancy in wheats of different genetic backgrounds. Lines from crosses between RL4137 and two sprouting susceptible cultivars (white seeded Timgalen and red seeded Neepawa) were developed (Dyck *et al.* 1986) to determine the inheritance of dormancy as measured by falling number values. The heritability estimates for the falling number results were 74% and 78% for the respective crosses. Gordon (1983) estimated a heritability value of 44% for α-amylase activity in grains subjected to a wetting treatment. Soper *et al.* (1989) found heritability estimates for sprouting score and α-amylase activity in intact spikes of durum wheat subjected to wetting to be moderate to high. These heritability estimates remained relatively unchanged over two cycles of intermating, which suggests that selection for dormancy in a plant breeding program would be feasible. DePauw and McCaig (1991) used various dormancy assays to measure the heritability of dormancy in 26 hexaploid wheat cultivars. These cultivars were subjected to artificial wetting treatments. They found that the genotype effects accounted for 44 to 90% of the phenotypic variation observed. Heritability, was expressed on a genotype mean basis, was highly significant for all the assay methods used and ranged from 0.59 to 0.93.

Noll *et al.* (1982) investigated the inheritance of the RL4137 type of dormancy in reciprocal crosses between the dormant RL4137 and four less dormant cultivars. It had been reported previously that reciprocal differences occurred in certain crosses (Belderok

1963, Freed 1972) suggesting that dormancy is conditioned by the maternal parent and that the embryo did not play a role in dormancy. Noll *et al.* (1982) found that F_1 seeds having RL4137 as the maternal parent were more dormant than the reciprocals, supporting previous reports that the seed coat and/or endosperm have a significant influence on seed dormancy. The embryo component however was partially dominant since F_1 seeds with RL4137 as the pollen parent were more dormant than seeds of the less dormant parents. They suggest that the partial dominance of dormancy in F_1 seeds should facilitate breeding for dormancy. Selection in a backcross program such as the one used for the production of the cultivar Columbus (Noll and Czarnecki 1980), could be effectively performed on the F_1 seeds. Progeny testing could then be performed to confirm F_1 selections.

Bhatt *et al.* (1983) found that dormancy in Kenya 321, a white seeded dormant wheat, was controlled by two recessive genes in two crosses. No maternal effects were detected in this study. Mares and Ellison (1990) reported that dormancy in white seeded wheats was recessive and partially dominant in red seeded wheats, confirming the findings of Noll *et al.* (1982). In a recent study, Sharma *et al.* (1994) found that dormancy in F_1, F_2, and backcross lines from crosses between red seeded sprouting tolerant parents and a white seeded susceptible parent was controlled by one dominant gene.

Paterson and Sorrells (1990) found that dormancy from the white cultivar, Clark's Cream and the white dormant line, NY6438-18 was a dominant trait in crosses to two non dormant white wheats. They found dispersed frequency distributions which was taken to indicate that dormancy was expressed as a quantitative trait. This is in agreement with Upadhyay and Paulsen (1988) who also studied the inheritance of dormancy in Clark's Cream. They obtained high heritability estimates for falling number and α-amylase activity, with lower estimates for visual sprouting. In contrast Paterson and Sorrells (1990) found quite low heritability estimates for grain dormancy using parent-offspring regression. They also found that dormancy from the two sources was strongly influenced by the environment. Large variation in dormancy among F_5 single seed descent lines

derived from two white seeded dormant parents indicated that multiple dormancy mechanisms exist in the genetic pool of domestic white seeded wheats.

Hagemann and Ciha (1987) also reported that preharvest sprouting resistance was expressed as a quantitative trait and was affected by the environment and genotype x environment interaction. Allan (1992) found that high temperature dormancy was expressed as a quantitative trait since they found a normal distribution, transgressive segregants and low heritability in a cross between the two resistant lines, Brevor and Clark's Cream. Anderson *et al.* (1993) identified eight regions of the wheat genome which were significantly associated to dormancy using quantitative trait analysis (QTL) with molecular markers. Taken together, eight markers accounted for 44 and 51% of the genetic variance in the two populations studied. It was suggested that these markers would be useful in a breeding program for the selection of preharvest sprouting resistance.

Dormancy has therefore been reported to be recessive (Mares and Ellison 1990), partially dominant (Noll *et al.* 1982, Mares and Ellison 1990), monogenic dominant (Sharma *et al.* 1994), digenic recessive (Bhatt et al. 1983) and quantitative (Upadhyay and Paulsen 1988, Paterson and Sorrells 1990). These results and the others presented in the review clearly show that dormancy is a complex trait. Dormancy has been shown to be highly influenced by the environment during and after maturation which makes it a difficult trait to evaluate.

3. POLYPHENOL OXIDASE

3.1 Introduction

Polyphenol oxidase (PPO) (tyrosinase, E.C. 1.14.18.1) is widely distributed in the plant kingdom and despite intense study of the enzyme since its first description in 1895 (Mayer and Harel 1979), biochemical and physiological studies have not elucidated the function and expression of PPO in plants. A short summary of research on the proposed physiological functions of PPO in plants will be presented followed by a summary of research pertaining to PPO in wheat kernels including: effects on end-use quality, testing

methodologies, cereal variety identification, levels in germinating seeds and hormonal regulation, genetics and chromosome location. The proposed role of PPO in dormancy has been discussed elsewhere in this review.

3.2 Function of Polyphenol oxidase in plants

PPO is a copper containing enzyme which catalyses the hydroxylation of monophenols (such as tyrosine) to diphenols which are then oxidized to benzoquinones. Proteins also contain phenolic tyrosine groups which can serve as substrates for PPO in the absence of low molecular weight phenolic substrates (Matheis and Whitaker 1984). Quinones are very reductive and are converted to black melanin pigments or covalently crosslink proteins and free amino acids (Matheis and Whitaker 1984). The formation of black quinone products represents the principle detrimental effect of PPO in post harvest physiology and processing of crop plants and is the primary reason for the great interest in PPO in food and cereal technology today.

Although the physiological function of PPO is poorly understood, roles for PPO in disease resistance and insect resistance and oxygen metabolism have been proposed (Ludlum *et al.* 1991, Felton *et al.* 1989, Lax and Vaughn 1991, Mayer and Harel 1979, Mayer 1987, Vaughn and Duke 1984, Farkas and Kiraly 1965). In general these roles are based either on the oxygen reduction activity of PPO (Vaughn *et al.* 1988, Arora and Wagle 1985, Sempio *et al.* 1975) or on the ability of PPO-generated quinones to covalently modify plant proteins thereby decreasing the nutritive availability of amino acids to herbivores and pathogens (Felton *et al.* 1989, Mayer 1987, Ludlum *et al.* 1991).

Some reports indicate that PPO increases in activity following infection by virus, fungi or mechanical injury (Balasurbramani *et al.* 1971, Batra and Kuhn 1975, Mayer and Harel 1979) while in other studies the levels of activity was found to decrease (Brune and Van Lelyveld 1982). Mohan and Khanna (1988) found that isolines of wheat carrying leaf rust resistance genes had greater PPO activity as compared to the susceptible cultivars indicating they may have a role to play in disease resistance. Arora and Wagle (1985) suggest that the levels of preformed phenol and PPO in wheat seeds indicate that

the phenolic oxidation system maybe involved in the expression of resistance to loose smut. Tyagi and Khanna (1987) however did not find a significant pattern of variation for PPO activity between resistant and susceptible isolines in relation to powdery mildew in wheat. The levels of total phenols may provide an indication of their involvement in resistance through oxidation to quinones which are more toxic to microorganisms (LeTourneau et al. 1957, Vaughn and Duke 1984).

3.3 Polyphenol oxidase and wheat end-use products

The undesirable action of PPO in food is of major concern not only because of the unacceptable brown colour produced but also because of changes in taste and nutritional quality as a result of the reactions (Matheis and Whitaker, 1984). A number of end products of wheat are commonly prepared from flours milled to high extraction rates in which the presence of PPO may play a role in affecting product colour. In India, wheat is a major food source and is consumed primarily in the form of chapattis, an unleavened pan-baked bread. Dough prepared from Indian varieties produces the desired cream coloured chapattis, while dough prepared from high yielding dwarf Mexican varieties turns brown upon standing and subsequently produces undesirably brown chapattis (Singh and Sheoran 1972, Abrol and Uprety, 1970, Abrol *et al.* 1972) Higher levels of PPO in the high yielding dwarf varieties are considered to be responsible for the darkening of whole wheat dough and chapattis (Abrol and Uprety 1970, Abrol *et al.* 1972, Tikoo *et al.*1973, Singh and Singh 1974a). Faridi (1988) found similar results from research on Middle East flat breads.

The colour of pasta products made from semolina is considered to be an important indicator of quality. The amber-yellow colour of these products is more attractive to the consumer and is due to the presence of carotenoid pigments. Browning of pasta products is therefore an undesirable trait. High levels of PPO activity in semolina have been correlated to browning of pasta (Kobrehel *et al.* 1974, Laignelet *et al.* 1972) indicating an important role for PPO in the undesired browning reactions that can occur in pasta. Kobrehel *et al.* (1972) suggested that a breeding program aimed at the improvement of

durum wheat products should concentrate on the selection of varieties having low levels of PPO.

Colour is also an important criterion for the quality of oriental noodles (Kruger *et al.* 1992, Kruger *et al.*1994b, Baik *et al.*1994, Baik *et al.* 1985). Both the alkali conditions and the long storage times of the dough may cause browning of noodles (Miskelly 1984), however this browning was found to be aggravated by elevated levels of PPO (Kruger et al 1992). Kruger *et al.*(1994b) suggest that the rate of change in brightness as measured with a HunterLab colorimeter can be a measure of enzymatic darkening. They found that as the level of PPO activity increased the rate of change in both brightness and yellowness of noodles also increased (Kruger *et al.*1994b). Protein content also contributed to the quality of noodle colour, and was found to be inversely related to noodle brightness (Miskelly and Moss 1985). As protein content increases, the eating quality improves but the colour becomes undesirable. Baik *et al.* (1994) found however, that across cultivars that varied widely in protein content, discolouration was affected more by genotype governed PPO levels than by protein content. The protein content of a flour affected the water activity of the dough which in turn resulted in discolouration of noodle dough.

Many of these end-use products of wheat are made from flours milled to higher extraction rates. This is due to the fact that the enzyme is concentrated primarily in the bran of wheat kernels. Hatcher and Kruger (1993) measured levels of PPO in different wheat flour millstreams to ascertain the levels of the enzyme on increased flour extraction rates. Up to a cumulative flour yield of 70% at least 90% of the PPO is removed with the bran layer. Whether the remaining 10% can bring about undesired browning reactions will depend on the initial PPO activity of the cultivar, the substrate availability and the particular end-product being made. These results were confirmed by Baik *et al.* (1994) who found that the PPO activities of a flour increased as the flour extraction rate increased. Extraction rates between 76 and 82% incorporated more bran in the flour resulting in increased PPO activity of the flour.

3.4 Polyphenol oxidase assays and distribution of the enzyme

Many different assays for PPO in cereal kernels have been developed over the years. One of the first tests measured the development of colour by seeds over time in the presence of phenol (Honold and Stahmann 1968). The pericarp of seeds with high levels of PPO developed more colour than those with little activity. Honold and Stahmann (1968) also used a modified method of Dawson and Magee (1955) which measured enzyme activity based on oxygen consumption. These were quantitative assays for PPO. Honold and Stahmann (1968) also used a qualitative PPO assay based on polyacrylamide gel electrophoresis. Levels of PPO activity were visualized in gels of seed extracts. Honold and Stahman (1968) found that PPO activity was too low in the wheat extracts to be detected in gels stained in a pyrocathecol solution.

Kobrehel *et al.* (1972) utilised acrylamide gel electrophoresis to separate the PPO isozymes and densitometry to measure levels of activity. Gel electrophoresis using cathecol as a substrate proved very effective in the identification of multiple forms of the enzyme in wheat (Taneja and Sachar 1974 a, b,c, Kruger 1976, Berry and Sachar 1982, Kobrehel *et al.* 1972).

The phenol colour development test is a simple qualitative test in which wheat kernels are placed on phenol saturated paper and the resulting colour development of the wheat kernels is scored on a scale of 0 (no change in colour) to 3 (dark brown) (Wrigley 1976, Wrigley and Sheperd 1974, Wrigley and Baxter 1974). The need to identify or verify the identity of grain samples with respect to cultivar arises at various stages of grain production from seed certification to milling. The phenol reaction has long been established as a cultivar identification test. The uniformity of the colour produced by the grains upon exposure to phenol provides an indication of cultivar purity. The intensity of the reaction provides a subjective assignment to one of four colour categories (scale 0 to 3) which is used in cultivar identification or verification. Wrigley and Baxter (1974) found that the phenol test was useful in identifying grain mixtures and was particularly effective when followed by electrophoresis of gliadins. In screening pure samples of grain, Wrigley and Sheperd (1974) found that the phenol test alone could not distinguish

between certain wheat varieties. The application of electrophoresis and the phenol test in combination was more useful in discerning varieties, however closely related varieties could still not be differentiated. Tao *et al.* (1992) used a modified phenol test which proved effective in the detection of changes in the composition of heterogeneous germplasm accessions after regeneration and storage in a germ bank. This test is non-destructive and easily applied to the screening of germ banks.

Crisp and Wrigley (1973) developed a quantitative phenol test where the seeds were left to react with the phenol and subsequently boiled. The absorbance of the resulting clear yellow solution was measured using a blue filter. The phenol ratio is the mean absorbance of the test solution divided by that of the distilled water blank. Phenol ratios for samples of the same cultivar ranged about 5% on either side of the mean. Thus a difference in phenol ratio of 10 to 15% was considered significant in distinguishing wheat samples. The authors found that several of the durum wheat varieties tested had phenol ratios of 1.0, indicating no colour reaction to phenol. Some degree of phenol colour reaction was obtained for all the common wheat varieties tested.

Mahoney and Ramsay (1992) developed a modified version of the simple phenol test using tyrosine as the substrate. Tyrosine is less toxic than phenol and is a safe alternative in the testing of PPO. The authors suggested that this test could be used to differentiate both hard and soft common wheat cultivars from durum wheat and would be useful as a test for the identification of common wheat contamination in durum wheat seed lots.

More recent PPO assays were also devised to be quantitative, allowing a more precise characterization than is possible using the simple phenol or tyrosine colour test. PPO activity can be measured by determining the rate of oxygen consumption by a ground wheat sample with an oxygen electrode (Lamkin *et al.* 1981, Marsh and Galliard 1986). The measurements are presented as nmol O_2 consumed/minute/gram of material. This assay was used to determine that cultivar differences in PPO activity could in many cases be used to distinguish wheat cultivars or wheat classes. Lamkin *et al.* (1981) found that durum wheats had quite low levels of PPO and could be distinguished from other

classes of wheat using the oxygen consumption assay. The soft white and club wheats were also on the average lower in PPO activity than the hard red winter, soft red winter and hard red spring wheats. Less variability was observed between the hard red winter and soft red winter wheats and between the white and club wheats making the PPO assay ineffective in distinguishing these classes of wheat. Although this is a quantitative test, it would by difficult to apply in a plant breeding program where high throughput is required.

A quantitative PPO assay was developed by Kruger *et al.* (1994a) which allows the testing of whole grain. The grains are steeped overnight then incubated in a cathecol solution for 30 minutes at 37 degrees. An aliquot of this mixture is then placed in a microtiter well and colour development is measured with a kinetic microplate reader. This whole grain assay was proposed for use in plant breeding programs aimed at reducing PPO levels because it allows rapid quantitative screening of whole grain.

3.5 Growth regulator effects on polyphenol oxidase during germination

Kruger (1976) studied PPO activities at various stages of growth and maturation in wheat. The enzyme was active during early kernel growth, remained active throughout development, and then activity decreased to a low level as the kernel matured. Up to 12 isozymes were identified in the durum wheat cultivar Hercules. A large part of the PPO activity was found in the endosperm in the early stages of development . This level dropped as maturity advanced while it increased in the germ and scutellum. Upon germination PPO activity increased 33 fold and a major isozyme which was present throughout development increased in intensity.

Tikoo *et al.*(1973) identified 3 to 5 isozymes in mature seeds of dwarf wheat varieties while Taneja *et al.*(1974) separated 10 isozymes in a dwarf wheat at 36 days post anthesis. Singh and Singh (1974b) showed that there was a change in isoenzymic components of PPO during germination and the levels of activity increased during germination. When using tyrosine as the substrate for PPO, Taneja and Sachar (1974a) found a 5 to 7-fold increase in enzyme activity in the kernel during germination. The

enzyme was confined to the endosperm. When using cathecol as a substrate for the enzyme they detected a 3-fold increase in activity in the kernel dissected from the seedling. A 50 to 100-fold increase in activity was observed in the excised coleoptiles and roots of the seedlings.

PPO was stimulated by the application of GA_3 to embryoless half seeds of wheat (Taneja and Sachar 1974b, Berry and Sachar 1982, Saluja and Sachar 1982, Jennings and Duffus 1977). The stimulated activity was found to be brought about by the activation of preformed enzyme molecules *in vivo*. Treatment of half-seeds with GA_3 modified the polyphenol oxidase and altered the electrophoretic pattern. Partially purified PPO showed enhancement of enzyme activity at pH 9.0 in GA_3 treated half seeds while in controls, negligible activity was detected at this pH (Berry and Sachar 1982, Saluja and Sachar 1982). These alterations in GA_3-stimulated PPO suggest that there is an activation of preformed enzyme molecules. The addition of inhibitors did not stop GA_3-stimulation of enzyme activity, therefore de novo synthesis of the enzyme was not required for the growth regulator effect on PPO activity (Taneja and Sachar 1974b).

3.6 Genetics of polyphenol oxidase and chromosome location of genes

A number of studies have reported on the genetics and chromosome location of genes encoding PPO. Initial studies by Gfeller and Svejda (1960) showed that the trait was monogenic. They crossed a plant with the black reaction to one with a light brown reaction. A one gene segregation ratio was obtained in the segregating progeny. Further genetic studies of PPO also showed monogenic control, but demonstrated the presence of a multiallelic series (Joshi and Banerjee, 1969) in emmer wheats. They found that T*riticum durum* lines and *Triticum carthlicum* lines had the black reaction, whereas the *Triticum dicoccum* lines had the brown reaction. The black reaction is dominant over either the brown or white reaction and brown is dominant over white. The reaction in tetraploid wheats was controlled by 2 dominant alleles at the same locus (Joshi and Banerjee 1969). These alleles differ in the speed at which the seeds turn black.

Bhowal *et al.* (1969) suggested that the A genome is the only source of PPO in emmer and bread wheats. Different diploid accession lines of the presumed progenitors of hexaploid wheat (*T. monococcum* AA, *Aegilops speltoides* BB, *T. tauschii* DD) and several tetraploid and hexaploid wheat cultivars were screened. Only the *Triticum monococcum* and *Triticum boeticum*, both AA genomes, had high PPO activity. The B and D genomes assayed had no PPO activity.

