The genetic improvement of wheat and barley for reproductive frost tolerance

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Chapter 1: Literature Review and Introduction to the Research

Introduction

The production of cereals, belonging to tribe *Triticeae*, extends from countries close to the polar circle to the equator in both high and low altitudes. The plant growth cycle is completed under a range of environmental conditions imposing abiotic stresses at various developmental stages. Low temperature is one of the major abiotic stresses faced by winter cereals and is a significant limiting factor on world production and distribution. Sub zero temperatures that promote ice formation in plants are often referred to as 'frost' events which can cause damage to plant cells significantly reducing yield.

Investigations into the physical, genetic and biochemical basis of frost tolerance at the vegetative stage have been extensive. In contrast very little is known about the tolerance to frost at the reproductive stages, hindering efforts to reduce the impact of the stress in Australia's major cereal production zones.

From a plant breeder's perspective, stress tolerance is the ability of a plant to maintain a high level of yield when grown under a range of 'stressful' conditions. Breeding for stress tolerance is aided by knowledge of sources of genetic variation, numbers of genes influencing the trait, and the effect of each gene and their interactions. An increased understanding of the genes controlling reproductive frost tolerance in cereals will aid plant breeders to incorporate this trait into Australian adapted varieties and potentially reduce the economic impact of this stress on Australia's cereal production industry.

Economic impact of frost damage

Damage at the vegetative growth stage (before the initiation of the floral primordia) is the most widespread type of frost damage and is a production constraint predominantly in cereal producing nations of the Northern hemisphere. In southern Australia, cereal production is centred on a winter dominant rainfall. Cereals with spring growth habit are planted in autumn with the majority of the growing season over winter. Winter temperatures are not low enough to cause considerable frost damage at the vegetative stage of development during these months. The predominant frost damage occurs from low temperatures (<-2^{o}C) in spring during the reproductive stages of cereal development. These frost events can cause floret and spike abortion as well as damage to the developing grain which can have a significant impact on yield and quality. This type of damage causes extensive economic losses to cereal crop production in Australia. Production is not only directly affected by reduced yield and quality but also indirectly, as a result of losses arising from strategies used to avoid frost events. Farmers in frost prone areas will delay seeding to reduce the risk of a frost event occurring during the most susceptible flowering stages. In southern Australia, for every 1 day that seeding is delayed past the optimal seeding date reportedly results in 1% decrease in yield, by postponing grainfill further in to late spring when heat and water stresses are more commonly encountered (McDonald *et al.*, 1983).

Cellular basis of cold and freezing damage

Low temperature can stress a plant in two ways – either through the direct effects of freezing, or through effects of low temperature in the absence of freezing. The effect of low, non-freezing temperatures on a plant cell can cause a reduction in efficiency of cell function. Low temperature can reduce membrane fluidity resulting in the inactivation of membrane-resident ion pumps (Huner *et al.*, 1993). As photosynthetic cells in plants accumulate energy after absorption of light, membrane bound ion-pumps cannot displace

the energy build up resulting in damage through oxidative stress. This type of stress can occur in tropical and subtropical plants at temperatures above 0^{o}C. For winter cereals, this type of low temperature stress occurs during a frost event in which the temperature drops below 0^{o}C but the plant cells remains unfrozen in a supercooled state (Huner *et al.*, 1993).

Most damage is caused when ice forms in plant tissues. As the temperature is lowered, ice first begins to form in the intercellular spaces. This lowers the water potential outside the cell, causing net movement of water from the apoplasm into the intercellular space (Campbell and Close, 1997). Cell death results when the dehydration tolerance of the cell is exceeded. Rapid dehydration of the cell can also cause lysis and loss of membrane function. Physical disruption of the plasma membrane by large ice crystal formation has also been reported (Campbell and Close, 1997). This usually occurs when ice forms in the intracellular space under extreme cold stress conditions.

Susceptibility of plant structures to frost

The susceptibility of cereal plants to freezing temperatures varies with growth stage (Figure 1). Different organs also have varying sensitivities to cold. Individual organ types, particularly those of reproductive nature, are more sensitive than others. These organs can also vary in susceptibility at different stages of their development (Saulescu and Braun, 2001). The point at which a plant will change from one developmental stage to the next depends on the environment and the developmental genes it possesses to respond to the environment. The main genes that control the changes in developmental phase can be grouped into 3 main types; earliness *per se*, photoperiod response and vernalisation response (Laurie *et al.*, 1995a). Earliness *per se* is the basic vegetative phase a plant must go through before it will undergo reproductive transition. It is driven by temperature and time and is often measured in degree days. Photoperiod response and vernalisation response are the

two main mechanisms that control the transition from vegetative to reproductive phase in cereals. Vernalisation response is the requirement of a plant to be exposed to a period of cold temperature before its will initiate flowering. Different genotypes can have a range of sensitivities to vernalisation depending on the number and type of vernalisation responsive genes they posses. Photoperiod response is the sensitivity of a plant to long day length. Short days will delay flowering in photoperiod sensitive plants producing more leaves and tillers as it will extend its vegetative growth. Photoperiod insensitive plants will not be delayed by short days and will initiate reproductive growth as soon as the vernalisation requirements are met. Once both photoperiod and vernalisation requirements are met, and the vegetative growth stage ends, cereals gradually become less frost tolerant as the plant develops through the reproductive growth stage (Mahfoozi *et al.*, 2001). Depending on the stage of plant development when a frost event occurs, the resulting damage can have little to severe impact on overall crop production.

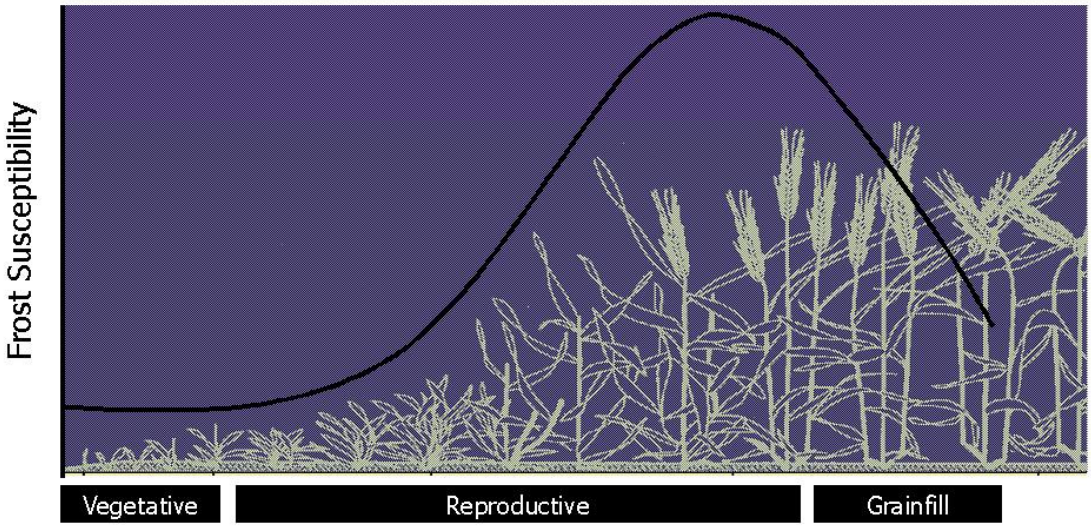


Figure 1- Frost susceptibility of cereals at different developmental stages. Adapted from Slafer and Rawson (1994), White (2000) and Zadoks *et al*. (1974).

Frost damage at the vegetative stage

During the vegetative stage, meristems are close to the soil surface and therefore relatively protected from frost events. Leaf tissue can be damaged at temperatures of –8 ^{o}C to - 11 ^{o}C (Fowler, 1983). Symptoms include chlorosis of leaves, leaf tip necrosis and regions of water-soaked appearance resulting from membrane damage and leakage of cellular contents. The impact of leaf damage during the vegetative growth stage on overall production can be low to moderate, depending on whether the plant can recover and produce more leaves. At temperatures of -15 ^{o}C to -25 ^{o}C, the buffering capacity of the soil loses its ability to protect the growing point from freezing (Fowler, 1983). At this temperature the soil freezes and young plants die leading to total crop loss.

Crop damage at the reproductive stage

During stem elongation, the sensitive growing point leaves the protection of the soil surface. Although the growing point has protection within the leaf sheath, temperatures as high as -5 ^{o}C can cause irreversible damage (Single, 1988). While a healthy growing point is bright yellow-green and turgid, a freezing damaged one will become white or brown and have a water-soaked appearance. Frost damage at this point can be moderate to severe, depending on the developmental stage of secondary tillers.

At the booting stage, the developing spike is enclosed by the leaf sheath which provides a level of protection from frost damage. At this stage the anthers and stigma, which are the most sensitive organs, are developing. Moisture can collect within the boot above the last node and freeze at temperatures as high as -2 ^{o}C (Single, 1988). This can irreversibly damage the stem, preventing water and nutrient flow to the developing spike. Symptoms include yellowing and cracking of the stem above the last node and white heads emerging

from the boot. This form of damage can have a moderate to severe impact on final crop production.

At the head emergence stage, several different tissues can be affected by frost. Stem damage can be common, but with the sensitive anthers and stigma exposed, these tissues are the most likely to be affected. Frost induced floret sterility can occur at temperatures as high as -1 ^{o}C (Single, 1988). Healthy anthers are usually green to yellow in colour (depending on their maturity) and turgid. Frost damaged anthers become shrivelled and greyish in colour. The stigma is white and feather-like when healthy but will become water-soaked in appearance and turn brown after frost damage. Frost induced sterility can have a severe impact on final production because the number of spikes and florets has been determined by this stage and no further compensation can be made for loss of grain set.

At the early grainfill stage, frost damage can stop grain development causing a shrivelled pinched appearance at maturity. Grain contents will become watery and greyish in colour when damaged at the early milk stage, instead of white and viscous as in a healthy grain. Frost damaged wheat and barley grains have reduced milling and malting quality and are subject to quality downgrading when delivered into the elevator system in Australia and attract a discounted price. A temperature of -2 ^{o}C can damage the grain and have a moderate to severe impact on production (Saulescu and Braun, 2001).