In a later study on the genetics of PPO using Chinese Spring (Hope) and Chinese Spring (Timstein) substitution lines (Zeven, 1972) the presence of loci for genes conditioning PPO in the caryopsis were identified on chromosome 2A of cv. Hope and 2A and 2D of cv. Timstein. This was the first report on the chromosome location of the PPO genes. No dosage affect was observed, since cultivar Timstein which carries two strong alleles (2A and 2D) was the same colour as CS(2A Timstein) and CS(2D Timstein). The author suggested that an allele is probably also present on chromosome 2B, although it was not identified.

Later work on the chromosome location of the PPO genes used monosomic analysis. Sadananda *et al.* (1977) evaluated F2 populations obtained from a cross between Red Bobs, its monosomic lines and Sharbati Sonora for PPO. In the segregation analysis, all the F2 populations segregated as 3 black : 1 white except the population with the 7B monosomic. This indicated the presence of a dominant gene encoding PPO on chromosome 7B. Also using monosomic segregation analysis, Bhat and Goud (1978) identified a major PPO gene on chromosome 6B with a modifier on 5B. They concluded that the production of PPO enzyme was conditioned by 2 pairs of independent factors, one dominant and the other recessive with each of them being capable of coding for PPO.

4. MOLECULAR MARKERS, LINKAGE ANALYSIS AND GENOME MAPPING

4.1 Introduction

Plant breeders have traditionally used selection based on phenotype for the improvement of plant varieties. With the development of genetic linkage maps for many

crop species a new method of selecting desirable genes has been established. The basic principle involves the selection for the character with easily detectable phenotypes, such as markers, to simplify the recovery of linked genes of interest. There are many reasons why marker based selection may be more effective or more desirable than selection based on the trait itself. Undesired individuals can be identified in the early stages of growth in order to conserve resources or to identify desired individuals for crossing before flowering such as in a backcross program. Some traits are difficult to evaluate due to the influence of the environment, presence of many genes (quantitative trait), or because of uneven inoculations or infections. It is also sometimes difficult to select for many traits at once. Markers can also be used to establish the unique genetic identity of individuals (genetic fingerprinting), to evaluate genetic relationships among individuals, and study genetic variation within and among populations (Dudley *et al.* 1991). The focus of this review will be on random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphic (RFLP) DNA markers in terms of their role as chromosome markers in cytogenetic analysis and their potential role as markers for specific genes.

4.2 Wheat genetics

Among plant species, bread wheat (*Triticum aestivum*) is one of the most important food crops in the world. Genetic analysis of wheat has been complicated by its polyploid inheritance. It is an allohexaploid ($2n = 6x = 42$) consisting of three genomes, A, B, and D combined by ancestral hybridization events involving three different but related diploid progenitors. The genome of wheat has been estimated at 17.3 picograms (pg) of DNA per haploid nucleus (Gale *et al.* 1989). This is equivalent to approximately 16×10^6 kilobase pairs. More than 75% of the genome consists of repeated DNA sequences and about 20% represents low-copy-number or unique sequences (May and Appels, 1987). An even smaller portion of the genome, probably no more than 1%, represents actual unique coding regions. Although polyploidy has slowed progress towards mapping of wheat, polyploidy does allow toleration of aneuploidy. The work of many cytogeneticists has resulted in the assembly of complete sets of nullisomic,

monosomic, tetrasomic and telosomic stocks. These cytogenetic stocks allow the mapping of traits and molecular markers to specific chromosomes. In nullisomic analysis, observations are made for the trait of interest in nullisomics for each chromosome and the normal euploid. The missing trait for a specific nullisomic line identifies the chromosome location. Alien substitution lines and alien addition lines are commonly used in the identification of chromosome location for molecular markers such as RFLPs (Chao *et al.* 1989, Devos *et al.* 1992, Devos *et al.* 1993, Sharp *et al.* 1989) and RAPDs (Wang *et al.* 1995). Substitution lines are lines in which specific chromosomes of a cultivar are replaced by those of alien species or of a different cultivar. These are produced by backcrossing to a series of monosomics so that each chromosome of a donor wheat is introduced separately into a recipient cultivar. Chromosome addition lines are lines in which specific chromosomes from an alien species or from a different cultivar are added separately. A list of alien substitution lines and addition lines available in wheat is presented by Shepherd and Islam (1988).

4.3 Molecular markers

The first marker loci available were those with an obvious impact on the morphology of plants such as genes affecting form, colouration, male sterility or disease resistance. Many of these morphological markers have been assigned to different chromosomes in different crops (MacIntosh 1988, O'Brien 1993). Morphological markers however can have large effects on phenotype and can mask the effects of linked minor genes making it difficult to identify desirable linkages for selection (Tanksley *et al.* 1989). They normally have alleles that interact in a dominant-recessive manner, making the identification of heterozygotes impossible and the number of morphological markers available in a population is also limited. The use of morphological markers can be made difficult by strong interactions with the environment and the genetic complexity of the trait. Also selection must often be delayed until the expression of the trait or postponed to the next generation for recessive traits.

The use of morphological markers to evaluate populations can be supplemented or even surpassed by a more direct study of the genome by means of biochemical or molecular markers. Two types of molecular markers have been developed and have been useful in improving our knowledge of the genetic variation of populations. These are protein markers and DNA markers.

Protein markers are generally available in the form of isozymes and storage proteins. These are expressed in a co-dominant manner, allow a complete description of the genotype, are free of epistatic interactions and are unchanged by the environment. Many isozyme markers have been mapped to specific loci in different crop species (Hart *et al.* 1980, McIntosh 1988, Landry *et al.* 1987, Sharp *et al.* 1988, O'Brien, 1993). So far, approximately 115 isozyme and storage protein loci have been located to wheat chromosomes (Hart *et al.* 1993).

A number of isoenzyme genes have been linked to major genes of interest. For example, esterase-2, acid phosphatase-3 and dipeptidase-2 have been shown to be linked to rust resistance genes in barley (Feuerstein *et al.* 1990). A more complete list of isozymes linked to important agronomic traits in several crop species is presented by Arus and Moreno-Gonzalez (1993).

It was suggested in 1980 (Botstein *et al.* 1980) that a large number of genetic markers might be found by studying differences in the DNA molecule itself rather than the expression of genes. The first DNA markers developed were restriction fragment length polymorphisms (RFLPs) and were first used in research on human diseases. Restriction enzymes are highly specific DNA cutters which cleave DNA at specific sequences called restriction sites. If two individuals differ by as little as one nucleotide in this site, the enzyme will cut the DNA of one but not of the other. Alterations which can occur in the recognition sites include point mutations, deletions, insertions or transpositions. Any such alteration will change the restriction pattern of the DNA, thereby generating restriction fragments of different lengths between the two individuals. These RFLPs are separated by electrophoresis and visualised by the specific binding of a radioactive probe. RFLPs are inherited in a co-dominant manner allowing the

identification of heterozygous individuals. They generally cover low-copy coding regions of the genome and are technically difficult to perform. Radioisotopes are usually required, however the development of non-radioactive detection methods (Ragot and Hoisington 1993) may eliminate the need for these. Large quantities of relatively pure DNA preparations are required for RFLP analysis and species-specific low-copy genomic DNA or cDNA clones are required for the visualisation (Waugh and Powell 1992).

Beckmann and Soller (1983) suggested that RFLPs could be applied to the genetic improvement of crop species. RFLPs can be used to establish linkage maps, to identify cultivars and to study genes controlling quantitative traits. Their potential uses in crop improvement as aids to plant breeders have been reviewed (Helentjaris *et al.* 1985, Tanksley *et al.* 1989, Paterson *et al.* 1991).

RFLPs have been used extensively for the creation of linkage maps which have enabled the development of indirect selection strategies for crop-improvement programs. Whole genome RFLP maps are now available for many crop species such as rice (Causse *et al.* 1994), tomato (Bernatzky and Tanksley 1986, Tanksley *et al.* 1992), potato (Bonierbale *et al.* 1988), maize (Helentjaris 1987, Burr *et al.* 1988), barley (Heun *et al.*1991, Graner *et al.* 1991, Kleinhoffs *et al.* 1993), lettuce (Landry *et al.* 1987) and oats (O'Donoughue *et al.* 1992). The physical size and the low level of polymorphism has generally held back the production of whole genome maps in wheat, however partial maps of wheat have been reported (Chao *et al.* 1989, Sharp *et al.* 1989, Liu and Tsunewaki 1991, Devos *et al.* 1992, Devos *et al.* 1993, Anderson *et al.* 1992, Nelson *et al.* 1995a, Nelson *et al.* 1995b, Nelson *et al.* 1995c, Van Deynze *et al.* 1995, Xie *et al.* 1993).

The analysis of nucleotide sequence variability has been revolutionized by the development of the polymerase chain reaction (PCR) (Saiki *et al.* 1988). A new class of molecular markers termed random amplified polymorphic DNAs (RAPDs) have been generated based on a modification of the polymerase chain reaction (PCR) (Williams *et al.* 1991). RAPDs are polymorphic sequences of DNA separated by gel electrophoresis after the polymerase chain reaction amplification using short random oligonucleotide

primers. The number of amplified DNA fragments depends on the length of the primer, the size of the genome and the fact that the complementary sequence of the primer must occur in the genome, on opposite strands and in opposite orientation within a distance that is amplifiable by PCR (Waugh and Powell 1992). Polymorphisms result from changes in either the sequence of the primer binding site which prevents the association with the primer, or from changes such as insertions, deletions or inversions, which alter the size or prevent the successful amplification of the target DNA. Amplification products are generally inherited as dominant markers, and as two alleles, either the absence or the presence of the band. The whole genome is surveyed using RAPD analysis compared to only low-copy coding regions surveyed using RFLP analysis. Genetic analysis with RAPD markers is rapid, involves no radioactivity or hybridization and requires only small quantities of crude DNA. RAPDs are well suited to the screening of large population such as those produced in plant breeding programs.

The RAPD technique has been applied to the construction of linkage maps (Williams *et al.* 1990), to identify varieties (He *et al.* 1992), to assess genetic diversity (Dweikat *et al.* 1993) to study taxonomic relationships (Tinker *et al.* 1993, Thorman *et al.* 1994) and to tag disease resistance genes (Paran and Michelmore 1993, Paran *et al.* 1991, Penner *et al.* 1993, Schachermayer *et al.* 1994).

Despite the speed and general usefulness of RAPD markers several shortcomings have been discussed such as reliability, dominant inheritance, low allele number (presence/absence of band), lack of homology between related taxa, and lack of specificity for unique regions of the genome (Paran and Michelmore 1993). A new type of marker has been developed which overcomes these disadvantages of RAPD markers. These are sequence characterized amplified region (SCAR) markers. A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primers (Paran and Michelmore 1993). SCARs are derived by cloning and sequencing the two ends of the amplified products of a RAPD marker. SCARs are advantageous over RAPD markers because they detect only one locus, their amplification is less sensitive to reaction conditions and they

can potentially be converted to co-dominant markers. SCARs are defined genetically and therefore can be used not only as physical landmarks in the genome but also as genetic markers. SCAR markers linked to downy mildew resistance in lettuce were the first developed (Paran and Michelmore 1993).

Various molecular separation techniques can be used to maximize the number of polymorphisms detected between genomes. These include denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* 1987, Reidel *et al.* 1990, He *et al.* 1992, Dweikat *et al.* 1993, Procunier *et al.* 1994) and temperature sweep electrophoresis (Penner and Betze 1994). A DGGE system which has recently been optimized (Procunier *et al.* 1994) can, when used in combination with RAPD analysis, greatly facilitate and enhance the detection of reproducible DNA polymorphisms among closely related plant lines. In comparing agarose, polyacrylamide and DGGE of identical RAPD products, little or no polymorphisms were detected on agarose gels. Polyacrylamide gel electrophoresis did improve the resolution of polymorphisms but the DGGE system was much superior for the enhanced and reproducible detection of DNA polymorphisms (Dweikat *et al.* 1993). Procunier *et al.* (1994) observed a four-fold increase in inheritable polymorphisms when RAPD products were separated on DGGE compared to agarose.

Genetic maps are valuable tools in crop breeding and genetic studies. The great strength of RFLP maps is that an almost unlimited number of markers can be mapped over the entire genome. The methods involved in the detection of RFLPs are however technically difficult. New techniques have been developed to convert RFLP clones into PCR based markers. These are sequence tagged sites, or STS. An STS is a short, unique sequence, amplified by PCR, which identifies a known location on a chromosome. Longer specific primers are made based on the sequence of a specific gene or anonymous RFLP probe. These are then used in the PCR reaction to amplify that region of the genome. D'Ovidio *et al.* (1990) and Weining and Langridge (1991) showed that PCR could be used to detect genetic polymorphisms in cereals with primer sequences derived from the sequence of a γ-gliadin and α-amylase gene respectively. Williams *et al.* (1991) designed six STS primer sets from RFLP probe sequences. Tragoonrung *et al.* (1992)

observed that 7 of the 8 RFLP markers converted into STS could be used to evaluate segregating populations. Inoue *et al.* (1994) converted 63 RFLP landmarks from the rice genome map into STS landmarks. Talbert *et al.* (1994) designed primer sets based on the sequence of 37 wheat RFLP clones. Of these 29 directed successful amplification of the wheat genome and 23 primer sets amplified products that mapped to the expected chromosome group. They concluded that in general, primer sets designed from RFLP clones will result in effective amplification in wheat. For the applied use of STS primers polymorphisms must be detected between wheat cultivars or lines.

CHAPTER 3

Inheritance of Seed Dormancy in Wheat Grown Under Controlled Environmental Conditions.

ABSTRACT: Mature wheat (*Triticum aestivum* L.) kernels exposed to high levels of moisture often suffer from pre-harvest sprouting. The inheritance of dormancy was characterized in the white seeded sprouting resistant genotypes RL4555 and Bihar 124. Plants were grown under controlled environmental conditions (16°C) and dormancy was assessed in germination tests performed at two temperatures (15°C and 20°C). One gene for dormancy was detected at 15°C and two genes were detected at 20°C in all crosses. Lines expressing dormancy at 15°C also expressed dormancy at 20°C, however a second gene was detected at this temperature. The use of the two germination temperatures allowed the identification of lines carrying either or both dormancy genes, thereby providing a well characterized population for subsequent molecular marker analysis. Dormancy was not associated with seed coat colour in white dormant x red non-dormant crosses. A RAPD marker was found to be linked in repulsion to the 15°C dormancy gene at a distance of 25.7 cM. The chromosome location of the marker could not be determined using cytogenetic lines as it consisted of repetitive DNA sequences.

INTRODUCTION

Germination of seeds in the heads before harvest is called preharvest sprouting and is primarily due to a reduced period of dormancy in the grain. Severe preharvest sprouting can reduce yields and seed viability and have detrimental effects on the quality of the grain due to the presence of germination specific enzymes such as α-amylase. Flour and meal from sprouted wheat contain high levels of α-amylase which adversely affect baking quality, resulting in a sticky crumb texture and an undesirable crust colour. Preharvest sprouting of wheat remains a problem in many regions of production. Although the physiological mechanisms and mode of inheritance of resistance of pre-harvest sprouting remain unclear, advancements in the introduction of resistance into new cultivars have been possible due to the identification of sources of genetic resistance in both red and white seeded wheat.

While many testing methods have been proposed and used in the past to evaluate levels of dormancy, no standard methods have been routinely accepted. Methodologies range from α-amylase testing on intact and on sprouted kernels (falling number and enzymatic assays), germination tests, sprouting indices, artificial rain testing, and field weathering. The use of an appropriate selection criterion for preharvest sprouting resistance is critical. Methods of evaluating sprouting resistance have been compared in a number of studies (Hagemann and Ciha, 1984, Paterson *et al.* 1989, McCaig and DePauw 1993, Scott *et al.* 1984). The testing of intact spikes measures sprouting susceptibility under natural conditions where head type and chaff influence the response. The use of threshed seeds may be advantageous, however, because the temperature and moisture conditions can be better controlled (Hageman and Ciha 1984). Germination tests on threshed seed are insensitive to physical traits such as the presence of awns, spike shape, and glume tenacity and are therefore a more direct assay of seed dormancy (Paterson *et al.* 1989). McCaig and Depauw (1993) found that both rain simulation on intact heads and germination tests on threshed seeds differentiated and ranked genotypes according to their rankings based on previous studies. Enzymatic tests on sprouted kernels do not necessarily measure the abililty of the seed to resist sprouting, however, they do provide an indication of the level of germination that has already taken place (Hagemann and Ciha 1984).

A white seed coat colour is a desirable quality characteristic for many uses of wheat. White wheat is preferred by the milling industry because higher flour extraction rates can be obtained compared to red wheat. White wheat is also preferred for the wheat based foods by people in India, Pakistan, and other Asian and African countries. Unfortunately white seed coat colour has traditionally been associated with low levels of tolerance to preharvest sprouting. There has been some evidence to show that there is relatively tight linkage or pleiotropic gene action between red kernel colour and some aspects of preharvest sprouting tolerance. Initial attempts at the incorporation of dormancy from red seeded germplasm into white seeded genetic backgrounds in

hexaploid wheat were unsuccessful (Gfeller and Svejda, 1960, Freed *et al.* 1976). In durum wheat, the relationship between red kernel colour and strong preharvest dormancy is either due to pleiotropy or extremely tight linkage (Soper *et al.* 1989). DePauw and McCaig (1983) identified recombinants possessing white kernel colour and seed dormancy in a cross between the dormant red seeded line RL4137 and a white nondormant line. As a results of these observations, they concluded that two mechanisms for sprouting resistance may exist in this germplasm, one associated with seed colour, the other independent of seed colour.

Although there is evidence that suggests there is relatively tight linkage or pleiotropic gene action between kernel colour and some aspects of preharvest sprouting tolerance, genetic variation for the trait does exist within colour classes and within spring and winter hexaploid wheats. Breeding for resistance to sprouting in white wheats has been aided by the development and identification of several genetic sources of resistance such as Ford, Kenya 321 (Derera *et al.* 1977, Bhatt and Derera 1980), Bihar 124 (Mares 1987) Clark's Cream and Brevor (McCrate *et al.* 1981). Recently the sprouting tolerant germplasms SC8019R1 and SC8021V2 were registered (Depauw *et al.* 1992). These lines both have Kenya 321 as one of the sources of sprouting tolerance. The mode of inheritance of dormancy in these and other genetic sources remains relatively unclear.

Inheritance of dormancy in wheats of various genetic backgrounds has previously been reported to be recessive (Mares and Ellison, 1990), partially dominant (Noll *et al.* 1982), digenic (Lawson *et al.* 1996), monogenic dominant (Sharma *et al.* 1994), digenic recessive (Bhatt *et al.* 1983, Mares 1996), digenic dominant (Gale, 1976) and quantitative (Upadhyay and Paulsen 1988, Paterson and Sorrells 1990, Anderson *et al.* 1993). The expression of dormancy within a genotype is conditioned by many factors but environmental conditions during embryogenesis and seed ripening appear to have a large impact on the development of dormancy (Georges 1967, Belderok 1968, Strand 1980). Since dormancy is highly influenced by the environment it is a difficult trait to evaluate. Expression of dormancy is greatest at physiological maturity and decreases during storage. In general, low temperatures (10°C) during seed development induce deep and

prolonged dormancy, while low temperatures during germination break dormancy of freshly harvested seeds (Gfeller and Svejda 1967). Reddy *et al.* (1985) proposed that if seeds of a given cultivar fail to germinate at 15°C, the cultivar has high genetic potential for producing dormant seeds.

Attempts to genetically characterize seed dormancy in field grown cereals have produced inconsistent results. Evaluation of dormancy in seed from plants grown under stable controlled environmental conditions could contribute some valuable information as to the number of major genes expressed under those conditions. This study was undertaken primarily to characterize the inheritance of grain dormancy from the white kernelled spring annual genotypes RL4555 and Bihar 124. The BiggarBSR/RL4555 population was used in a study on the identification of a RAPD marker associated with dormancy.

MATERIALS AND METHODS

Plant material:

The main population involved in these studies consisted of 115 F_6 recombinant inbred lines produced by single seed descent from the F_2 of a cross between a white dormant genotype (RL4555) and a red non-dormant genotype (cv. BiggarBSR). The pedigree of RL4555 is as follows: Kenya Farmer*2/Kenya 321 sib. Two doubled haploid populations were also evaluated, the first consisting of 27 lines from a cross between BiggarBSR a red, non-dormant cultivar and Bihar, a white dormant cultivar. The pedigree of the dormant parent, Bihar 124 is unknown. The second doubled haploid population was quite small and consisted of 13 lines from a cross between Timgalen, a white non-dormant cultivar, and RL4555, a white dormant line.

An additional part of this study involved an F_5 recombinant inbred population produced by single seed descent from the F_2 of a cross between two white lines, RL4555 (dormant) and cultivar Fielder (nondormant).

The following cytogenetic stocks were used in the RAPD, SCAR and RFLP analysis sections of this study: Barley chromosome additions into *Triticum aestivum* cv.

Chinese Spring (Islam et al. 1981), *Triticum aestivum* cv. Chinese Spring nullitetrasomic lines (Sears, 1966), *Aegilops umbellulata* chromosome substitutions into Chinese Spring (Miller 1988), *Triticum turgidum* var. *dicoccoides* (2n = 28, genomes A and B) chromosome substitutions into *T.t.* var. durum cv. Langdon (Joppa 1993), and *Triticum tauschii* additions to *Triticum aestivum* cv 'Thatcher' (Dr. Eric Kerber, unpublished results).

Cabinet testing

Plants were grown in replicate under controlled environmental conditions of 20°/18° day/night temperatures. At anthesis the temperature was reduced to 16°C day/night temperature. Individual heads were harvested at physiological maturity which was identified by the complete loss of chlorophyll from the glumes (Hanft and Wych 1982). This loss of green colour precedes maximum kernel dry weight by about 1 day. Heads were after-ripened at 30°C for 3 days then stored at -20°C, a temperature that has previously been shown to preserve dormancy (Noll and Czarnecki, 1980, Mares 1983, Paterson *et al.* 1989).

The heads were hand threshed (5 heads/line) and dormancy was evaluated by germination tests. Seeds from each line (25) were placed crease side down on filter paper (3MM) in glass Petri plates with 6 mL of water, in duplicate. The plates were incubated at 15°C and seeds with visible radicles were counted and removed every day for 8 days. At the end of the 8 day period seeds that had not germinated were treated with 1 ml of 10^{-3} M GA_3 (per plate) to ensure seed viability. Seeds that failed to germinate after GA_3 treatment were discarded from the test. Seeds with fungal growth were also discarded. The parental lines (RL4555 and BiggarBSR) as well as resistant (Bihar124) and susceptible (Roblin, Fielder) checks were also included in the germination tests. A subsample of 81 lines were germinated at 20°C in duplicate. All germination tests were duplicated for each replicate and the data is presented as the mean percent germination of two duplicates per replicate at day 8 of testing. Values for germination percentage after 4

days are also presented. A subsample of 81 lines from the population was also tested at a germination temperature of 20°C.

Data analysis utilized software of the Statistical Analysis System Institute Inc (1992) on a personal computer (IBM). The data was transformed using the arcsine of the square root transformation (Scott *et al.* 1984, Paterson and Sorells 1990) in order to control the variance of the data and to improve the normality of the data. Analysis of variance was performed using the General Linear Model Procedure and, given significant F values, the Dunnett's test was subsequently performed on the means of the 2 replicates using the SAS protocol (SAS Institute, 1992). While statistical analysis was performed on the transformed data the graphs of the germination frequencies represent non-transformed data for ease of visual interpretation.

Field Testing

The RL4555/Fielder population was planted on May 20, 1992 in replicate rows and May 14, 1993 at the Agriculture Canada research station at Glenlea. The population was seeded in 1m rows with the parental genotypes and other selected genotypes (RL4555, BiggarBSR, Fielder, Bihar, Timgalen and Roblin) as checks in the dormancy tests. Twenty heads from each line were harvested at physiological maturity as identified by the loss of chlorophyll from the glumes (Hanft and Wych 1982). The heads were stored at room temperature for 5 days then stored at -20°C. Replicate testing was done on bundles of ten intact heads per line in a rain simulator chamber. During this rain simulation treatment the samples received approximately 60 mm of moisture within 2.5 h, then were maintained at a constant temperature of 15°C and high humidity throughout the testing period. The number of sprouted kernels per head was counted after 7 days of treatment (Derera *et al.* 1977). The specific length of the rain simulation treatment was determined by the differential response of the check samples in terms of their degree of germination. The average number of sprouts/head was transformed using the log (x + 1) transformation (SAS Institute, 1992) to control the variance and improve the normality of

the data. The transformed data were subjected to the Dunnett's test to determine which lines were significantly different from the dormant parent, RL4555.

A subsample of 40 lines consisting of 20 very dormant and 20 very non-dormant lines, as determined after the first year of testing was selected for further evaluation. These together with the same checks were planted in the field in replicate rows on May 14 and May 28, 1993 and harvested and tested as described previously. The subsample was also grown under controlled environmental conditions

The lines grown in 1993 were tested for dormancy in germination tests done in Petri plates because in trial runs of sprouting in the rain simulator all heads developed severe mold. Dormancy could therefore not be reliably analyzed as the glumes and seed were severely infected with fungal diseases (black point and *Fusarium*) which may have interfered with the sprouting tests done on intact heads. . In using threshed seed, only visually sound seeds were selected for the germination tests. Although some mold did develop in the Petri plate test, these seeds were removed and were not included in the final germination analysis. The germination temperature was raised to 23°C for tests on the 1993 material since dormancy was not expressed at either 15°C or 20°C.

RFLP analysis

Major grain colour genes have been identified on all three group 3 chromosomes by aneuploid analysis (Metzger and Silbaugh 1970). The cDNA clone ABC174, provided by the North American Barley Genome Mapping Project (Kleinhofs *et al.* 1993), has been located on chromosome 3 of the barley genome and homoeoloci on the long arms of wheat chromosomes 3D and 3B were both shown to be highly significantly associated with grain colour (Nelson *et al.* 1995b). The RILs from the cross BiggarBSR/RL4555 were analyzed for RFLP linkage to dormancy using the clone ABC174.

DNA extractions for RFLP analysis were performed as described by Kleinhofs *et al.* (1993). The purified DNA (15 ug) was cut with 30 units of restriction enzyme overnight (HindIII, EcoRV, DraI, XbaI and BstEII, from Gibco BRL) as per the manufacturer's instructions. Electrophoresis was performed in a 0.9% agarose gel in TBE

buffer (54mM Tris, 54 mM Boric acid, 10 mM EDTA ,pH8.0) at 37 volts for 18-20 hours. Gel blotting and hybridization conditions were as described by Sharp *et al.* (1988). The DNA probe was labelled by the random hexamer method with [^{32}P] dATP according to the manufacturers instructions (BRL Random Primers DNA Labelling System, Gibco BRL).

RAPD analysis:

The 76 primers used for the initial screening were from the 300 and 700 series from the Biotechnology Laboratory, The University of British Colombia (UBC), Vancouver, B.C., Canada. PCRs were performed in a 50 uL reaction using the Hot Start technique (Procunier *et al.* 1995). This technique prevented the mixing of reactants until the temperature was high enough to melt the wax bead. This minimized the non-specific annealing of primers to DNA and reduced the formation of primer-dimers. For each 50uL reaction, the solution under the wax contained 1µl of 10X PCR buffer (0.50 M KCl, 100 mM Tris-Cl, pH 9.0 and 1% Triton-X-100), 3 µl of 24 mM $MgCl_2$, 5 µl of a 2.5 mM solution of each dATP, dCTP, dGTP and dTTP, and 5.0 µl of primer (10 ng/µl). A wax bead was added, the tube was heated at 75°C for 20 min. and then cooled. The upper volume contained 4 µl of 10X PCR buffer, 3 µl of template DNA (20 ng/µl), 0.5 µl *Taq* DNA polymerase (5 units/µl) and distilled water to make a total of 36 µl. An initial denaturation step of 3 min. at 94°C preceded 45 PCR cycles in a Thermolyne thermocycler. The cycle program consisted of a denaturation step of 1 min. at 94°C, an annealing step of 2 min. at 36°C and an extension step of 2 min. at 72°C. The last step of the last cycle was followed by a final extension-polymerization of 10 min. at 72°C.

Primers identifying polymorphisms between the parental lines BiggarBSR and RL4555 were used on a subsample of 40 lines. Any RAPD fragment showing potential linkage to dormancy was then tested on the remaining 60 lines of the population. PCR products from the initial parental screening were visualised in both agarose and denaturing gradient gels (DGGE). The primers OP-V4 and OP-W15 (Operon Technologies, Alemeda, Calif.) were also used in the RAPD analysis. These primers

have been shown to amplify fragments linked to dormancy in the cultivar Transvaal (Lawson et al. 1996).

Linkage analysis was performed using JoinMap computer software (Stam, 1993). pairwise recombination frequencies between loci were calculated and converted to map units (cM) using the Kosambi function.

Electrophoresis

PCR amplification products were initially separated in a 1.4% agarose gel in TBE buffer (54 mM Tris, 54 mM Boric acid, 10 mM EDTA, pH 8.0) run for 240 volt-hours. Polymorphic bands were noted and the sample was then subjected to denaturing gradient gel electrophoresis as described by Procunier *et al.* (1995). Electrophoresis was carried out in a 10% polyacrylamide gel with a linear gradient of 20-60% denaturant (consisting of deionized formamide and urea), for 1200 volt-hours after which gels were stained in ethidium bromide (0.5 μg/ml), destained and visualised under ultraviolet light. Both agarose and DGGE gels were photographed under ultraviolet illumination with a red filter using Polaroid (Cambridge, Mass) Type 57 positive film.

Cloning and sequencing of the RAPD marker linked to dormancy:

The UBC primer 303 (5'GCGGGAGACC-3') amplified an 800 bp RAPD fragment ($UBC303_{800}$) that was loosely linked to a dormancy locus. The RAPD band was present in the non-dormant parent and absent in the dormant parent. This RAPD band was eluted from a 1.4% agarose gel using a Spin-X filtration device (Costar, Cambridge, MA). The purified DNA fragment was cloned using the TA cloning kit (Invitrogen Corporation, San Diego CA). Transformation of competent cells (*E. coli* strain DH5α) was as per the TA Cloning instruction manual. White recombinant colonies were picked for plasmid isolation and further PCR analysis and sequencing.

Selected white colonies were grown in 5 mL of LB media overnight. Plasmids were isolated using the Wizard Mini Preps DNA purification system from Promega. Isolated plasmids were reamplified by 30 cycles of PCR as described previously but with

an annealing temperature of 52°C using universal M13 forward and reverse primers. The size of the amplified band was compared to the original RAPD band via agarose gel electrophoresis.

Double strand sequencing was done by the dideoxy chain termination method using an automated sequencer with universal M13 primers (Plant Biotechnology Institute, Saskatoon, Sask. Canada). The first 450 bases from the 3' end of the$UBC303_{800}$ fragment were sequenced.

Primers for SCAR amplification and analysis

Oligonucleotide primer pairs were designed for the cloned RAPD products for use for locus specific amplification based on the sequence information. A forward primer was synthesized to include the original 10 bases of the RAPD primer (forward primer) in addition to the next 10 3' bases. The primer sequence from the 5' end was selected using the computer software PRIMER (Version 0.5, 1991, Whitehead Institute for Biomedical Research) based on the criteria of 60% GC content, amplification product size of at least 400 bp and melting temperature of between 50°C and 65°C. Primers were synthesized by an Applied Biosystems DNA synthesizer model 391. PCR amplification using the locus specific primers proceeded as described for RAPD analysis with the exception that 35 cycles were performed with the annealing temperature varying from 55 °C to 65°C. Primer sequences were as follows: 5'GCGGGAGACCATTCCAACCC-3' forward, 3' GTCTGTTAGGCCATCAGAAC-5' reverse.

RESULTS

Cabinet Study

The recombinant inbred lines (BiggarBSR/RL4555) were tested for dormancy at two germination temperatures and percent germination was determined after 4 and 8 days. After 4 days at 15°C only 9 lines were significantly different from the resistant parent and the susceptible parent was only 35% germinated. This indicated that the differentiation between dormant and non-dormant lines was not complete at 4 days and

therefore all germination tests proceeded for 8 days. The frequency distribution of the replicate means for the RIL population germinated at 15°C at day 8 is presented in **Figure 3.1**. Using Dunnett's test, 51 of the 115 lines were identified as being significantly different from the dormant parent RL4555 with a χ^2 (1:1) value of 1.47 (P >0.2) supporting a one gene segregation model. For the Dunnett test, lines with less than 20% germination were scored as dormant. All other lines were scored as non-dormant. The correlation analysis of the two replicates shows a Pearson's correlation coefficient of 0.82 indicating that the two replicates were closely related.

A subsample of this population consisting of 81 lines was tested for dormancy at a germination temperature of 20°C. The frequency distribution for dormancy evaluated at 20°C is presented in **Figure 3.2**. At this temperature 62 lines were scored as dormant and 19 as non-dormant using the Dunnett analysis. The non significant chi square test (χ^2 (3:1) = 0.123, P >0.7) supports a 2 gene segregation ratio for dormancy at this germination temperature. Within the 81 lines tested, 40 lines were dormant at both 15 and 20°C and 18 were non-dormant at both temperatures. Only one line was found to be non-dormant at 20°C and dormant at 15°C, and 22 lines were found to be dormant at 20°C but non-dormant at 15°C (**Table 3.1**). These 22 lines presumably carry a second gene for dormancy that is expressed only at 20°C while 40 lines expressed dormancy at both temperatures. Both red and white lines were among the 22 lines dormant only at 20°C.

One major gene was also detected in the BiggarBSR/Bihar DH population tested at 15°C with 15 dormant lines and 12 non-dormant lines, χ^2 of 0.15, P>0.7. When this population was tested at 20°C, 19 were dormant and 8 were non-dormant, with a chi-square value of 0.308, P >0.5. The BiggarBSR/Bihar population was also segregating for red and white seed colour, but because of the small size of the population it was not possible to determine the segregation of dormancy with seed coat colour since only two lines were white. These two white lines were dormant. The Timgalen/RL4555

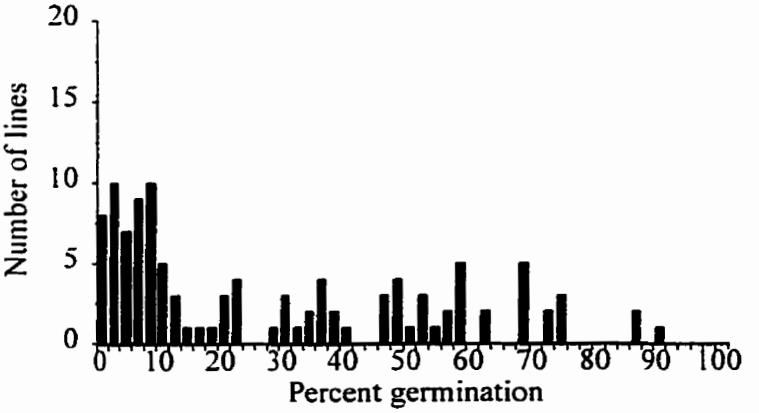

Figure 3.1. Frequency distribution of germination percent at day 8 scored at 15°C for Biggar BSR x RL4555 population.

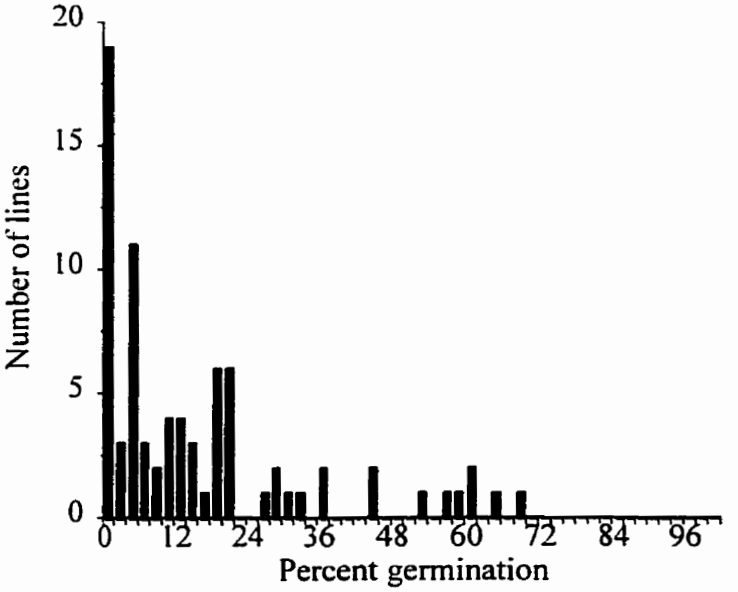

Figure 3.2. Frequency distribution of germination percent at day 8 scored at 20°C for a subsample of 81 lines from the Biggar BSR x RL4555 population.

population consisted of 6 dormant lines and 7 non-dormant lines ($\chi^2 = 0.08$, P>0.8) at 15°C. At 20°C this population had 9 dormant lines and 4 were non-dormant ($\chi^2 = 0.23$, P>0.7).

The segregation of dormancy scored at 15°C and at 20°C with seed coat colour in the BiggarBSR/RL4555 population was also analyzed (**Table 3.2**). In the population of 115 lines tested at 15°C, 5 lines were segregating for seed colour, 83 were red seeded and 27 were white seeded. The chi-square analysis for a 3 red:1 white segregation ratio supports the hypothesis that there are two red seed coat colour genes in the parent BiggarBSR. Within both the red seeded lines and white seeded lines dormancy segregated as expected for a one gene ratio. The non-significant chi square for independence supported the hypothesis of independence of seed coat colour and dormancy (**Table 3.3**).

Table 3.1. Segregation for dormancy within a subsample consisting of 81 lines from the BiggarBSR/RI4555 population, scored at 15°C and 20°C germination temperatures and a chi square to test deviation from the expected ratios.