Frost tolerance in cereals

The Triticeae tribe have evolved two main mechanisms to overcome low temperature stress. One of the mechanisms is to avoid having sensitive tissues exposed during the times when frost events are prevalent. The avoidance mechanism which prevents the switch from vegetative to reproductive growth is called vernalisation response. Genotypes which carry

vernalisation responsive genes (winter types) need an extended period of cold before they will switch from vegetative to reproductive development (Takahashi and Yasuda, 1971). In environments with extended winters and sub zero conditions, winter cereals will remain at a vegetative growth stage until the onset of spring when frost damage to sensitive reproductive tissue is less likely. Two major loci control vernalisation in wheat and barley, *vrn1* and *vrn2*. The genes at both of these loci have been cloned from diploid wheat (Yan *et al.*, 2004b, Yan *et al.*, 2003). *Vrn1* encodes a MADS-Box transcription factor required for flowering. The dominant *Vrn1* spring allele is transcribed regardless of vernalization, while the recessive *vrn1* winter allele is transcribed only after vernalization (Yan *et al.*, 2003). *Vrn2* encodes a zinc finger-CCT domain transcription factor which is down-regulated by vernalisation and which is hypothesised to act as a negative regulator of *vrn1*, binding the promoter and/or intron 1 region only of *vrn1* winter alleles but not that of *Vrn1* spring alleles (Yan *et al.*, 2004b, Fu *et al.*, 2005). The *vrn2* locus has dominant winter alleles (*Vrn2*) and recessive spring alleles (*vrn2*). The two loci are epistatic, with flowering being influenced by *vrn2* locus genotype only in the presence of the homozygous *vrn1* winter allele.

Plant breeders have attempted to reduce the impact of frost mainly by focusing on improving tolerance (rather than by manipulating vernalization requirement). The ability of a plant to resist cold stress is influenced by cold accumulation (Fowler, 1996). Cold accumulation or hardening occurs when plants are exposed to low but non-freezing temperatures prior to frost exposure (Hayes *et al.*, 1993). Compounds observed to accumulate during cold acclimation are believed to serve a protective function against subsequent frost exposure (Andrews *et al.*, 1973)

Genetic control of frost tolerance

Major tolerance to cold stress in winter cereals appears to have co-evolved with the vernalisation response frost avoidance mechanism. It has been well documented that genotypes of wheat, barley and other winter cereals that have winter growth habit (i.e. require vernalisation to flower) are more tolerant to cold stress than those with spring growth habit (Sutka, 2001, Pan *et al.*, 1994, Toth *et al.*, 2003). One of the frost tolerance loci that explains a high proportion of genetic variation observed in wheat and barley has been designated *Fr1*. All previously published studies of this locus have focused on frost damage to the vegetative tissues.

Genetic control of vegetative frost tolerance in barley

Hayes *et al.* (1993) mapped genes controlling vegetative frost tolerance in a barley doubled haploid mapping population derived from the frost tolerant cultivar Dicktoo and frost susceptible cultivar Morex. The mapping study was completed using field survival data from Oregon and Montana, and LT50 (lethal temperature where 50% of plants survive) measured in a temperature controlled growth cabinet. A region on chromosome 7(5H), also containing the *vrn-H1* vernalisation response locus, was found to contain QTL for both field based and controlled environment cold tolerance measurements. This locus was designated *Fr-H1* (Figure 2). Francia *et al*. (2004) identified two QTL for frost tolerance in a population derived from the frost tolerant winter barley cultivar Nure and the frost susceptible spring barley cultivar Tremois. One QTL was coincident with the *vrn-H1* locus, and may be the same as *Fr-H1* identified by Hayes (1993). In neither study was recombination observed between the *vrn-H1* locus and the frost tolerance locus. A second QTL identified by Francia *et al*. (2004) was located proximal to the *vrn-H1*/*Fr-H1* locus, and was designated *Fr-H2* (Figure 2). In this population the two QTL had an additive effect on frost tolerance, with positive alleles contributed by the winter parent Nure.

Genetic control of vegetative frost tolerance in wheat

The group 5 chromosomes in wheat have also been found to influence vegetative frost tolerance. Loci for traits associated with vegetative frost tolerance have been mapped to corresponding locations on chromosomes 5A, 5D (Sutka, 2001) and 5B (Toth *et al.*, 2003), in regions corresponding to *Fr-H1*/*Vrn-H1* in barley as well as a second locus distal to *Fr1*/*Vr1* now designated *Fr2* (Snape *et al.*, 2001) (Figure 2). Sutka *et al.* (1994) mapped *vrn-A1* and *Fr-A1* loci using recombinant substitution lines made from a cross between the frost sensitive spring line 'Chinese spring (T. spelta 5A)' and the frost tolerant winter line 'Chinese spring (Cheyenne 5A)'. A single recombinant was identified in this population, carrying the winter *vrn-A1* allele and the frost susceptible *fr-A1* allele. Interestingly, the genetic location of the *Fr-A1* gene was reported as being located proximal to the *vrn-A1* gene in this paper and distal to the *vrn-A1* gene in the paper published by Sutka (2001). There has been a question raised over this single recombinant between the *vrn-A1* and *Fr-A1* loci, something that has not been reported since (J. Dubcovsky, personal communication, 2004). Sutka (2001) also identified a second frost tolerance locus on chromosome 5D using a 'Chinese Spring' × Chinese Spring (Cheyenne 5D) segregating population. *Fr-D1* (which is now designated *Fr-D2*) was mapped 10cM proximal to the *vrn-D1* locus on chromosome 5D, confirming that these two loci are linked but distinct. Toth *et al.* (2003) mapped the *Fr-B1* locus (now designated *Fr-B2*) on chromosome 5B. A population was developed from a cross using the frost susceptible line 'Chinese Spring' and the frost tolerant line 'Chinese Spring (Cheyenne 5B)'. The *Fr-B2* mapped approximately 41cM proximal to the *vrn-B1* locus.