Score/temperature	Dormant 20°C	Non-dormant 20°C	Total # lines
Dormant 15°C	40	1	41
Non-dormant 15°C	22	18	40
Total	62	19	81

Trait*	Ratio	Chi square	df	Prob. = 0.05
D-15°C: ND- 15°C (40:41)	1:1	0.0123	1	3.84 NS
D-20°C: ND- 20°C (62:18)	3:1	0.123	1	3.84 NS
D-20°C + ND-15°C : ND-20°C + ND-15°C (22:18)	1:1	0.2	1	3.84 NS

*D = dormant, ND= non dormant. NS is non significant.

Seed coat colour and dormancy at 20°C also appeared to be inherited independently (χ^2 = 0.43, df = 1, P >0.5) (**Table 3.3**) Five lines were segregating for seed colour and were therefore not counted in the analysis. Within the red and white lines tested at 20°C the results were in good agreement with the expected 3:1 Mendelian segregation.

Table 3.2. Segregation of dormancy within red and white lines germinated at 15°C and 20°C.

	15°C		20°C	
Population	Dormant	Non-dormant	Dormant	Non-dormant
Whole population	64	51	62	19
Red sub-population	50	33	43	11
White sub-population	12	15	16	6

Trait	Ratio	Chi square	df	Prob. = 0.05
Red:white	3:1	0.0123	1	3.84 NS
Red-D: Red-ND, 15°C	1:1	3.48	1	3.84 NS
Red-D: Red-ND 20°C	3:1	0.616	1	3.84 NS
White-D:White-ND, 15°C	1:1	0.333	1	3.84 NS
White-D: White-ND, 20°C	3:1	0.0604	1	3.84 NS

Table 3.3 - Chi-square test for independence for seed coat colour and dormancy at 15°C and 20°Cin BiggarBSR/RL4555 population.

	15°C			20°C		
Class	Dormant	Non-dormant	Total	Dormant	Non-dormant	Total
Red	50 (46.78)[1]	33 (36.22)	83	43 (41.92)	11 (12.08)	59
White	12 (15.22)	15 (11.78)	27	16 (17.08)	6 (4.92)	17
Total	62	48	110	54	22	76

χ^2 independence 15°C = 2.067 NS, df = 1, Prob.(0.05) = 3.84

χ^2 independence 20°C = 0.43 NS, df = 1, Prob. (0.05) =3.84

[1]Expected values

The location of the probe ABC174 on specific chromosomes was confirmed by hybridization on DNA blots from Giza(RL4137) group 3 substitution lines cut with 6 different restriction enzymes. A locus on chromosome 3A was not identified however 2 loci on chromosome 3B were identified, one of high molecular weight and the other of lower molecular weight consisting of a band on top of a consistently present doublet. A locus on 3D was also identified. When DNA from the parents was cut with the restriction enzyme Hind III and hybridized to the RFLP marker ABC174 polymorphism for 3D locus was revealed between the two parents. A second polymorphism was identified however it consisted of a doublet which was not present on the Giza substitution lines. This doublet is approximately the same molecular weight as the second 3B band of the Giza lines. The cDNA clone was hybridized to DNA from the segregating population to determine the mapping distance between the marker loci and the actual seed coat colour genes. The two polymorphic loci segregated as a 1:1 ratio, indicating that there was no aberrant segregation of the marker loci. Within the SSD population tested, 52 had the RL4555 3B allele and 49 had the BiggarBSR 3B allele, giving a χ^2 value of 0.089, $p > 0.7$. Also, 50 lines had the RL4555 allele on 3D and 51 had the BiggarBSR allele on 3D, with a chi-square value of 0.01, $p > 0.9$.

Both dormant and non dormant plants had either or both of the BiggarBSR red gene marker alleles indicating that the red seed coat colour is not linked to dormancy. The following table (**Table 3.4**) present the data for dormancy at 15°C relative to seed colour and alleles for the two marker loci. Similiar results were obtained for dormancy at 20°C (data not shown).

We observed 76 lines that had either or both BiggarBSR alleles (3BL and 3DL) and 25 which had only the RL4555 alleles for both loci. Within the 76 red seeded lines of the population tested for dormancy at 15°C only 7 did not have at least one BiggarBSR marker allele, and within the 25 white seeded lines 7 had one or more BiggarBSR alleles. These lines therefore represent crossovers which occurred between the marker loci and the actual red seed coat colour genes. Since it was not possible to precisely visually determine the number of actual red genes in each individual line, markers to the 3D and

3B genes were considered both together and individually and subjected to linkage estimation with the computer program JoinMap. The two red gene markers taken together are linked to red seed coat colour by 8.24 units of recombination. Neither of the markers, taken together or independently, were linked to dormancy. Nine lines were not tested for the marker because of the lack of seed or plant material for DNA extraction and 5 lines were segregating for seed colour and were not included in the analysis. This data also confirms the segregation analysis for seed colour which determined that BiggarBSR has two genes for red seed coat colour, one on 3D and one on 3B. Dormancy was not linked to the seed colour as determined by the non significant χ^2 test for independence and by the linkage analysis of the data with the Join Map program.

Table 3.4 Distribution of the number of lines scored for the 3B and 3D marker alleles for dormancy scored at 15°C. (3B-RL4555 indicates the lines carrying the RL4555 marker band for the 3B chromosome, 3B-Biggar indicates the lines carrying the BiggarBSR marker band for chromosome 3B etc. ND is non-dormant)

	ABC174 marker alleles				
Score	3B-RL4555 3D-RL4555	3B-Biggar 3D-Biggar	3B-RL4555 3D-Biggar	3B-Biggar 3D-RL4555	Total
Red-dorm	4	14	16	11	45
Red-ND	3	6	9	13	31
White-dorm	7	1	1	1	10
White-ND	11	3	1	0	15
Total	25	24	27	25	101

Within the sample tested for dormancy at 20°C, 51 lines had either or both of the BiggarBSR red seed coat colour marker alleles and a total of 20 had neither BiggarBSR red seed coat colour marker alleles. Within the 51 red seeded lines, only 6 did not have a red gene RFLP band, and within the white seeded lines 6 of the 20 lines did not have the white RFLP band. These lines once again represent the crossovers between the marker and the actual red gene loci.

The two RFLP loci (on chromosomes 3BL and 3DL) identified by the cDNA clone ABC174 were linked to red seed coat colour by 8.27 cM with a standard error of 3.03. The marker was used to identify lines carrying one or two red genes in order to evaluate the influence of these genes on the level of dormancy in these lines. The presence of two red genes did not influence the level of dormancy within those lines as both very dormant and very non-dormant lines with one and two red genes were observed.

Field study:

In 1992 a total of 159 RILs from a cross between Fielder and RL4555 were sown in replicate at the Glenlea research station. Heads from only 100 lines were harvested from each replicate row and evaluated for dormancy from this total population, 59 lines were not harvested as they failed to mature before major frosts. The susceptible parent had an average of nine sprouts per head while the resistant parent RL4555 had an average of 0.75 sprouts per head after seven days of testing. After subjecting the transformed means of the replicated germination data to the Dunnett's test 56 lines were significantly different from the resistant parent RL4555 and 44 were not significantly different. This gave a non significant chi square value of 1.44 (1df) which supported the hypothesis that this variation is controlled by a single gene.

Dormancy for the 1993 field material was evaluated as germination tests on hand threshed seed. The germination test was performed at 23° C because at 15°C and 20°C, the temperatures previously used in germination tests, dormancy was not being expressed in either the resistant parent, the resistant checks or the segregating lines. Only 60 lines could be harvested during this field season, the other lines did not fully mature before the major frosts. One gene for dormancy was detected in the population of 60 lines (**Table 3.5**). Twenty dormant and twenty non-dormant lines were selected for a cabinet study of dormancy. These plants were grown under the same conditions as described for the BiggarBSR/RL4555 population. Germination tests performed at 15°C in Petri plates allowed the expression of dormancy within the seeds. Eighteen of the 20 dormant lines

were now scored as dormant, and twenty-two lines were scored as non-dormant. These 40 lines were grown in the field in 1993 and after evaluation of dormancy at 23°C in germination tests on threshed seed 8 of the 20 dormant lines were dormant.

Table 3.5. Dormancy data for field grown Fielder/RL4555 population.

Score	1992[1]	1993[2]
Dormant	44	23
Non-dormant	56	37
Total	100	60

Trait	Ratio	Chi square	df	Prob. = 0.05
Dorm: Nondorm 1992	1:1	1.44	1	3.84 NS
Dorm:Nondorm 1993	1:1	3.26	1	3.84 NS

[1] Simulated rain chamber
[2] 23°C germination tests.

RAPD analysis:

RAPD analysis was performed on the BiggarBSR/RL4555 population to identify a marker linked to either or both dormancy genes identified. With the 76 RAPD primers assayed, 12 polymorphic loci were identified between the two parents. Only 5 of the 12 polymorphic loci were distinguishable on agarose gels. Primers that amplified polymorphic RAPD bands were selected and tested for linkage to the dormancy loci in 40 of the SSD lines. Promising RAPD primers were then applied to the rest of the 100 SSD lines. The chi square value for goodness of fit for the dormancy gene expressed at 15°C and the RAPD band amplified by the primer UBC303 (800 bp) was significant at the 0.01 level ($\chi^2 = 14.91$, df = 3). This marker was not linked to the gene that was expressed at the higher germination temperature (20°C).

This segregation data was subjected to linkage analysis using the computer program JoinMap. The RAPD marker is linked in repulsion to a gene controlling dormancy by 25.74 cM (Kosambi) with a LOD score of 3.03. **Figure 3.3** shows the

segregation of the RAPD polymorphic band that showed linkage to dormancy. The band segregated as a 1:1 ratio as supported by the non-significant chi-square value (χ^2 = 0.2174, df = 1). None of the other polymorphic loci were linked to dormancy. Operon primers V4 and W15 were assessed for linkage of the amplified fragments to dormancy in the SSD population as these were shown to be linked to dormancy in the cultivar Transvaal (Lawson *et al.* 1996). On the parental lines Biggar BSR and RL4555 the primers were not however polymorphic.

The polymorphic fragment from BiggarBSR amplified by primer UBC303 was cloned and partially sequenced. The clone was successfully sequenced at the 3' end only. It is possible that the 5' end contained a hairpin loop that was not opened with the sequencing protocols used. Locus specific primers were designed based on the 450 bases that were sequenced from the 3' end. These primers amplified a fragment of the expected size (400 bp).

At an annealing temperature of 55 degrees, a series of bands were produced using the locus specific primers on both the parental lines (**Figure 3.4**). A 600 bp polymorphic band was identified, however this band was not associated with dormancy when the segregating population was screened. The expected 400 bp band was the most prominent band however it was not polymorphic between the parents BiggarBSR and RL4555 at this temperature. Different primer and DNA concentrations were tried for the amplifications with no effect on the 400 bp band (**Figure 3.4**). The annealing temperature of the PCR protocol was increased to 60°C and 65°C at which temperatures only a 400 bp band was present in both parents. Further increases in annealing temperature resulted in the loss of amplification from both parents. Separation of PCR products on denaturing gradient gels did not reveal polymorphisms.

The locus specific primers were also applied to the Chinese Spring nulli-tetrasomics, Langdon (*T. dicoccoides*) substitution lines and wheat-barley addition lines to identify the chromosome location. No polymorphism was observed in the 400 bp amplicon between the various cytogenetic stocks and therefore the chromosome location of the marker could not be established.

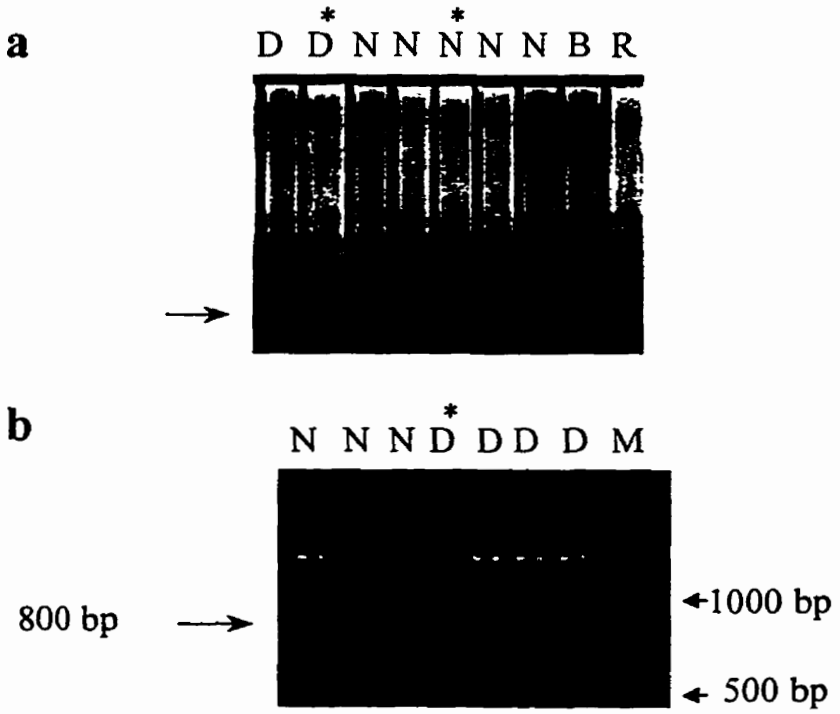

Figure 3.3 Electrophoretic banding pattern of RAPD bands produced with primer UBC303 separated on DGGE (a) and agarose (b). The arrow indicates the polymorphic band. N= Non-dormant, D = dormant, M = size standards, * = recombination between marker and dormancy, R = RL4555, B = BiggarBSR

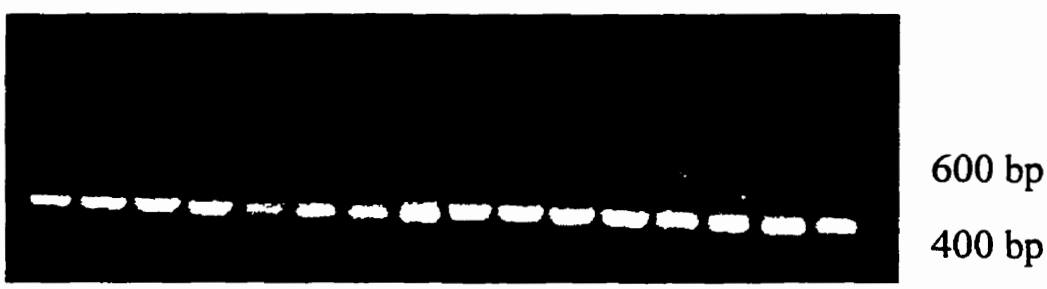

Figure 3.4 Banding pattern of locus specific amplification of parental DNA at annealing temperature of 55° C. Lanes 1-4 and 9-12 are 20, 40, 60 and 80 ng DNA from RL4555 with 50 and 100 ng of primers respectively. Lanes 5-8 and 13-16 are 20, 40 60 and 80 ng of DNA from BiggarBSR with 50 and 100 ng of primers respectivley.

The cloned RAPD marker was used as a radioactive probe on Southern blots of DNA from these various substitution lines and cytogenetic stocks. The clone identified 6 bands on the parental DNA indicating that it was a low copy number probe, an essential characteristic of an RFLP probe. A polymorphism was not observed between the parents. Southern blot analysis using the cloned RAPD marker as a probe was performed on DNA from a series of different alien substitution lines and the Chinese Spring nullitetrasomic lines. These lines were not polymorphic when hybridized with the cloned RAPD fragment.

DISCUSSION

A factor that contributes to the complexity of assessing seed dormancy is the fact that different methodologies exist for evaluating dormancy levels. Various approaches to evaluating seed dormancy have been assessed (Scott *et al.* 1984, Hageman and Ciha, 1984, Paterson *et al.* 1989) and for the purpose of evaluating sprouting resistance germination tests on threshed seed or intact heads have been found to be well suited to plant breeding objectives (McCaig and DePauw 1993, Hageman and Ciha 1984). In this study Dunnett's test was applied to the germination data to identify lines which were significantly different from the dormant parent. All other lines were considered dormant and therefore considered to be resistant to preharvest sprouting. This provided two distinct classes of lines, dormant and non-dormant. The testing methodology used here maintained two important environmental conditions by using a controlled maturation environment and by using two germination temperatures.

An important criteria for germination tests is the point in the test at which genotypes are compared. To assess the level of dormancy possessed by the individual lines of the populations germination tests were performed at specific temperatures and sprouted kernels were counted and removed daily. In order to establish the level of

dormancy possessed by a specific line the percent germinated seed was determined after 4 and 8 days and the data was compared to the dormant parent using the Dunnett analysis. In comparing germination percentage for day 4 and day 8 almost all the lines were not significantly different from the dormant parent after 4 days therefore differentiation between the dormant and non-dormant lines had not fully occurred at this time. After 4 days of germination the susceptible parent, BiggarBSR was only 35% germinated. After 8 days of germination the susceptible control cultivars (Roblin, Timgalen and Fielder) and the susceptible parent (Biggar BSR) were fully sprouted (80 -100%) while the dormant parents (RL4555 and Bihar) remained relatively unsprouted (0-10%).

The main temperature selected for the germination test was 15°C, a temperature which permits a very stringent selection for dormancy. At this temperature all but the very susceptible lines should germinate (Reddy *et al.* 1985) as dormancy tends to be broken at low temperatures. This was an important factor in the testing protocol since we wanted to identify lines carrying specific dormancy genes that are expressed at different temperatures. Under the controlled environmental conditions provided during the maturation of the wheat (16°C) and with stringent germination temperature of 15°C approximately 50% of the lines were not significantly different from the dormant parent. This indicated the presence of one major gene which is expressed in these genotypes when germinated at 15°C. When the temperature was raised to 20°C a second gene was clearly expressed. Dormancy has recently been shown to be mediated by two recessive genes in the white seeded wheat AUS 1408 (Mares, 1996) and a two gene model has been postulated for sprouting resistance in the red seeded Australian genotypes Transvaal and AUS 1490 (Lawson *et al.* 1996) and the white seeded genotype Kenya 321 (Bhatt *et al.* 1983).

A Kenya 321 sib was identified as a source of dormancy for the development of new varieties tolerant to preharvest sprouting (Bhatt and Derera, 1980). The results presented here demonstrate that the genotype RL4555, which derives its dormancy from Kenya 321, has at least two dormancy genes. This does not limit the number of genes to two in the dormant parent RL4555 as only two germination temperatures were tested and

plants were matured at one temperature. The data from the field grown material suggests that more than two genes may be present in the genotype RL4555. In 1992 the major gene expressed at 15°C was also identified in the Fielder x RL4555 population grown in the field. In 1993 however, the two genes previously identified from the cabinet studies were not detected in the selected lines. A gene was however being expressed at 23°C suggesting that this may be a third gene encoding dormancy in the genotype RL4555. This demonstrates one of the difficulties in the analysis of dormancy, that the environment under which the plants mature is critical to the level of dormancy expressed. The late summer of 1993 was quite cool and very wet, conditions which contributed to the reduction in the expression of dormancy. Another aspect of this experiment was the analysis of seed coat colour within the dormant and non-dormant lines. Dormancy segregated independently from seed coat colour in the RIL population. The use of the molecular marker ABC174 linked to red seed coat colour allowed the identification of lines carrying one, two or no red genes. Dormant and non-dormant lines with either one or both red genes were identified. This DNA marker identified two loci, one on chromosome 3D and one on 3B. Red lines had either one or both of the red gene markers (3D or 3B) with 7 recombinant lines among these (red lines with neither red gene marker). Within the white seeded lines 7 were identified as recombinants and therefore had either or both red gene marker alleles. This therefore represents a recombination percentage of 8.24 with a standard error of 3.03.

The incorporation of high levels of dormancy into new white seeded cultivars has been slowed by the lack of information concerning the mode of inheritance of the trait. The results presented here identified lines that carry individual genes which could be selected for in breeding programs aimed at reducing levels of preharvest sprouting. The red gene marker could also be applied to the selection of white seeded lines without the practical and logistic problems associated with the selection of a trait that does not develop until full maturity.

The RAPD marker identified here is loosely linked to a major dormancy gene. The polymorphic band amplified by primer UBC303 was cloned and partially sequenced

in an attempt to synthesize specific primers for the locus. The length of the wheat genome has been estimated at 4600 cM (Gill *et al.* 1991). Theoretically the identification of a linked marker with a maximum recombination distance of 30 cM would require approximately 160 polymorphic markers to cover the whole genome. A linkage distance to 10 cM would require 460 polymorphic markers. Assuming an average of approximately 8 bands per random primer amplification and considering that only 12 of those 608 bands were polymorphic, we observed a 2% level of polymorphism. This level drops considerably if we take into account those polymorphisms observed only on agarose gels (0.8%). With the maximum level of polymorphism observed in this study 8000 primers would theoretically have to be screened to find a marker linked by 30cM. It was therefore surprising that a marker was identified after screening only 76 primers. Therefore rather than continuing the screening of random primers to find a more closely linked marker, the loosely linked marker was used in various studies in an attempt to identify the chromosome location of the marker. Knowledge of the chromosome location would enable us to exploit the chromosome specific maps to target the location and find a tightly linked marker. The polymorphic fragment identified in the segregating population could not be assigned to a specific chromosome when the RAPD primers or the locus specific primers were applied to nullitetrasomic lines of Chinese Spring.

The results presented here strongly suggest that the amplification product from the random 10-mer primer UBC303 contains repetitive DNA. The chromosome location of the tagged dormancy gene could not therefore be determined. The results presented here confirm the findings of Devos and Gale (1992) who suggest that the cloning of RAPD bands from wheat and their use in Southern hybridization will often not be possible because the majority of the amplified products contain repetitive DNA sequences.

Operon primer V4 and W15 were assessed for linkage to dormancy in the SSD population as these were shown to be linked to dormancy in the cultivar Transvaal (Lawson *et al.* 1996). The parental lines Biggar BSR and RL4555 were not however polymorphic with these primers therefore no conclusion could be drawn as to linkage to dormancy in the population used on this study. One of the limitations of RAPD markers

is the lack of applicability to other populations. Although a population may be segregating for the trait, the marker may not be polymorphic in that population and therefore can not be applied.

Dormancy is a trait that is very difficult to assess because it is influenced by the environment. The incorporation of dormancy into new cultivars could benefit greatly from marker assisted selection. The marker identified here is loosely linked to a major dormancy gene in the genotype RL4555. Because the distance between marker and dormancy loci is quite large (26cM) the RAPD would be of limited use in a plant breeding program. Generally a marker should be linked by at least 5 cM to be of use in marker assisted selection in order to limit the number of recombinants selected (Tanksley 1983). The identification of a marker on the other side of the dormancy locus would increase the efficiency of the RAPD marker in breeding programs.

CHAPTER 4

RFLP Analysis of Group 2 Chromosomes in Wheat and Their Association with Dormancy

ABSTRACT: RFLP analysis of the group 2 chromosomes was undertaken in segregating wheat populations to verify the potential role of these chromosomes in dormancy. The 21 probes used in this study had been previously mapped in barley. While dormancy has traditionally been associated to red seed coat colour in wheat (group 3 chromosomes), genetic sources of dormancy have also been identified in white seeded genotypes. A search of the group 2 chromosomes of wheat genome for markers associated to dormancy genes not yet located to specific chromosomes. This approache has proved to be very inefficient due to a lack of polymorphism detection with the barley probes. Only nine of the 21 probes revealed polymorphisms. These were located on chromosomes 2D and 2BL.

INTRODUCTION

Increased resistance to pre-harvest sprouting is an important objective of plant breeding programs in many wheat producing areas of the world. Seed dormancy is the main factor involved in pre-harvest sprouting resistance (Derera *et al.* 1977). Initial research indicated that there was a strong association between red seed coat colour and pre-harvest sprouting resistance. Since white seeded sprouting resistant progeny were not recovered in crosses of dormant red lines by non-dormant white lines the association was proposed as being due to very tight linkage or pleiotropy (Soper *et al.* 1989, Freed *et al.* 1976, Gfeller and Svejda, 1960). Initially the variability for sprouting resistance among white seeded genotypes was believed to be quite low (Derera *et al.* 1977) however a number of white seeded genotypes have been identified or developed that exhibit significant levels of resistance to sprouting (DePauw and McCaig, 1983, Sorrells and Paterson 1986, DePauw *et al.* 1985, 1992) suggesting that mechanisms unrelated to red seed coat colour may be involved.

Evidence supporting the presence of factors influencing dormancy other than seed coat colour has been provided. Factors such as differential embryo response to abscisic acid (ABA) (Stoy and Sundin 1976), the interference of gas exchange by the enzyme tyrosinase (Côme *et al.* 1984), gibberellic acid (GA) insensitivity (Gale and Marshall 1973), and a proteinaceous inhibitor of α-amylase (Weselake *et al.* 1983a) have all been proposed as physiological mechanisms involved in pre-harvest sprouting resistance. Furthermore, embryo and aleurone specific dormancy associated transcripts have been identified in barley (Aalen *et al.* 1994).

Information regarding the chromosome location of genes associated with seed dormancy could assist plant breeders in developing strategies for the manipulation of this complex trait. Two ABA-inducible genes have been mapped to chromosome group 2S and 2L (Rayfuse *et al.* 1993) and a dormancy associated transcript (B15C, Aalen *et al.* 1994) has been mapped to the short arm of chromosome 2H of barley (Kleinhofs *et al.* 1993). Three α-amylase inhibitor loci have been identified in wheat and are located on the long arms of chromosomes 2A, 2B and 2D (Masojc *et al.* 1993) and genes for tyrosinase have been identified on chromosomes 2A and 2D of wheat (Chapter 5 this thesis). In a recent study on the analysis of quantitative trait loci associated to dormancy in wheat, several loci on the group 2 chromosomes were found to be associated with dormancy (Anderson *et al.* 1992). Analysis of sprouting resistance in the population derived from a cross between BiggarBSR and RL4555 failed to demonstrated linkage to the red gene loci. Marker analysis of the group 2 chromosomes within this population was performed to evaluate the potential role of these chromosomes in sprouting resistance.

MATERIAL AND METHODS

Plant material

The segregating families used for linkage analysis included a RIL population (115 lines) from a cross between the white seeded dormant line RL4555 and the red seeded non-dormant line BiggarBSR. A population of 20 resistant and 20 susceptible RIL from

a cross between RL4555 and Fielder (white non-dormant) was also used. Two small doubled haploid (DH) populations were also evaluated for dormancy. One DH population consisted of 13 lines from a cross between Timgalen (white non-dormant) and RL4555, the other consisted of 27 lines from a cross between BiggarBSR (red non-dormant) and Bihar (white dormant). Dormancy in these lines was evaluated as previously described (Chapter 3).

RFLP analysis

The RFLP-based genetic map of chromosome 2H of barley (Kleinhoffs *et al.* 1993) was the source of DNA probes that were used in this RFLP analysis of linkage to dormancy. Three probes were from the German Barley mapping project (Graner *et al.* 1991) and two probes were from Sharp *et al.* (1989). The cDNA from the barley alpha-amylase inhibitor (Leah and Mundy 1989) and the dormancy related cDNA probe B15C (Aalen *et al.* 1994) were also evaluated for linkage to dormancy. Most probes used have been mapped in barley and while they cover the barley chromosome 2 they may not reveal polymorphisms on all three wheat group 2 chromosomes.

DNA extraction was performed as described by Kleinhofs *et al.* (1993) and methods for blotting and hybridizations were as described by Sharp *et al.* (1988). Chinese Spring nullisomic-tetrasomic (Sears 1966) lines were used to identify the chromosome location of loci identified by individual probes.

The probes were first evaluated on blots of parental DNA cut with 5 restriction enzymes (HindIII, EcoRV, XbaI, BstEII, DraI). Probes identifying RFLP between the parents were hybridized to DNA from the segregating populations for linkage analysis. Segregation analysis and estimation of pair-wise recombination distances between loci were performed using JoinMap computer software using the Kosambi function (Stam 1993).

RESULTS AND DISCUSSION

The probes used in this study had previously been mapped to chromosome 2H in barley. In order to identify informative probe and restriction enzyme combinations in wheat, the 21 group 2 probes were hybridized to DNA from the parental lines from the four segregating populations, cut with five restriction enzymes. The results, summarized in **Table 4.1,** indicate that nine probes revealed polymorphisms between the dormant and non-dormant parents of at least one of the three populations.

Table 4.1. Restriction enzymes used to detect polymorphisms in three mapping populations on group 2 chromosomes with 21 probes and arm assignments to informative probes.

Probe	Polymorphism	Population	Location	Source
ABC454	H,E,Bs,D	BxR, FxR	2DS	NAGMP
B15C	H	BxR	2DS	Aalen *et al.* 1994
Rubisco	H	FxR	2DS	unknown
135	D	BxBh	2DS	Sharp *et al.* 1989
ABC153	H, E	BxR BxBh	2BL	NABGMP
GLN2	H,E,Bs,D	BxR, FxR	2BL	NABGMP
MWG720	Bs,D	FxR	2BL	Graner *et al.* 1991
KSUD022	NP			NABGMP
ABG019	NP			NABGMP
ABG356	NP			NABGMP
ABG358	NP			NABGMP
ABG008	NP			NABGMP
ABG005	NP			NABGMP
ABG002	NP			NABGMP
α-amylase inhibitor	NP			Leah and Mundy, 1989
MWG858	NP			Graner *et al.* 1991
MWG520	NP			Graner *et al.* 1991
101	NP			Sharp *et al.* 1989
21A1	NP			NABGMP
21A8	D, E	BxR, FxR, BxBh	2DS	NABGMP
22B8	H, E,Bs,D	FxR	2DS	NABGMP

S= short arm, L= long arm, H= HindIII, Bs=BstEII, D=Dra1, E= EcoRV, B= BiggarBSR, R= RL4555, Bh= Bihar F= Fielder

the mapping populations. Levels of polymorphisms between cultivars of hexaploid wheats is reported to be low (Sharp *et al.* 1989, Chao *et al.* 1989). Kam-Morgan *et al.* (1989) report that only one in three loci was polymorphic. Levels of polymorphism are reported to be higher (50%) between synthetic hexaploids and common wheat cultivars (Nelson *et al.* 1995b). In the larger BiggarBSR x RL4555 population seven polymorphic loci were identified. The nine informative probes were applied to DNA from the appropriate segregating population cut with the appropriate restriction enzyme to verify association with dormancy. Hybridizations were then carried out on restriction digests of DNA from Chinese Spring group 2 nulli-tetrasomics to assign these probes to specific chromosome locations. **Figure 4.1** shows an example of such an analysis which allowed the identification of bands on chromosomes 2A, 2B and 2D.

All polymorphic loci segregated as Mendelian alleles with the expected 1:1 ratios. The clone ABC153 identified 2 loci that mapped to the long arm of chromosome 2B. A number of probes in a study by Devos *et al.* (1993) detected more than one locus per chromosome arm. They suggest that these probes should be used with caution as some multigene families are dispersed over large genetic distances. For example two copies of XEmbp were identified on the long arm of chromosome 2R and the distance between these two loci was approximately 30 cM (Devos *et al.* 1993). Duplicate loci have also been reported by Nelson *et al.* (1995a) in the production of a dense group 2 chromosome map in wheat. The distance between the two loci of ABC153 on the long arm of chromosome 2B was 35 cM. These two loci are flanking a gene which conditions resistance to the loose smut race T10 (Procunier *et al.* 1997).

A small linkage group on the short arm of chromosome 2D was identified and is presented in **Figure 4.2.** All linked loci had LOD scores greater than 3. No other linkage groups were found, demonstrating the lack of coverage of the wheat genome using this limited number of barley markers. Polymorphic loci on chromosome 2A and the short

arm of 2B were not identified in either populations. There was very poor coverage of the long arm of 2D with only one polymorphic locus identified. The alpha-amylase inhibitor did not reveal polymorphisms in either population and therefore association with dormancy could not be determined. The dormancy related cDNA clone B15C, also shown to be associated with tyrosinase activity (Chapter 5), was not associated with dormancy in the crosses used here. All other polymorphic RFLP loci also segregated independently from dormancy as determined by X^2 analysis and linkage analysis.

Problems were encountered with the RFLP analysis in the Timgalen x RL4555 DH population. A very large linkage group was identified on chromosome 2B of this population.

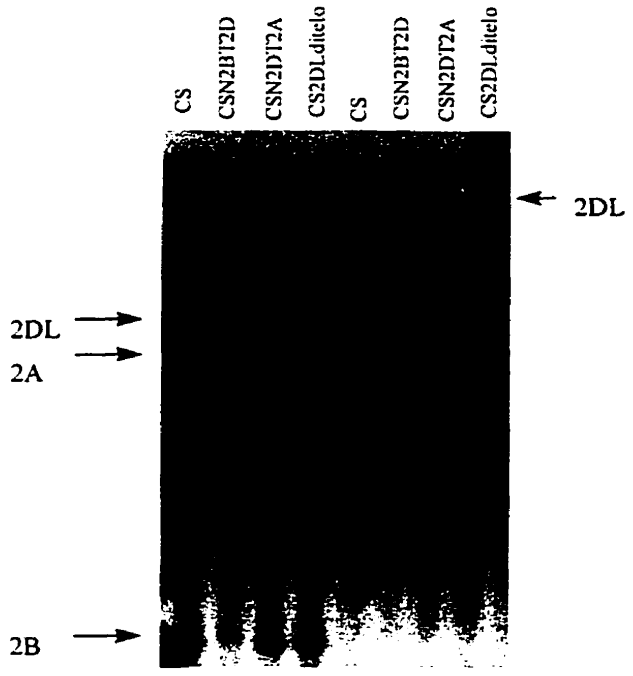

Figure 4.1 - Chromosome location of α-amylase inhibitor loci in CS nulli-tetrasomics DNA digested with HindIII (lanes 1-4) and EcoRV (lanes 5-8).

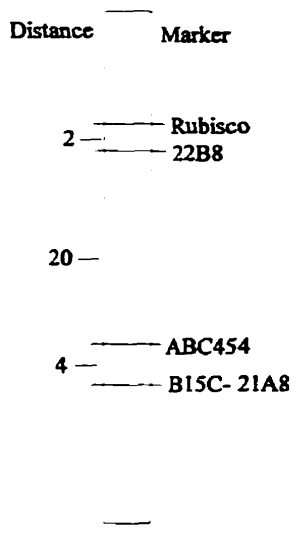

Figure 4.2. Recombinant linkage map of chromosome 2DS, showing the location of linked markers. All distances are given in cM.

The probes DO22, 101, 135, 22A8, the α-amylase inhibitor, ABG019, GLN2 and 356 were all linked together. Distorted segregation ratios with preferential inheritance of the Timgalen alleles was observed. All polymorphic loci segregated as three Timgalen alleles to one RL4555 allele. These probes were located on both the short and long arm of chromosome 2B. Most of the 2B chromosome from Timgalen is of alien origin, carrying an introgressed segment from *T. timopheevi* (Devos *et al.* 1993). Although this was a very small population and could not have effectively been used for mapping the group 2 chromosomes, the preferential inheritance of the Timgalen 2B chromosome also limited the use of this population in this study.

Although there was no association between the group 2 markers and dormancy, we can not conclude that this chromosome group is not involved in dormancy. Only a very small portion of the group 2 chromosomes was covered by this study, primarily the short arm of 2D and the long arm of 2B. A more in depth study would require a much larger number of probes to cover the potential length of 200 cM for each chromosome.

As the wheat genome map becomes more developed more probes will become available to further evaluate the role of the group 2 chromosomes in dormancy.

This study clearly shows the problem associated with the use of existing mapped RFLP probes to map novel populations. The level of polymorphism was extremely low and therefore the coverage of the group 2 chromosomes was also low. Genetic maps have traditionally been made using populations from genetically diverse parents such as synthetic hexaploids in order to increase the level of polymorphisms within the population. The application of such maps to common wheat cultivar populations may be limited.

CHAPTER 5
Kernel Tyrosinase in *Triticeae* spp. and the Inheritance of Genes Controlling its Expression in Common Wheat.

ABSTRACT: A quantitative colorimetric microtitre plate assay was developed to measure levels of tyrosinase activity in individual wheat kernels. The test was performed on 85 durum and 55 common wheat cultivars. The majority of the durum cultivars had low levels of tyrosinase activity, while most hexaploids had high levels of activity. There was an overlap of activity levels between the two, however, so that it was not always possible to distinguish durum wheat lines from common wheat lines. This assay is simple and non-destructive and would be a suitable test for screening of material in plant breeding programs to improve noodle and pasta quality. This tyrosinase assay was also used to screen several alien substitution and addition lines resulting in the identification of genes encoding tyrosinase on chromosome 2A and the short arm of chromosome 2D. A synthetic hexaploid (RL5710) identified as a source of zero tyrosinase was used as a parent in a study of the mode of inheritance of tyrosinase in wheat. The genetic studies performed on two segregating F_2 and F_3 populations demonstrated the presence of two genes of different effect that interacted epistatically. Molecular markers flanking one tyrosinase locus were identified.

INTRODUCTION

Tyrosinase (E.C. 1.14.18.1) or monophenol monooxygenase, is a copper containing enzyme found in most higher plants. The enzyme catalyses the hydroxylation of monophenols to diphenols which are oxidized to benzoquinones. These molecules are very reductive and readily convert to black melanin pigment or covalently cross-link proteins and free amino acids (Matheis and Whitaker 1984). The post harvest enzymatic browning of many fruits and vegetables that is caused by the catalytic action of tyrosinase is a serious problem in food technology (Matheis and Walker 1984). In cereals it is the kernel tyrosinase that has received the most attention as this enzymatic reaction is known to cause the browning of many wheat products such as Middle East flat breads (Faridi 1988), chapattis (Abrol and Uprety, 1972), Oriental noodles (Kruger *et al.* 1992, Baik *et al.* 1995) and pasta products (Kobrehel *et al.* 1974). The formation of the black quinone

products represents the primary detrimental effect of tyrosinase in the post-harvest processing of crop plants and is the primary reason for the great interest in tyrosinase in food technology. The undesirable action of this enzyme in food is of major concern not only because of the unacceptable brown colour produced but also because of changes in taste and nutritional quality as a result of the reactions (Matheis and Walker, 1984). Tyrosinase can be as important as α–amylase in the deterioration of end use quality of wheat (Kruger and Hatcher 1992).