In all cases, vegetative frost tolerance was mapped using field and controlled environment screening methods. Only a single QTL was identified in each of the populations, linked to

the *vrn1* locus either in coupling (*Fr1*) or separated by recombination (locus now designated *Fr2*). Also, unlike the barley mapping studies, the *vrn* and *fr* genes were separated by at least one recombination event in most of the populations studied.

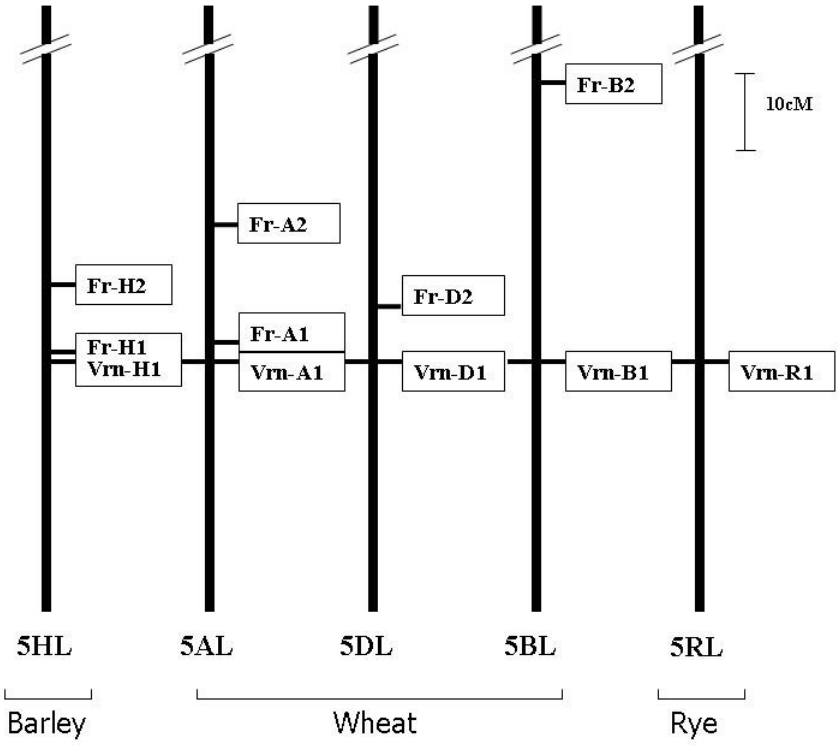


Figure 2 – Schematic diagram of syntenous *vrn1/fr* regions on group 5 chromosomes in wheat, barley and rye. Adapted from (Borner *et al.*, 2002, Sutka, 2001, Toth *et al.*, 2003, Francia *et al.*, 2004).

Reproductive frost tolerance in cereals

Since the 1930s, attempts have been made to identify genetic variation for reproductive frost tolerance in wheat and incorporate the associated genes into commercially relevant adapted material with very limited success (Marcellos, 1988). The unreliability of previous field

based screening methods due to confounding factors such as spatial variation, seasonal variation and overriding maturity effects, has made progress slow (Single, 1988). Single (1988) collected a set of genetic material from a number of locations around the world that experienced similar spring radiation frost events to Australia. He also collected winter growth habit/vegetative frost tolerant wheat lines to include in a radiation frost screening nursery. He concluded that the vegetative frost tolerant lines were so late in heading and maturity that they avoided the spring frost events and were of no use in Australian conditions (Single, 1988). He was also unable to identify any spring or facultative growth habit wheats that showed any useful increased level of reproductive frost tolerance.

Freezing tolerance genes and mechanisms

As the majority of studies into freezing tolerance have focused on the ability of a plant to cold acclimate under cold but non-freezing conditions, the effort to isolate genes associated with freezing stress has been largely restricted to those that are induced during this acclimation process.

The dehydrin family of genes are among the genes most strongly induced during cold acclimation and also accumulate in response to ABA application, drought and salinity (Campbell and Close, 1997). The biochemical role of dehydrins is yet to be determined. Choi *et al.* (1999) sequenced and genetically mapped 11 dehydrin genes in the Dicktoo × Morex barley mapping population. Three dehydrin genes, *Dh1*, *Dhn2* and *Dhn9*, mapped to a cluster on the long arm of chromosome 5HL. These genes have also been located on the group 5 chromosomes in wheat (Cattivelli *et al.*, 2002a). Recombination events in both wheat and barley have shown that the *Dhn* genes are separate to the frost tolerance locus on group 5 chromosomes in triticeae. Other dehydrin genes have been assigned to

chromosomes 3H, 4H and 6H in barley but are not coincident with any other frost tolerance locus detected in wheat or barley mapping studies (Choi *et al.*, 1999).