While the detrimental effects of the enzyme in the food processing industry are well documented, the physiological function of tyrosinase in plant tissues and grain is still poorly understood. Tyrosinase has been studied in a number of plant varieties and plant tissues in relation to its involvement in disease and pest resistance and oxygen metabolism (Farkas and Kiraly 1965, Ludlum *et al.* 1991, Batra and Kuhn 1975). In cereal grains it has been studied particularly in terms of its regulation of expression during germination and its effect on seed dormancy (Tikoo *et al.* 1973 , Côme *et al.* 1984, Corbineau *et al.* 1981). The pericarp of a seed can interfere with the germination process by limiting the exchange of gases by the embryo. This interference with gas exchange may occur by the enzymatic consumption of oxygen by the tyrosinase enzyme in the pericarp, consequently depriving the embryo of oxygen and inhibiting its germination (Côme 1984, Corbineau *et al.* 1981). Although there is no direct evidence for a causal relationship between tyrosinase and dormancy every physical or biochemical treatment which reduces the oxidation reaction by tyrosinase and facilitates oxygen passage to the embryo leads to better germination (Côme *et al.* 1984).

Conflicting results have been reported as to the chromosome location of the genes encoding tyrosinase and the inheritance of the trait. Genes have been identified on chromosomes 2A and 2D (Zeven 1972), on chromosome 7B (Sadananda *et al.* 1974), and on chromosome 6B with a modifier on 5B (Bhat and Goud 1978) in wheat lines of different genetic backgrounds. Initial genetic studies have proposed that tyrosinase is monogenetically inherited and involves a multiallelic series (Joshi and Banerjee 1969).

Various methodologies have been developed to evaluate levels of tyrosinase activity in wheat grains. Several qualitative tests have been established in which the darkening of whole seeds treated with a phenolic substrate (phenol, tyrosine or cathecol) is assessed (Mahoney and Ramsay 1992, Wrigley 1976). This phenol colour development test is a simple qualitative assay used frequently to test for varietal purity (Wrigley and Shepherd 1974, Wrigley 1976, Wrigley and Baxter 1974, Crisp and Wrigley 1973). Mahoney and Ramsay (1992), using a modified phenol test, reported that kernels of common wheat (*Triticum aestivum* L.) cultivars have high tyrosinase activity while durum wheat (*Triticum turgidum* L. var *durum*) cultivars have low levels of this enzyme. The phenol reaction test remains a subjective test where rankings between degrees of darkening can be difficult to make. Quantitative testing methodologies that measure the change in oxygen consumption of a suspension of ground grain in the presence of a phenolic substrate have been used (Lamkin *et al.* 1981, Marsh and Galliard 1986, Hatcher and Kruger 1993). These assays are time consuming and not suited to a plant breeding program where rapid screening techniques are required. Recently a quantitative assay that is more suited to the high throughput testing of a plant breeding program was developed (Kruger *et al.* 1994a). This assay measures the development of the black pigmentation over time in intact grain using a kinetic microtitre plate reader.

This paper describes a quantitative colorimetric microtitre plate assay based on a modification of the method of Mahoney and Ramsay (1992) which can be used to measure levels of tyrosinase activity in single wheat kernels. This assay was used to determine the levels of tyrosinase in individual kernels of 85 durum wheat and 55 common wheat cultivars. The quantitative assay was also used to determine the mode of inheritance of tyrosinase and the chromosomal location of genes encoding tyrosinase. This was accomplished by screening cytogenetic stocks and two segregating F_2 and F_3 populations. RFLP analysis using clones mapped in barley was performed on the segregating F_2 populations to tag the genes and to identify the specific chromosome location of the genes.

MATERIALS AND METHODS

Tyrosinase assay

Tyrosine substrate solution (0.01 M disodium tyrosine in 0.1 M Tris-HCl buffer, pH 9.0) was prepared according to the method of Mahoney and Ramsey (1992) with the exception that Tween 80 was added to a final concentration of 0.2 % to ensure complete and even wetting of the individual kernels. Single wheat kernels to be assayed were placed in rigid, flat-bottomed 96-well microtitre plates (Corning) containing the substrate solution (0.2 ml) and the plate was incubated at 37 °C for 2.5 h. After incubation, seeds with high tyrosinase activity had a dark brown pericarp and those with little or no activity remained unchanged. Kernels treated in this way retained full viability. The brown pigment found in the substrate solution was measured by transferring aliquots (0.1 ml) from each well to a second 96-well plate. Absorbance was measured with a Titertek Multiscan MCC/340 plate reader using a 405 nm filter. For some durum cultivars a yellow pigment appeared in the substrate solution although the kernels did not develop a brown pericarp pigmentation. The yellow pigment was not a product of the tyrosine in the substrate as it was also present when these kernels were incubated in Tris-Cl pH 9.0 without the tyrosine. The absorbances of the plates containing this yellow pigment were read using a 690 nm filter, as at this wavelength the yellow pigment was not detected.

Thirty-five cultivars (20 durums and 15 common wheats) were also assayed using phenol (2 mM) as the substrate (Lamkin *et al.* 1981) and incubated for 4 h at 37 °C. Absorbance was read at 340 nm with substrate blanks.

Tyrosinase activity was evaluated by incubating intact seeds of three cultivars (5 and 10 seeds) in 1 ml tyrosinase substrate buffer. The seeds were incubated for 3 hours at 37°C and the absorbance of 100ul aliquots was read at 405 nm. Tyrosinase levels were also measured on the whole wheat fraction of two wheat genotypes, Genesis and HY361. Whole wheat (100 mg) was incubated with 1 mL of tyrosine buffer for 16 hours, after which 100 uL aliquots were transferred to a microtiter plate. Absorbance was read at 405 nm.

Plant material

For each cultivar and cytogenetic stock five individual kernels were evaluated. Each microtitre plate included four replicate positive controls (*Triticum aestivum* L. cv. Roblin) and four negative controls (*Triticum turgidum* L. var *durum*, cv. Langdon). Data for the cultivar survey are presented as activity relative to that of cv. Roblin. Most hexaploid wheats were field grown in 1986 at Glenlea, Manitoba and were kindly provided by Dr. R. McKenzie. The durum wheats were from various sources, either from the Plant Gene Resource Collection grown in 1979 or grown in cabinet conditions and were kindly provided by Dr. D. Leisle. Although these seeds were stored for over 10 years, cultivars with both high and low activities were observed.

Cytogenetic stocks were from various sources. The *Triticum monococcum* stocks were received from the Percival collection, England, *Aegilops speltoides* lines were from M. Tanaka, Japan and the *Triticum turgidum* var *durum* cv Langdon (*T. turgidum* var '*dicoccoides*') chromosomes substitution lines were from Dr. L. R. Joppa, U.S.D.A. Agricultural Research Service, Agronomy Department, North Dakota State University, Fargo, North Dakota (Joppa 1993). The wheat-barley addition lines were from Dr. A. Islam, (Islam *et al.* 1981). Chinese Spring(Hope) substitution lines, Chinese Spring ditelosomics and Chinese Spring nullitetrasomics were from Dr. E.R. Sears, U.S.D.A., University of Missouri, Columbia, Missouri (Sears *et al.* 1957, Sears and Sears 1978, Sears 1966). The synthetic hexaploids were kindly provided by Dr. Eric Kerber, Agriculture and Agri-Food Canada, Cereal Research Centre, Winnipeg.

Data for the tyrosinase assay of the addition and substitution lines are presented here as activity relative to that of the recurrent parent of the lines. For example, in the Chinese Spring (Hope) substitution lines, Hope chromosomes were substituted for Chinese Spring chromosomes. Tyrosinase activity is therefore presented as activity relative to that of cv. Chinese Spring. The data was analyzed using a SAS General Linear Models procedure and with significant F values, means from each line were compared with each other using the Ryan-Einot-Gabriel-Welsh Multiple F test (REGWF) with a significance level of 5% (SAS Institute, 1992).

Two F2 populations were produced to study the inheritance of tyrosinase. The first population consisted of 43 F_2 individuals from a cross between the zero tyrosinase synthetic hexaploid RL5710 and the CPS line Alpha. The second population consisted of 70 F_2 individuals from a cross between the line 334-5 (a zero tyrosinase line from Roblin/RL5710) and the cv. Roblin. Two to three seeds from F_2 lines were sown to produce the F_3 plants. Seeds from F3 were bulked.

RFLP and linkage analysis

Genomic DNA was isolated according to the method of Kleinhofs *et al.* (1993). Quantitation of the DNA from each parental line and the segregating F_2 and F_3 lines was determined by spectrophotometry. Sets of mapped barley clones were kindly provided by the North American Barley Genome Mapping Project (Kleinhofs *et al.* 1993), by Dr. A. Graner (Institute for Resistance Genetics of the Federal Biological Research Center for Agronomy and Forestry, Grunbach, FDR; Graner, A. 1991) and Dr. P.J. Sharp (AFRC Institute of Plant Science Research, Cambridge Laboratory, Cambridge, UK; Sharp *et al.* 1989). The probe B15C was kindly provided by Dr. Aalen (Plant Molecular Biology laboratory, Department of Biotechnological Sciences, Agricultural University of Norway, Aalen *et al.* 1994). Cloned inserts were amplified by PCR and purified by phenol/chloroform extraction. The inserts were labelled by the random hexamer method with [^{32}P]dATP according to the manufacturers instructions (BRL Random Primer DNA labelling System, Gibco BRL). Southern blotting of DNA and hybridizations were performed as described in Chapter 3 of this thesis.

The tyrosinase data and the RFLP data from the F_2 populations were analyzed using the JoinMap computer software (Stam, 1993) to determine the linkage relationships between markers and the tyrosinase gene. The Kosambi function (Kosambi 1944) was used to calculate the recombination value (cM).

RESULTS

Tyrosinase assay and cultivar survey

A microtitre plate assay was developed and used to evaluate the levels of tyrosinase activity in durum and common wheat cultivars from various regions of the world. Activity in the durums varied from a low of 0.03 (cv. Quilafen) to a high of 1.77 (cv. Matarrese) with the majority of the cultivars in the 0-0.5 range of activity (**Table 5.1**). Common wheats had higher levels of tyrosinase activity, with the majority of the cultivars in the 0.5 to 1.0 range of activity (**Table 5.2**). When groups of 5, 10 and 20 seeds were assayed, the CV. between six replica means was 9.6%, 10%, and 8% respectively for the cultivar Roblin.

Thirty-five cultivars (20 durums and 15 common wheats) were also assayed using phenol as the substrate. The enzyme activities obtained using the two substrates (tyrosine and phenol) were closely correlated ($r^2 = 0.75$, $P < 0.0001$) demonstrating that both assays were probably measuring the same enzyme (phenol oxidase, tyrosinase E.C. 1.14.18.1).

Tyrosinase activity was measured in bulks of five and ten seeds incubated in 1 ml of tyrosine buffer. Aliquots of 100 ul were traferred to a microtitre plate and absorbance was read at 405 nm. The data indicates that there is a linear relationship between the assay of five and ten seeds, that is that absorbance increases as the number of seeds per volume of substrate increases (**Table 5.3**). Levels of tyrosinase activity measured in whole meal fractions of two wheat cultivars compared to the microtitre plate assay are presented in **Table 5.4.**

Table 5.1. Relative tyrosinase activities[1] of durum wheat kernels of various origins.

Cultivar	Origin	Act.	St. dev	Cultivar	Origin	Act.	St. dev.
Quilafen	Chile	0.03	0.03	Ramsey	USA	0.03	0.015
Simeto	Italy	0.03	0.011	Maliani	Italy	0.04	0.028
Trinakria	Italy	0.04	0.017	Velbelice	Italy	0.04	0.011
Duilio	Italy	0.05	0.015	Leeds	USA	0.05	0.018
Medora	Canada	0.05	0.02	Messapia	Italy	0.05	0.006
Mexicali	Mexico	0.05	0.026	Valnova	Italy	0.05	0.014
Valaniene	Italy	0.06	0.09	Valsacco	Italy	0.06	0.055
Vespro	Italy	0.06	0.11	Wakooma	Canada	0.06	0.03
Ranger	USA	0.08	0.027	Flodur	France	0.09	0.07
Vic	USA	0.09	0.008	Amaralejo	Portugal	0.1	0.044
Conte Morando	Italy	0.1	0.038	Rio	USA	0.1	0.045
Lambro	Italy	0.11	0.043	Sentry	USA	0.11	0.012
Hymera	Cyprus	0.12	0.012	Langdon	USA	0.12	0.024
Hercules	Canada	0.13	0.017	Kamilaroi	Australia	0.13	0.067
Biodur	France	0.14	0.014	Golden Ball	Canada	0.14	0.03
Valnera	Italy	0.14	0.04	Arcola	Canada	0.15	0.075
Bidi 17	Algeria	0.15	0.12	Ward	USA	0.15	0.04
Wascana	Canada	0.17	0.024	Yavaros	Mexico	0.17	0.07
Riente	Italy	0.19	0.04	Valgiorgio	Italy	0.19	0.08
Produra	USA	0.2	0.08	Rugby	USA	0.2	0.064
Valoriolo	Italy	0.2	0.07	Cappelli	Italy	0.21	0.095
Edmore	USA	0.21	0.075	Orgaz	Portugal	0.21	0.08
Tomclair	Germany	0.21	0.07	Valfiora	Italy	0.21	0.07
Valselva	Italy	0.22	0.09	Attila	Germany	0.23	0.124
Nugget	USA	0.23	0.089	Montferrier	France	0.24	0.096
Rikita	France	0.24	0.094	Ambral	France	0.25	0.06
Appulo	Italy	0.26	0.034	Giorgio 449	Italy	0.26	0.017
Crane 3	Mexico	0.28	0.007	Gabbiano	Italy	0.28	0.007
Sel Romana	Italy	0.28	0.02	Maristella	Italy	0.3	0.015
Westbred	USA	0.3	0.1	Valle Zisa	Italy	0.31	0.04
Isa I	Italy	0.32	0.024	Kid	Italy	0.32	0.015
Capeiti	Italy	0.33	0.06	Grifoni	Italy	0.33	0.03
Guasila	Italy	0.33	0.03	Tito	Mexico	0.33	0.2
Giorgio 324	Italy	0.34	0.06	Russello	Italy	0.34	0.036
Frances Ibrido	Italy	0.36	0.04	Giorgio 534	Italy	0.37	0.05
Giorgio 571	Italy	0.39	0.046	Sabato Visco	Italy	0.4	0.08
Pepe	Italy	0.44	0.06	Cresso	Italy	0.52	0.136
Cando	Canada	0.54	0.043	Valgerardo	Italy	0.6	0.1
Triminia	Italy	0.62	0.09	Berillo	Italy	0.74	0.09
Granato	Italy	0.76	0.15	Taganrog	Arg.	0.84	.055
Marzuolo	Italy	0.8	0.19	Nummin	Italy	1.0	0.16
Minitula	Italy	0.89	0.065	Matarasse	Italy	1.77	0.4
MarzuolCervone	Italy	1.22	0.04				

[1] Tyrosinase enzyme activities measured as absorbance at 405 nm or 690 nm over 2.5 hours at 37°C and expressed relative to the absorbance of c.v. Roblin. Results are the mean and standard deviation of measurements on 5 individual kernels of each cultivar. Roblin had a mean absorbance of 0.4 and a standard deviation of 0.04.

Table 5.2 Relative tyrosinase activities[1] in common wheat cultivars

Cultivar	Origin	Act.	St. dev	Cultivar	Origin	Act.	St. dev.
Cadet	Canada	0.33	0.06	Pitic 62	Mexico	0.4	0.04
Little Club	USA	0.41	0.07	Tincurrin	Australia	0.44	0.07
Fielder	USA	0.45	0.07	Meering	Australia	0.46	0.06
Bihar 124	India	0.47	0.08	Bayonnet	Australia	0.49	0.086
Condor	Australia	0.51	0.09	Egret	Australia	0.51	0.09
Reno	Norway	0.52	0.011	Cranbrook	Austalia	0.52	0.09
Marquis	Canada	0.54	0.04	Drabant	Sweden	0.57	0.014
Ralle	Germany	0.57	0.019	Kite	Australia	0.58	0.1
Vernon	Canada	0.58	0.08	Sunkota	Canada	0.59	0.06
Millewa	Australia	0.6	0.06	Eradu	Australia	0.61	0.06
Hyden	Australia	0.61	0.077	RL4555	Canada	0.65	0.15
Sunstar	Australia	0.63	0.06	Tahti	Finland	0.67	0.013
Amy	Sweden	0.67	0.03	Runnar	Norway	0.67	0.036
Oxley	Australia	0.67	0.06	Kenya Farmer	Canada	0.68	0.13
Laval	Canada	0.68	0.08	Prelude	Canada	0.69	0.04
Casavant	Canada	0.69	0.15	Suneca	Australia	0.71	0.04
Sylva	Czech.	0.74	0.047	Monopol	Germany	0.75	0.21
Marshall	USA	0.76	0.11	Cajeme	Canada	0.77	0.07
Niva	Czech.	0.79	0.05	Vega	Czech.	0.79	0.02
Sinton	Canada	0.8	0.1	Owens	Canada	0.81	0.2
Neepawa	Canada	0.82	0.05	Sun 43A	Australia	0.83	0.1
Halbert	Australia	0.85	0.06	Glenlea	Canada	0.85	0.09
Biggar BSR	Canada	0.87	0.15	Ruso	Finland	0.88	0.04
Inia 66	Canada	0.88	0.08	Kadett	Sweden	0.89	0.05
Tapio	Finland	0.89	.037	Canthatch	Canada	0.91	0.2
Wildcat	Canada	0.91	0.14	Genesis	Canada	0.94	0.16
Timgalen	Australia	1.04	0.08				

[1] Tyrosinase enzyme activities measured as change in absorbance at 405 nm or 690 nm over 2.5 hours at 37°C and expressed relative to the absorbance of c.v. Roblin. Results are the mean and standard deviation of measurements on 5 individual kernels of each cultivar. Roblin had a mean absorbance of 0.4 and a standard deviation of 0.04.

Table 5.3. Levels of tyrosinase activity in 5 and 10 seeds incubated in 1 ml of tyrosinase substrate.

Cultivar	5 seeds	10 seeds
Genesis	0.455	1.1
HY361	0.222	0.39
Roblin	0.62	1.4

Table 5.4. Tyrosinase activity in intact seed and in whole meal.