The C-repeat Binding Factor (CBF) family of genes are transcription factors that are inducible by cold, dehydration and salt stress (Choi *et al.*, 2002). They promote the transcription of genes having a Dehydrative Responsive Element (DRE) in their promoters (Choi *et al.*, 2002), and several have also been shown to regulate COld-Regulated genes (COR). Galiba (2001) mapped *Cor14b* on the long arm of 2A in diploid wheat, and a QTL regulating *Cor14b* expression on chromosome 5A. Vagujfalvi *et al.* (2003) mapped *CBF3* in the middle of the regulatory QTL on 5A, identifying it as a candidate for the gene underlying the QTL effect.

COR genes are induced by abiotic stresses such as drought and cold (Galiba, 2001). Up to 20 genes induced by cold acclimation conditions have been identified in barley (Cattivelli *et al.*, 2002b). *COR* mRNA's usually reach peak levels 2-3 days after the beginning of cold exposure and become less abundant within a few hours of returning to non-acclimation temperatures. Although the accumulation of *COR* mRNAs correlates with cold hardening, the function of these genes is still unknown. It has been speculated that *Cor14*, one of the proteins most strongly induced by cold, has a role in cold hardening plants because it only accumulates in wheat and barley at a well defined minimum temperature (Galiba, 2001).

Concentrations of cell osmolytes have been correlated with resistance to osmotic stress. It is generally accepted that osmolyte accumulation reduces the efflux of cell water under dehydrative stress and enables cells to maintain cell turgor (Beck *et al.*, 2007). The main osmolytes found to accumulate under dehydrative stress include the soluble carbohydrates sucrose and fructan, the amino acid proline and the dipolar ion glycine betaine. These osmotically active solutes are likely to act as cyro-protectants by reducing cell dehydration

during extracellular ice formation (Thomashow, 1990). A locus controlling sucrose accumulation has been mapped close to the *vrn*-A1 locus in wheat (Galiba, 2001). Crown fructan accumulation was mapped the long arm of chromosome 5H in barley, coincident to the *Fr-H1*/*vrn-H1* locus by Hayes (1993). Although it can be assumed that these osmolytes provide dehydrative stress tolerance there is no evidence that the increased osmotic potential these osmolytes provide is the single contributing factor to the observed increased cold tolerance in cold tolerant genotypes.

Molecular genetic tools for gene discovery

The discovery, characterization, and exploitation of agriculturally important traits is critical for fundamental understanding of plant biology. Stress tolerance genes are important in an agricultural sense as they contribute to yield stability under a range of production environments. The cloning of stress responsive genes may provide insights into gene function on a fundamental level and a starting point for studying regulatory mechanisms and gene-gene interactions. The genetic tools and information becoming available to assist in isolating and manipulating economically important genes may enable the engineering of stress resistance to levels not achieved by naturally observed genetic variation.

Many of the world's most economically important crop species are from the Poaceae family including maize, wheat, barley, sugarcane and rice. The complete sequencing of larger plant genomes such as barley (5,000 Mb) and wheat (16,000 Mb) has not yet been practical. Although the lineages leading to cereal crop species diverged almost 65 Million years ago, comparative mapping studies have revealed that gene order conservation between these species is very high (Sorrells *et al.*, 2003). The relationship between chromosomes from different grass species enables the use of one species as a template for comparison to other Poaceae species. Rice serves as such a model species, as it has a relatively small diploid

genome of approximately 430Mb which has been sequenced (Sasaki, 2005). The small genome size of rice is mostly due to a reduced content of repetitive DNA sequences.

Another potentially useful model species is *Brachypodium sylvaticum*, a diploid species with a genome size similar to rice (Foote *et al.*, 2004). *Brachypodium sylvaticum* diverged later than the tropical grasses such as rice and just prior to the temperate cereals such as wheat and barley. Genes that evolved post this divergence are more likely to be present in the *Brachypodium* genome.

Break down in co-linearity between the Poaceae species has been reported on several occasions. The rearrangement of genes has been attributed to insertion/deletion of transposable elements, deletion, insertion, inversion, duplication of genes as well as gene movement (Scherrer *et al.*, 2005). This observed rearrangement has not only occurred between species but between genotypes within a species of the Poaceae family indicating a more recent evolutionary event. Most reported genes showing lack of conservation are those of secondary importance, such as disease resistance genes. An example of this is the *Rpg7* gene in barley. Scherrer et al (2005) found that there was no conservation of the genomic region containing the *Rpg7* gene in several genotypes of barley and concluded that this was a rapid and recent divergence in the barley genome.

A tool to aid in gene discovery in large genome crop species is the development of Expressed Sequence Tag (EST) databases. ESTs are normally partial sequences of cDNA clones representing transcribed mRNAs. As the expressed part of the genome in the family Poaceae is highly conserved, it is possible to use the EST sequences from the target species to search the full genome of a template species (such as rice) to investigate coliniarity in the target genomic region (Sorrells *et al.*, 2003). In efforts to tag and isolate genes of interest from large genome cereals, SNP-based PCR markers can provided an efficient means of

mapping genes that have been predicted from the rice genome sequence to be present in particular chromosomal regions.