Cultivar	Microtiter plate assay - seeds[1]	Whole meal assay[2]
Genesis	0.606	1.389
HY361	0.344	0.644

[1] Tyrosinase microtitre plate assay on 5 individual seeds presented as absorbance at 405 nm

[2] Results are presented as the absorbance at 405 nm of 100 uL aliquots from the whole meal assay performed on 100 mg of whole meal incubated in 1 mL tyrosine buffer

Germplasm survey

A quantitative microtitre plate assay was used to survey levels of activity in various diploid and tetraploid species of *Triticeae*. These species represent the three genomes of common hexaploid wheat (McIntosh, 1988). The survey showed that accession lines from the three diploid genomes tested carried genes for tyrosinase. In all, nine accessions of *Triticum tauschii* (D genome), four lines of *Triticum monococcum* (A genome) and two lines of *Aegilops speltoides* (B genome) were tested. The data, presented as the mean absorbance of each line relative to that of the cultivar Roblin, are presented in **Table 5.5A**. Of the nine D genome accession lines, five had high levels of tyrosinase activity while four exhibited a complete absence of the enzyme. All the A and B genome accession lines tested had high levels of enzyme activity. In addition, high levels of tyrosinase activity were found in the two cultivars of rye that were tested indicating the presence of a gene on the R genome (**Table 5.5B**). Two cultivars of triticale were tested, with one exhibiting high levels of activity and the other segregating.

Presented in **Table 5.5C** are the results from a survey of four synthetic hexaploids that have a tetraploid parent (AABB) and a diploid parent (*Triticum tauschii* DD). One of these synthetics (RL5710) was a zero tyrosinase line, having the *Triticum durum* cultivar Stewart and the *Triticum tauschii* accession line RL5261 as its parents. The three other synthetic hexaploids had significant levels of enzyme activity.

Chromosome location of tyrosinase gene(s)

A series of substitution lines and other cytogenetic stocks were screened in order to identify the chromosome location of the gene(s) encoding tyrosinase. The mean absorbance values of 2 replicates of either 5 or 10 seeds were analyzed using the REGWF means comparison analysis. For all the data presented means that were not significantly different are designated with the same letter.

Seeds from the wheat-barley addition lines were assayed for tyrosinase activity. These results are presented in **Table 5.6** as activity relative to Chinese Spring. It was found that the addition of chromosome 2 of barley significantly increased the levels of tyrosinase compared to the wheat parent and the other addition lines. The 5H chromosome addition line is not available.

Similar results were obtained when Chinese Spring (Hope) substitution lines were assayed (data not shown). These lines have individual chromosomes from the cv Hope replacing the Chinese Spring chromosomes, consequently information on the specific chromosome can be determined rather than just the chromosome group. Substitution lines having the Hope chromosomes 2A and 2D were significantly higher in tyrosinase activity than the cultivar Chinese Spring. Tyrosinase activity for Langdon (*T. dicoccoides*) substitution lines is presented in **Table 5.7**. The line carrying chromosome 2A had significantly more tyrosinase activity than the other substitution lines.

Table 5.5A - Tyrosinase activity in diploid and tetraploid accession lines

Species and genome	Accession number	Relative[1] activity
Triticum monococcum AA	RL5234	0.74
	RL5235	0.83
	RL5236	1.07
	RL5237	1.19
Aegilops speltoides BB	RL5298	0.86
	RL5299	1.03
Triticum tauschii DD	RL5271	1.45
	RL5495	1.25
	RL5288	1.52
	RL5496	1.21
	RL5261	0.25
	RL5266	0.36
	RL5003	0.31
	RL5499	0.11
	RL5289	0.20
Triticum dicoccum AABB	cv. Vernal	1.08
AADD synthetic	735/5	3.11
	738/L	3.71
T. monococcum X T. carthlicum AAAABB		2.03

[1]Mean absorbance of 5 individual kernels of each accession line expressed relative to the absorbance of c.v. Roblin.

Table 5.5B. Tyrosinase activity in rye, triticale and two hexaploid cultivars and their extracted tetraploid components.

Cultivar	Genome	Tyrosinase activity[1]
Canthatch	AABBDD	0.85
Tetracanthatch	AABB	0.79
Prelude	AABBDD	0.7
Tetraprelude	AABB	0.59 and 0.23
Rye- cv. Rosen	RR	1.08
Rye- cv. Prolific	RR	0.6
Triticale- cv. Carman	AABBRR	1.7
Triticale- cv. Frank	AABBRR	1.7 and 0.36

[1] Mean absorbance of 5 individual seeds and expressed relative to the absorbance of the cv. Roblin.

Table 5.5C. Tyrosinase activity for synthetic hexaploids.

Accession number	Cross	Relative[1] activity
RL 5334	*T. durum* cv. Stewart X *T. tauschii* var. *strangulata* RL 5271	1.31
RL 5435	*T. durum* cv. Hercules X *T. tauschii* var. *strangulata* RL 5271	1.7
RL 5436	*T. dicoccum* cv. Vernal X *T. tauschii* var. *strangulata* RL 5271	3.07
RL 5710	*T. durum* cv. Stewart X *T. tauschii* var. *strangulata* RL 5261	0.25

[1] Activity is expressed as mean absorbance relative to that of cv. Roblin.

Table 5.6. Tyrosinase assay of Chinese Spring (Betzes) addition lines (BAL).

Line	Activity[1]	Score[2]
CS	1	a
Betzes	1.92	b
BAL1	0.77	a
BAL2	1.58	b
BAL3	0.95	a
BAL4	0.96	a
BAL6	0.95	a
BAL7	0.98	a

[1] Activity relative to that of cv. Chinese Spring.
[2] Means with the same letter are not significantly different.

In the assay of Chinese Spring group 2 nullitetrasomics and ditelosomics (**Table 5.8**) the ditelosome for the long arm of chromosome 2D (2DL ditelo) exhibited significantly less activity than the 2DS ditelosomic line. The Nulli 2B tetra 2D line had significantly more activity than the other lines.

Inheritance of tyrosinase activity

Individuals from two F_2 populations segregating for tyrosinase were analyzed for tyrosinase activity using the microtitre plate assay. The F_2 populations segregated into three phenotypes: High, medium and zero (**Table 5.9**). The highs were characterised by the development of black pigment after 3 hours of incubation. The mediums were characterised by the development of a brownish pigment only after 24 hours of incubation at room temperature. After 3 hours of incubation the mediums remained unchanged. The zero lines did not develop the pigmentation even after 24 hours. Both hypotheses had non-significant chi square values. The analysis of F_3 data from both populations is presented in **Table 5.10A** and **5.10B**.

Table 5.7. Tyrosinase activity for *Langdon dicoccoides* substitution lines.

Line	Acitivty[1]	Score[2]
Langdon	1	b
1A	1.13	b
2A	5.73	a
3A	0.67	b
4A	1.2	b
5A	0.87	b
6A	1.67	b
7A	0.80	b
1B	1.00	b
3B	0.67	b
4B	0.67	b
5B	0.67	b
6B	0.67	b
7B	0.67	b

[1] Data is presented as activity relative to cv. Langdon.
[2] Means with the same letter are not significantly different.

Table 5.8. Tyrosinase assay of group 2 Chinese Spring nulli-tetrasomics and ditelosomics.

Line[1]	Mean[2]	Score[3]
CSN2BT2D	1.43	a
CS	1	b
CSN2DT2A	0.93	b
CSN2AT2B	0.9	b
CS2DS ditelo	0.885	b
CS2DL ditelo	0.76	c

[1] CS = Chinese Spring, N = nulli , T = tetra, 2DSditelo = ditelosomic short arm of 2D.
[2] Data is presented as activity relative to cv. Chinese Spring
[3]Means with the same letter are not significantly different.

Table 5.9. Segregation of kernel tyrosinase activity in two F_2 populations: RL5710/Roblin and RL5710/Alpha.

Tyrosinase score	Roblin population	Alpha population
Zero	11	7
Medium	12	9
High	47	27

Population	Ratio	Chi square	df	Prob. = 0.05
Roblin	9 high:3 medium:4 zero	3.67	2	5.99 NS
Alpha	9 high:3 medium:4 zero	1.8	2	5.99 NS
Roblin	9High:4 medium: 3 Zero	3.55	2	5.99 NS
Alpha	9High:4 medium: 3 Zero	0.75	2	5.99 NS

Table 5.10A Segregation ratios tyrosinase activity for the F_3 population from the RL5710 X Alpha cross.

F_2 lines	F_3 Highs	F_3 Mediums	F_3 zeros
Highs	24	5	5
Mediums	0	12	5
Zeros	0	0	28

Trait	Ratio	Chi square	df	Prob. = 0.05
F_2 Highs- F_3 Highs:Med: Zero	25:5:6	0.102	2	5.99 NS
F_2 Mediums- F_3 Med: Zero	5:1	1.99	1	3.84 NS

Table 5.10B Segregation ratios for tyrosinase activity in the F_3 population from the RL5710 x Roblin cross.

F_2 lines	F_3 Highs	F_3 Mediums	F_3 zeros
Highs	34	6	8
Mediums	0	22	7
Zeros	0	0	30

Trait	Ratio	Chi square	df	Prob. = 0.05
F_2 Highs- F_3 Highs:Med: Zero	25:5:6	0.077	2	5.99 NS
F_2 Mediums- F_3 Med: Zero	5:1	1.16	1	3.84 NS

Table 5.11 presents putative genotypes and their corresponding phenotypes for tyrosinase activity for the proposed 9:3:4 segregation ratio. The zeros should all breed true when selfed since the resulting progeny will be homozygous recessive for the first gene (aa) and carry either the dominant or recessive allele of the second gene (bb or B_). The mediums should segregate as 5 mediums: 1 zeros and the selfed highs should segregate into the three phenotypes in the ratio of 25 highs: 5 mediums: 6 zeros.

Approximately 40% of the F_1 and F_2 plants in both populations died due to hybrid necrosis. This reduced the population size considerably, and another 20% of the plants had milder necrosis which resulted in very poor seed set.

Table 5.11. Putative genotypic classifications for tyrosinase

Phenotype ratio[1]	Genotype ratios
4 Zeros	1 aabb : 1 aaBB : 2 aaBb
3 Mediums	1 AAbb : 2 Aabb
9 Highs	1 AABB : 2 AaBB : 2 AABb : 4 AaBb

[1] F_2 phenotypes based on the 9:3:4 ratio.

Molecular markers and linkage analysis

Eighteen probes previously mapped to the *Hordeum* chromosome 2 were used to screen the parents of both crosses in order to identify those showing polymorphisms. Eight of the clones detected polymorphisms between the parental lines. **Table 5.12** lists probes used in this study and indicates the enzyme that the polymorphism was identified with as well as the chromosome location of the polymorphic locus and the source of the probe. To verify that the RFLPs produced by these probes were derived from chromosome 2D or 2A (those involved in coding for tyrosinase) the clones were hybridised to DNA membranes of nullitetrasomic and ditelosomic lines of Chinese Spring wheat (**Figure 5.1**). The restriction patterns indicated that these probes identify loci on chromosomes 2B and 2D. Probes that identified loci on chromosome 2A were not identified.

Linkage analysis of the RFLP loci identified on the short arm of chromosome 2D relative to the high and medium tyrosinase activity was conducted on 39 and 55 F_2 individuals from the Alpha and Roblin crosses respectively. The two RFLP loci identified by the probes ABG019 and B15C, were segregating in a 1:2:1 ratio in both F_2

populations. **Figures 5.2A and 5.2B** show an example of the segregating RFLP pattern obtained with the probes ABG019 and B15C in the F_2 individuals. Data was analysed for linkage to high, medium and zero tyrosinase and the RFLP loci were found to be associated with both the high and medium tyrosinase lines **(Table 5.13)**. The chi square analysis for this association showed significant deviation from the expected 9:3:3:1 ratio for the two markers in both populations except for the probe B15C in the Alpha population where the chi square value was significant at the 0.1 level only. When subjected to linkage analysis using the computer program JoinMap the DNA marker ABC019 mapped 15.5 cM away from the tyrosinase locus on chromosome 2D in both the F_2 populations screened. The B15C locus mapped to 21.3cM from the tyrosinase in the Alpha population and to 15.5 cM from the tyrosinase locus in the Roblin population. The B15C locus mapped to 36.75 cM and 31 cM from the ABC019 locus in the Alpha and Roblin populations respectively which suggests that the two marker loci flank the tyrosinase locus on the short arm of chromosome 2D. LOD scores for the linkage data was greater than 5 for all mapped distances.

Table 5.12. List of probes used in the tyrosinase linakge analysis.

Probe	Polymorphic enzyme (NP= not polymorphic)	Chromosome location[1]	Source of clone
ABG356	E D	2BS	NABGMP
ABG019	H	2DS	NABGMP
B15C	H	2DS	Aalen *et al.* 1994
KSUD022	H, E, B, D	2DL	NABGMP
ABG306	NP	2HS	NABGMP
ABC162	NP	2HS	NABGMP
ABG005	HEBD	2BS	NABGMP
ABG002	NP	2HS	NABGMP
135	NP	2DL	Sharp *et al.* 1988
ABC468	NP	2HS	NABGMP
ABG358	NP	2HS	NABGMP
ABG014	NP	2HS	NABGMP
ABG459	NP	2BS	NABGMP
MWG858	NP	2HS	Graner *et al.* 1991
ABC454	NP	2HS	NABGMP
ABG008	NP	2HS	NABGMP
GLN2	H E D	2DL	NABGMP
22B8	NP	2DS	NABGMP

[1] The location of markers identified as 2HS (Hordeum) was not determined in wheat.

Table 5.13 Linkage analysis between DNA markers on the short arm of chromosome 2D and the tyrosinase locus in the F_2 populations.

DNA marker	F2 population	n	Expected segregation	Segregation[1] T_M_	T_mm	ttM_	ttmm	Chi-square	Recomb. fraction
ABC019	Roblin	55	9:3:3:1	38	7	2	8	15.34 **	0.16
B15C	Roblin	55	9:3:3:1	39	6	3	7	12.77 **	0.16
ABC019	Alpha	39	9:3:3:1	27	5	2	5	8.45 *	0.18
B15C	Alpha	39	9:3:3:1	26	6	2	5	7.51 NS	0.21

** Significant at 0.01 level, * Significant at 0.05 level, for 3 df, P(0.05)=7.92.

[1] T = high or medium tyrosinase activity, t = zero tyrosinase activity, M = marker band present, m = marker band absent.

Figure 5.1. RFLP analysis of Chinese Spring nullitetrasomic and ditelosomic lines for the group 2 chromosomes locating the polymorphic band on the short arm of chromosome 2D. DNA was digested with HindIII and hybridised to the clone ABG019. The chromosome location of each band is indicated on the left.

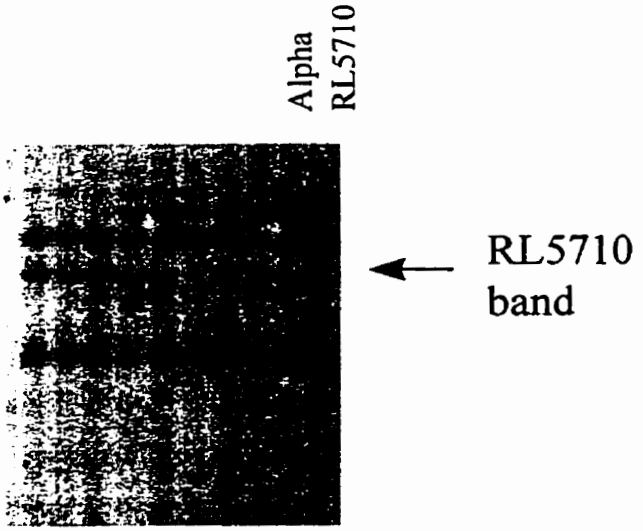

Figure 5.2A. RFLP analysis of F_2 plants and their parents digested with HindIII and hybridised to clone the ABG019. The arrow indicates the polymorphic band on the short arm of chromosome 2D. Lanes 1 to 4 are zero tyrosinase lines and lanes 5 to 10 are high or medium lines.

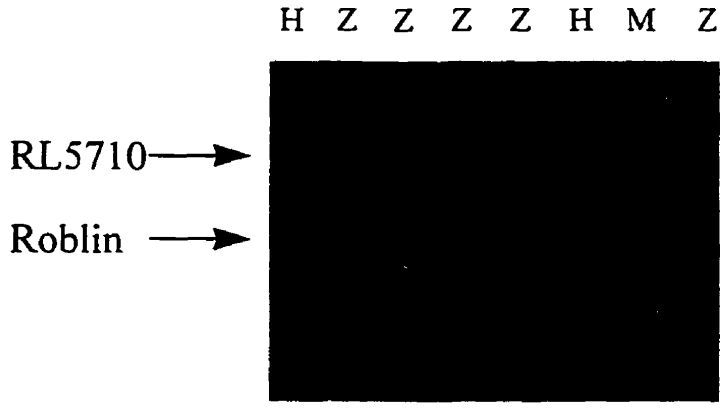

Figure 5.2B. Southern blot of F_2 plants and their parents digested with HindIII and hybridised to clone the B15C. The arrows indicate the parental alleles on the 2DS chromosome. The tyrosinase score is indicated above each lane, Z is zero, M is medium and H is high level of tyrosinase activity.

DISCUSSION

Browning of pasta and white common wheat noodles is due to phenol oxidase and is an undesirable quality characteristic. Since genetic variation of tyrosinase/phenol oxidase activity exists amongst durum and common wheats it should be possible to reduce browning of pasta and noodle products by selection of germplasm having low levels of the enzyme. All currently registered Canadian durum cultivars were found to have very low levels of tyrosinase activity, possibly reflecting a selection for low pasta browning and/or kernel brightness in breeding programs. Italian durum wheat cultivars were found to generally have very high levels of the enzyme and most Canadian common wheat cultivars used for making white noodles had higher levels of activity. This assay could not necessarily be used to distinguish all durum wheats from common wheats as there is a wide overlap of activity levels between the two. None of the hexaploids had very low activities comparable to the Canadian durums, however.

The presence of tyrosinase can affect the colour of many common wheat products that are traditionally prepared from flours milled to higher extraction rates. The simple and rapid microtitre plate assay described here allows a quantitative evaluation of tyrosinase levels in wheat. It is therefore well suited to the large scale screening of tyrosinase in breeding programs for the improvement of white noodle and pasta quality. The test is non destructive thereby permitting the seeds tested to be recovered and grown to maturity. The microtitre plate assay is reproducible and reliable as shown by the low coefficients of variation for replicate means.

The results of the tyrosinase assay on bulks of five and 10 seeds indicates that the assay is linear in terms of increase of absorbance relative to the number of seeds tested. Although the levels of tyrosinase activity in whole meal and in the microtitre plate assay were linear in response (high levels in the microtiter plate assay gave high levels of activity in the whole meal) varietal differences in the microtiter plate assay may occur due to differences in the permeability of the seed coat (Table 5.4).