SNPs are the most abundant forms of sequence variation in most genomes (Kota *et al.*, 2003). SNP frequency has been reported from around 1/60 to 1/600 base pairs in various crop species but can vary significantly depending on the genotype, genomic location and species relatedness (Kota *et al.*, 2003). This variation provides an opportunity to develop a high frequency of SNP based markers for fine mapping. The almost limitless number of SNP's coupled with new technologies in DNA extraction and electrophoresis have progressed to a point where high throughput genotyping is possible to saturate genetic regions of interest and to identify rare recombination events in large mapping populations.

Bacterial Artificial Chromosome (BAC) libraries have provided a useful tool for characterising genomes. Physical mapping, structural analysis and map based cloning can be aided with the availability of a large insert genomic library of the crop species of interest (Foote *et al.*, 2004). The barley cv. Morex BAC library was constructed with a 6.3x coverage, allowing a 99% probability of recovering any specific sequence of interest (Yu *et al.*, 2000). The barley cv. Haruna Nijo BAC library has also recently been constructed with similar coverage as the Morex library (Saisho *et al.*, 2002). One of the main applications of these barley BAC libraries is positional cloning of genes controlling traits of interest. Several disease resistance genes have already been isolated using these genomic resources. Sequencing BACs containing the gene of interest after probing with markers in the genomic region containing the desired gene, it is possible to obtain the full gene sequence information. This can usually only be achieved when the genomic region that contains the gene of interest is narrowed to a relatively small genetic distance via fine mapping using large populations.

Conclusion

The research into cold tolerance at the vegetative stages of cereal development has revealed that genetic variation does exist and that the trait is controlled by few genetic loci with major effects. The current understanding of the underlying mechanisms of cold tolerance is limited. New molecular genetics technologies will help improve our understanding of the genetic control of cold tolerance and provide selection tools for breeding.

Research Aims

The proposed study aims to investigate the genetic control of reproductive frost tolerance in barley and to use this knowledge, coupled with the understanding of gene co-linearity between members of the family Poaceae, to investigate the genetic control of frost tolerance in wheat. It is also aimed to design and implement a reproductive frost tolerance breeding strategy that incorporates the latest technologies to efficiently introgress the target loci. The ultimate aim of this study is to provide plant breeders with germplasm, knowledge and tools that will be useful in delivering varieties with improved yield stability in frost prone environments.

Specific aims addressed within each chapter

Aim 1: Identify and characterise genetic variation for reproductive frost tolerance in barley

Chapter 2: QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.)

Description: Identification of genetic variation, development of screening methods and mapping QTL for reproductive frost tolerance in barley.

Chapter 4: Fine mapping the reproductive frost tolerance locus on chromosome 5HL in barley

Description: Development of populations that can be used to map the 5HL reproductive frost tolerance locus and maturity locus at higher resolution than in Chapter 2. Development of molecular markers for refining the map locations. Phenotype the populations for reproductive frost tolerance and days to head emergence to better define the location of the genes controlling these loci.

Aim 2: Determine if wheat genomic regions syntenous to the barley 5HL reproductive frost tolerance locus affect reproductive frost tolerance.

Chapter 3. The effect of the *Vrn1* genes on reproductive frost tolerance in hexaploid wheat

Description: Testing of associations between *vrn1* alleles and reproductive frost tolerance in two sets of hexaploid wheat germplasm.

Aim 3: Develop and execute a breeding program to rapidly introgress reproductive frost tolerance into Australian adapted barley germplasm.

Chapter 5. A strategy to rapidly introgress frost tolerance genes into adapted barley germplasm

Description: Design and implement a breeding strategy to rapidly introgress identified reproductive frost tolerance loci into Australian adapted germplasm

Chapter 6. Evaluation of the RFT barley breeding strategy

Description: Evaluate the developed germplasm for reproductive frost tolerance and adaptation to barley growing areas in Australia.

Reinheimer, J.L., Barr, A.R. and Eglinton, J.K. (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (Hordeum vulgare L). *Theoretical and Applied Genetics, v. 109 (6), pp. 1267-1274, October 2004*

Chapter 3. The effect of the Vrn1 genes on reproductive frost tolerance in hexaploid wheat

Introduction

Genetic linkage between *vrn*-H1 and Reproductive Frost Tolerance (RFT) has been established in barley (Chapter 2). Homoeologs of this major developmental locus in barley have been identified in diploid, tetraploid and hexaploid wheat at the syntenous locations on the group 5 chomosomes in the A, B and D genomes (Yan *et al.*, 2004a). These loci in wheat have been reported to control vernalisation response and to be linked to frost tolerance at vegetative stages of development (Galiba, 1995, Koemel *et al.*, 2004, Reddy *et al.*, 2006, Snape *et al.*, 2001, Sutka, 2001, Toth *et al.*, 2003). The vernalisation response genes on chromosome arm 5AL in wheat and 5HL in barley have been isolated, revealing that the genes and gene products are very similar. Homologues to this gene have also been identified on chromosomes 5BL and 5DL in hexaploid wheat (Fu *et al.*, 2005). The 5L chromosomal region on the H genome of barley, and the A, B and D genomes of wheat show strong coliniarity, indicating a high level of conservation in this region among wheat, barley and other species from the tribe *Triticeae* (Lindelaursen *et al.*, 1997).

In bread wheat, winter growth habit has been associated with frost tolerance at the vegetative stage of development. The effect of the 5L chromosome regions carrying the *vrn*1 winter alleles on frost tolerance in bread wheat has been studied at the vegetative stage of development on a set of isolines containing a cv. Dirk background (Koemel *et al.*, 2004). Not all winter alleles of the group 5 vernalisation response genes contribute equally to tolerance to frost, with *vrn*-A1 contributing the highest level of frost tolerance, followed by *vrn*-D1 and *vrn*-B1. The three wheat *vrn*1 loci also have an unequal effect on vernalisation response. The *vrn*-A1 locus has the largest effect on vernalisation response and also shows

higher transcript levels when compared to *vrn*-B1 and *vrn*-D1 when cold treated (Loukoianov *et al.*, 2005). The Triple Dirk isoline set represents only 5 out of the possible 8 combinations of alleles at the 3 *vrn*1 loci, meaning that some combinations of winter and spring alleles were not tested for their contribution to VFT.

The majority of Australia's wheat production is from varieties with a spring growth habit (Brougham 2006). Prospects for utilizing the *vrn*1-associated source of frost tolerance in spring wheat breeding will be limited if the RFT is a direct effect of the winter allele or winter growth habit. The association of RFT with the winter allele on chromosome 5H in barley can be overcome by the use of the spring allele at *vrn*-H2 which produces a spring phenotype irrespective of *vrn*-H1 locus genotype. As the functional allele of *Vrn*2 is a dominant repressor of flowering, a spring allele would need to be present on all three genomes in wheat to have the same effect as in barley. Spring alleles have not yet been identified for all 3 *Vrn*2 loci of hexaploid wheat (J. Dubcovsky, personal communication, 2004).

A recent study by Brougham (2006) indicated that the winter alleles of all three *vrn*1 loci are present in Australian hexaploid wheat germplasm. Only the triple winter genotype was not represented in mainstream cultivars. The other seven combinations of vernalisation alleles were present. For example, the cultivar Wyalkatchem which is one of the most broadly adapted and widely grown 'spring' cultivars in southern Australia, has the winter allele at the *vrn*-A1 and *vrn*-D1 loci and the spring allele at *Vrn*-B1 but flowers at an earlier time than most other mainstream cultivars (Brougham, 2006). If frost tolerance at the reproductive stage can be demonstrated to be associated with winter *vrn*1 alleles in wheat, as it has been in barley, it may be possible to utilise this tolerance in varieties adapted to Australia's major wheat production zones.

Tools to better assess the effect of the group 5 vernalisation response genes and/or associated loci on frost tolerance at the reproductive stages of development are now available. This includes perfectly linked molecular markers, germplasm and phenotyping facilities such as a radiative frost chamber and field based screening nurseries.

The potential of the three syntenous *vrn*1/*Fr*1 regions in wheat to provide RFT will be investigated in this chapter. Each of the syntenous *vrn*1/*Fr*1 loci on the group 5 chromosomes will be assessed independently and in combination. This information may uncover sources of tolerance suitable for utilisation in Australian varieties.

Materials and Methods

Germplasm - Australian bread wheat varieties

A set of Australian current or soon-to-be-released varieties were selected for the study and provided by the wheat breeding company Australian Grain Technologies. These lines had been genotyped for each of the *vrn*1 loci by Brougham (2006) using diagnostic markers. All 8 genotype combinations for the three group 5 *vrn*1 vernalisation response genes were represented in this set. The alleles were subsequently confirmed using diagnostic molecular markers (Yan *et al.*, 2004a, Fu *et al.*, 2005) and are listed in Table 1.

Table 1. *Vrn*1 genotypes of Australian bread wheat lines. 'S'= Spring allele. 'W'= Winter allele

Name	*Vrn*-A1	*Vrn*-B1	*Vrn*-D1
YOUNG	S	S	S
YITPI	S	S	W
H46	S	W	S
JANZ	S	W	W
VENTURA	W	S	S
WYALKATCHEM	W	S	W
KENNEDY	W	W	S
SUN503B	W	W	W

Germplasm - Triple dirk DH population

Pugsley (1971) developed several Near Isogenic Lines (NILs) in which 5 different combinations of winter alleles at the three *vrn*1 loci were combined in a 'cv. Dirk' bread wheat background. The NILs were developed using a minimum of three backcrosses, and are predicted to contain 94–97% Dirk genetic background.

Two NILs carrying complementary *vrn*1 alleles were selected as parents for population development: 'Triple Dirk A' (spring-*Vrn*-A1, spring-*Vrn*-B1, winter-*vrn*-D1) and 'Triple Dirk E' (winter-*vrn*-A1, winter-*vrn*-B1, spring-*Vrn*-D1). These were crossed to produce 150 F_1 derived doubled haploid lines with an aim to produce approximately 18 lines with each of the 8 possible *vrn*1 combination.