To maximize milling yields, new cultivars should preferably have low tyrosinase activity to maintain good flour grade colour and be suitable for bread and noodle end-use products. A new cultivar with the absence of this enzyme activity could increase flour yield potential without the risk of containing contaminating bran carrying the tyrosinase enzyme. The identification of the zero tyrosinase synthetic hexaploid wheat RL5710 was important as it can now be used as a source of zero tyrosinase in breeding programs aimed at the reduction or elimination of tyrosinase in hexaploid wheats. This synthetic hexaploid wheat was produced from hybrids between the tetraploid wheat (2n = 28 = AB) cv. Stewart and the *T. tauschii* accession line RL5261 (2n = 14 = D) (Kerber, personal communication). Rather than simply selecting for reduced levels of tyrosinase the complete elimination of this enzyme activity can be achieved. Medium-hard white wheats suitable for the production of alkali noodles in Asia have been identified as a growing market. A new Canada Prairie Spring (CPS) white seeded wheat cultivar with the absence of tyrosinase would be perfectly suited to such a market.

Various accession lines representing the diploid progenitors of hexaploid wheat were assayed for tyrosinase activity to verify the existence of genes for tyrosinase on these genomes. This germplasm survey clearly shows that *Triticum tauschii, Triticum monococcum* and *Aegilops speltoides* all carry genes encoding tyrosinase indicating that a tyrosinase gene could be present on either or each of the genomes of hexaploid wheat. These results contradict findings by Bhowal (1968) who reported that only *Triticum monococcum* and *Triticum boeticum,* both considered to have the A genomes, had high tyrosinase activity. A gene was also present on the genome of *Secale cerealis* (R) since both rye cultivars tested had high levels of activity. Triticale also had high levels of activity with one cultivar segregating for the trait.

TetraCanthatch is the tetraploid (AABB) extraction of the hexaploid wheat cultivar Canthatch. Both these lines had high levels of tyrosinase activity. This would seem to indicate that the A or B genome is involved in coding for tyrosinase. The extracted tetraploid component of the cultivar Prelude produced conflicting results. This tetraploid was found to be segregating for tyrosinase. Seeds from TetraPrelude with high tyrosinase activity and zero tyrosinase activity were grown out and found to breed true to their initial phenotype. This would indicate that the A or B genome may be involved in coding for tyrosinase. The synthetic tetraploid accession lines (AADD) had extremely high levels of tyrosinase activity, suggesting that the D genome may be contributing to the levels produced by the gene on the A genome. The synthetic hexaploid AAAABB also had very high levels of activity perhaps due to the double dose of the A genome.

A number of wheat lines which contain added or substituted alien genetic material have been produced by cytogeneticists taking advantage of the capacity of hexaploid wheat to tolerate aneuploidy. Complete sets of lines have been obtained with whole chromosomes, chromosome arms or segments of arms from other species.

Wheat lines in which single barley chromosome pairs are added were assayed for tyrosinase activity using the quantitative microtitre plate assay. The tyrosinase assay of the wheat/barley addition lines showed that the addition of chromosome 2 of barley to wheat significantly increased the level of tyrosinase activity in the wheat cultivar to that

of the barley cultivar Betzes (**Table 5.3**). The evaluation of the Langdon (*T. dicoccoides*) substitution lines identified chromosome 2A as the location of a gene encoding tyrosinase. The fact that TetraCanthatch and Canthatch have high levels of activity also suggests that the A or B genome must carry a gene for tyrosinase in durum wheats, which do not have the D genome. The evaluation of the Chinese Spring (Hope) substitution lines also identified chromosomes 2A and 2D as the locations of genes for tyrosinase. This contradicts the finding of Zeven (1972) who showed that the 2D Hope sustitution did not increase the level of tyrosinase activity. There may have been an error in the labelling of these lines in either study thereby producing the conflicting reports.

Analysis of Chinese Spring nullitetrasomic lines for the group 2 chromosomes (**Table 5.5**) identified chromosome 2D as the location of a gene encoding tyrosinase with the analysis of the 2D ditesomic lines locating the gene more specifically to the short arm of chromosome 2D. There was a significant increase in activity when the 2B chromosomes were replaced by a pair of 2D chromosomes. This would indicate that the tyrosinase gene on 2D may be the principle gene involved in coding for tyrosinase since the substitution of 2D chromosomes by a pair of 2A chromosomes (nulli2Dtetra2A) did not significantly affect the level of activity.

The results presented here conflict with reports that identified a gene on chromosome 7B (Sadananda *et al.* 1977) and a report of a major tyrosinase gene on chromosome 6B with a modifier on 5B (Bhat and Goud, 1978). Our study suggests the presence of a gene on chromosomes 2A and chromosomes 2D with the gene on 2D being stronger than the gene on 2A.

Initial genetic studies of tyrosinase have shown that tyrosinase is monogenetically inherited and involves multiple alleles in emmer wheats (Joshi and Banerjee, 1969). Bhat and Goud (1978) concluded from their studies that the production of tyrosinase enzyme is conditioned by 2 pairs of independent factors, one dominant, the other recessive, each one capable of coding for tyrosinase. The results of the genetic study presesnted here showed that tyrosinase in wheat is probably encoded by two dominant genes, a strong gene on 2D and a weaker gene on 2A, that interact epistatically. The high tyrosinase phenotype is the

result of the presence of dominant alleles for both the strong and weak genes (A_B_) while the medium phenotype would be the result of the dominant allele of the strong gene only (A_bb). Zero tyrosinase lines would therefore be characterised by the presence of the weak gene (aaB_) or the absence of either of these these (aabb). Analysis of the F_3 populations supported this proposed mode of inheritance. The F_2 zero tyrosinase lines produced only zero tyrosinase lines in the F_3 as expected. The mediums segregated into mediums and zeros and the highs segregated into the three phenotypes in the expected ratios. In the crosses used for the study the zero tyrosinase parents had the double recessive genotype and the high tyrosinase parents had the double dominant genotype. If the zero parent had been aaBB crossed with the high AABB then no mediums would have occurred in the F_2. This absence of mediums in crosses has subsequently been observed when using zeros resulting from crosses with other lines. (Howes, personal communication). Had the hypothesis of 9Highs: 4 mediums:3 zeros been correct the zeros would not have bred true in the F_3 and would have segregated into mediums and zeros. The mediums in this case would have bred true to type in the F_3.

A large portion (approximately 40%) of the F_1 plants showed severe hybrid necrosis. No seed was obtained from these plants. Approximately 20% of the plants that survived displayed a milder form of necrosis where only a few seeds were produced. The necrosis did not appear to be linked to the zero tyrosinase trait as non-necrotic zeros were observed. Hybrid necrosis is the premature gradual death of leaves and leaf sheaths in certain wheat hybrids (Hermsen 1963). It is based on two complementary genes, Ne_1 and Ne_2, located on chromosomes 5B and 2B respectively (Tsunewaki 1992). The degree of necrosis in the F_1's varies greatly due to the occurrence of multiple alleles of Ne_1 and Ne_2. Three general classes of necrosis have been identified. In the first class the F_1 of some crosses is lethal while in the second class termed moderate necrosis, too few and too small kernels are produced. The third group of F1's exhibit only weak necrosis symptoms where most plants survive and set seed. The synthetic hexaploid used in the crosses for the genetics study is derived from the hybridization of the *T. durum* cultivar Stewart and the *Triticum tauschii* accession lines RL5271. The durum cultivar Stewart carries the Ne_2

gene, however the strength of the allele is unknown (Hermsen 1963). The Ne_1 gene must therefore be carried by the two hexaploid wheats used in the crosses (Roblin and the CPS line Alpha). The genotypes from the necrotic hybrids would therefore be Ne1ne1Ne2ne2.

In a study which utilised 14 synthetic hexaploids in crosses with 14 common wheat cultivar, May and Lagudah (1992) found that one-half of the hybrids showed severe hybrid necrosis. In one cross the hybrids were segregating for the presence or absence of hybrid necrosis. The hexaploid parent in this case (cultivar Millewa) must therefore have been heterogeneous for one of the necrosis genes (Ne2 and ne2 in this case) since there were both necrotic and normal F_1 hybrids. In the results presented here we also observed necrotic and normal hybrids within the same F_1 families, therefore the hexaploid parents were probably heterogeneous for Ne1 and ne1. Little information is available as to the hybrid necrosis alleles carried by current cultivars. The experimental line Alpha has the following pedigree: NB406/Era*4/ct932//Glenlea*6/CT932//Verio. The line CT932 has in its background the cultivar Lemhi that possesses the weak allele of the Ne1 gene (Hermsen 1963), therefore it is possible that this gene was carried over into the experimental line Alpha.

Problems have often been encountered when crossing synthetic hexaploids with commercial wheats (May and Lagudah 1992, Cox *et al.* 1990). Some of the deleterious characters encountered in such crosses in addition to hybrid necrosis are dwarf grass clumping, head shape and length variations from almost club to speltoid, and threshability from almost impossible to easy. Plants also varied enormously in height with some measuring up to 2 m. Other morphological traits that were observed in studies with synthetic hexaploids include coleoptile colour varying from green to dark red, head and glume colour varying from white to black, awns that varied from none to fully awned and seed colour varying from white to dark (May and Lagudah 1992, Cox *et al.* 1990). Because of these large variations in morphology and mainly because of the occurrence of the deleterious traits, the direct crossing of *T. tauschii* (DD genome) with commercial wheats has been recommended as being more effective for the introduction of desirable genes rather than producing and intercrossing synthetic hexaploids with commercial

wheats (Cox *et al.* 1990, May and Lagudah 1992). In the study presented here similar deleterious traits were observed with hybrid necrosis being the most severe trait. This trait as well as tenacious glumes did however appear less frequently in the F_3 families and zero tyrosinase lines with normal growth habit have been identified and could be used as sources of zero tyrosinase.

RFLPs were used in our study to tag a tyrosinase locus. This confirmed the chromosome location of one gene on chromosome 2D and identifed markers that could be used for marker aided selection during the development of new wheat cultivars. RFLPs were very well suited to this study since the chromosome location of the genes encoding tyrosinase was identified by the analysis of cytogenetic stocks. Knowledge of the chromosome location before attempting to tag genes permitted us to exploit the RFLP maps that have been contructed in barley, using chromosome specific markers to target specific chromosome locations.

The two RFLP markers which mapped to the short arm of chromosome 2D were found to be flanking the strong tyrosinase gene by 15.5 cM on either side in the Roblin population. Since both the high and medium tyrosinase lines possess the strong gene these were analysed together and the strong tyrosinase gene was found to be associated with both RFLP markers. Although the chi square value for linkage between the tyrosinase locus and the probe B15C was not significant at the 0.05 level for the Alpha population, linkage analysis did map the marker at a distance of 21.3 cM from the tyrosinase locus. The LOD score for this linkage analysis was greater than 3.0.

The clones ABC019 and B15C have been mapped on the chromosome 2H where the loci are located near the centromere at approximately 5 cM from each other (Kleinhoffs *et al.* 1993). This map distance was not conserved in the results presented here. In a comparative RFLP mapping study of the homoelogous group 2 chromsomes in wheat, rye and barley, Devos *et al.* (1993) found that although gene orders were highly conserved in the three genomes the genetic distances between mapped loci varied greatly. A comparison of the map distances between the same loci on chromosomes 2A, 2B and

2D of wheat suggested that chromosome 2D recombines more frequently that either chromosome 2A or 2B (Devos *et al.* 1993).

Since the markers are on either side of the tyrosinase locus, only four plants out of 55 in the Roblin population and five out of the 37 in the Alpha population show the absence of both markers. These would be the results of a double crossover within the ABC019- Tyr and Tyr-B15C intervals. Selecting plants for both markers would give approximately an 8% error rate for tyrosinase selection compared to approximately 15% for one marker alone.

CHAPTER 6
GENERAL DISCUSSION

The quality of wheat flour is affected by the soundness of the grain, colour of the pericarp, milling method and extraction rate, presence of contaminants and tyrosinase activity (Baik *et al.* 1994). Genetic studies of two traits associated with flour quality were undertaken in this project; dormancy which influences the level of preharvest sprouting and the enzyme tyrosinase which is responsible for the discolouration of end-use products. Different approaches were used in the studies which had the ultimate goal of identifying the number of genes involved in coding for the traits and identifying molecular markers associated with these genes.

Two dormancy genes were identified in segregating populations having the white seeded genotype RL4555 as the dormant parent. The usual method of evaluating dormancy involves the testing of plants grown under several different environments because dormancy is influenced by the conditions under which the grain matures. The methodology presented here standardizes the growing conditions and allows the separation of individual genes based on their expression at different temperatures. The testing protocol allowed the identification of lines carrying either or both genes by testing for germination at two different temperatures. The results of the study indicate that the incorporation of these two genes may be sufficient to increase the level of preharvest sprouting resistance to that found in the genotype RL4555. The characterized segregating populations were used for the development of molecular markers associated to either or both genes. A search of the group 2 chromosomes using markers from the barley RFLP linkage map (Chapter 4) showed that a chromosome by chromosome search for molecular markers is a very ineffectual approach to tagging specific genes when the location is unknown. The level of polymorphism associated with the mapped RFLP markers limited the identifiable loci to small areas of chromosomes 2BL and 2DS. Polymorphic group 2 markers were not associated with the dormancy genes. Random

amplified polymorphic DNA (RAPD) markers were better suited to this study because the chromosome location of the genes was not known and existing chromosome maps could not effectively be exploited. A RAPD marker was found to be linked to one of the dormancy genes by 25.74 cM. This is considered loose linkage and such a marker would be of limited use to plant breeders. The chromosome location of this marker was investigated using the RAPD band as a probe on DNA from different cytogenetic stocks. The location of genes to chromosomes is part of the process used to identify and characterize a gene. It makes mapping easier and allows the efficient determination of linkage with markers already mapped to the same chromosome. The location of the dormancy marker could not however be determined as it contained repetitive DNA. The identification of the chromosome location of the marker would have allowed us to use the chromosome linkage maps to target the specific location of the dormancy gene. Dormancy is a trait for which the application of molecular markers would be very useful because the evaluation of the trait itself is difficult and time consuming.

The majority of Canadian durum cultivars have low levels of tyrosinase activity while most Canadian common wheat cultivars have high levels. In a survey of cytogenetic stocks a synthetic hexaploid that had no tyrosinase activity was identified. This synthetic was used as the zero tyrosinase parent to produced two segregating populations in which two genes encoding tyrosinase were subsequently identified. The genes interact epistatically with one being stronger than the other. They are located on chromosomes 2A and 2DS as determined by the analysis of alien substitution and addition lines and nullitetrasomic lines. Since the chromosome location of the genes was determined by genetic analysis it was then possible to exploit the barley chromosome 2 linkage map. RFLP markers flanking the strong tyrosinase gene on chromosome 2DS were identified. Taken together these markers would have a selection error rate of 8%.

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IMAGE EVALUATION
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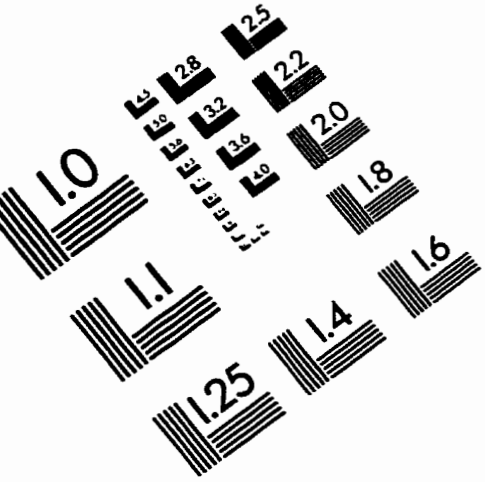

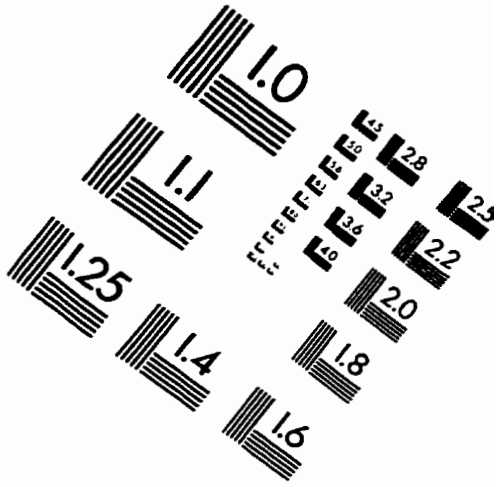

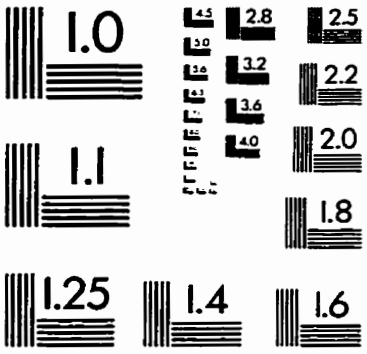

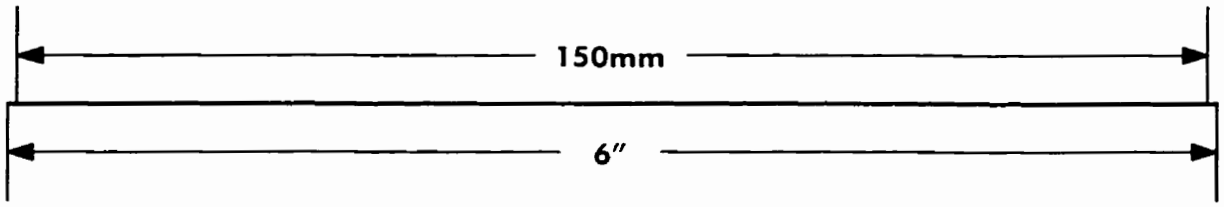

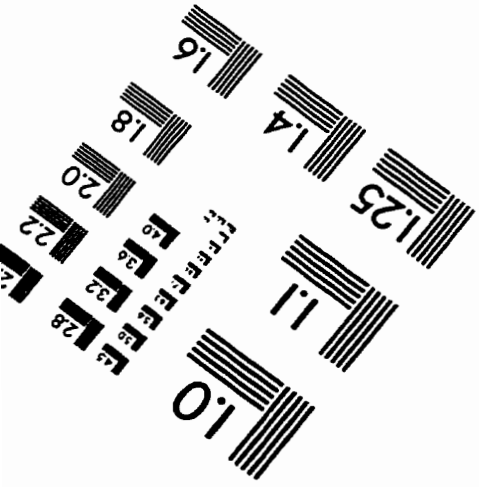

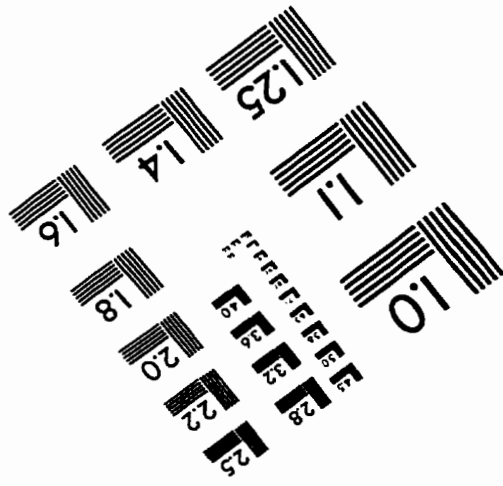

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