Tolerance alleles at the *Fr*2 locus also located on chromosome 5L may have also been transmitted into the NILs during backcrossing. However, based on the reported genetic distance between *vrn*1 and *Fr*2 (Chapter 1) and the number of backcrosses used to produce the isolines 'Triple Dirk A' and 'Triple Dirk E', it is unlikely that tolerance alleles of *fr*2

would be present in either of these lines used as parents. No analysis was carried out to determine whether *Fr2* was segregating in this population.

Genotyping lines for vrn1 alleles

Diagnostic molecular markers for each of the three wheat *vrn*1 loci were used to determine the *vrn*1 genotype. The *vrn*1 molecular markers have been developed based on sequence variation observed between alleles of the cloned genes (Yan *et al.*, 2004a, Fu *et al.*, 2005). The lines in this study were scored for all of the markers. Variation at the *vrn*-A1 locus has been attributed either to sequence differences in the promoter or to insertion/deletions in intron 1. Both the promoter and intron 1 markers were used to determine allele type at the *vrn*-A1 locus. No variation was observed for *vrn*-A1 within the germplasm for intron 1, so it was assumed that variation at this locus was being controlled by the promoter variants described by Fu *et al.* (2005) and Yan *et al.* (2004a). The intron 1 deletion correlated with variation at *vrn*-B1 and *vrn*-D1. The *vrn*1 markers segregating in this material were used for genotyping, using primers and conditions for PCR and electrophoresis described by Fu *et al.* (2005) and Yan *et al.* (2004a).

LT50 screening

LT50 values were determined by a modified version of the method of Fowler and Limin (2004). Seeds were germinated on moistened filter paper in a Petri dish for 2 days at 4^{o}C followed by 1 day at 25 ^{o}C. Seeds were then transferred to a hydroponics raft on a modified Hoagland's solution (Fowler and Limin, 2004) and grown at 20^{o}C for 3 days with a 12hr day/12hr night cycle. Plants were then cold acclimated for 28 days at 4^{o}C with a 12hr day/12 hr night cycle. Acclimated seedlings were cut approximately 2cm above the crown and 0.5cm below the crown, bundled into groups of 10, wrapped in a moistened tissue wipe and placed in a 10cm glass test tube. Glass tubes were incubated for 12 hours in a

polyethylene glycol bath at -3 ^{o}C. An ice chip was then added to each test tube to prevent supercooling by aiding ice nucleation. The temperature was reduced at -1 ^{o}C/hour until the temperature was -8 ^{o}C. After each hour a bundle/replicate of each genotype was removed (2 bundles/replicates per line). Treated crowns were left at 4 ^{o}C for approximately 12 hours and then planted into UC soil mix at 20 ^{o}C with 12hr day/ 12hr night cycle. After 3 weeks, plants from each temperature treatment were assessed for regrowth and the lethal temperature where 50% of plant survived (LT50) was determined using a Gompetz standard curve analysis (Genstat© Version 8).

Field based screening for reproductive frost tolerance

The field based screening experiment was conducted at Loxton, South Australia under conditions similar to that described in Chapter 2. Four Triple Dirk (TD) DH lines with each of the 8 allele combinations, parents and Australian commercially grown varieties were planted in a randomised complete block design with 2 replicates at each of the 4 seeding times. Each entry was grown in a 3-row, 1.5 meter plot. The 4 seeding dates were approximately 1 week apart and on 13th, 20th and 27th of April, and 4th of May 2007. Multiple seeding times were used to allow for maturity differences between lines. After the occurrence of a frost event (< -2^{o}C at canopy height) when the majority of lines were in head, individual tillers were tagged with coloured tape at the head emerged/pre anthesis/anthers yellow growth stage (decimal growth stage 59) (Zadoks *et al.*, 1974) to mark those tillers that were at the same developmental stage at the time of the frost event. Approximately 3 weeks after the frost event, tagged tillers were harvested and used to determine Frost Induced Sterility (FIS) levels. Data were analysed using REML (Genstat© Version 8), fitting spatial terms to the model to take into account any spatial temperature variation that may have occurred during the frost event, and trends in FIS caused by other environmental factors.

Controlled environment screening for reproductive frost tolerance

The parents of the Triple Dirk DH population were planted in three replicates at 6 seeding times in 8" pots containing a UC soil mix. These seeding times were approximately 7 days apart, starting on the 24/04/07 and concluding on the 29/05/07. On the 3rd (08/05/07) and 4th (18/05/07) seeding dates, TD DH lines representing all 8 *vrn*1 allele combinations (four lines per combination), were planted in triplicate in 8" pots containing a UC soil mix. Pots were placed on the ground in the field at the Waite campus of the University of Adelaide to approximate growth in field conditions. Seeding times were chosen to be similar to those used for commercial production, so the plants were exposed to temperature and photoperiod conditions close to that experienced by local wheat crops.

When the majority of plants from the TD DH population were at the head emergence stage, 3 replicates of each line were selected for RFT screening along with parents and commercial checks. All plants were tagged with coloured tape at 3 discrete stages of development so direct comparisons between could be made:

These were:

Stage 1: Head emerging/pre anthesis/anthers green (Z49)

Stage 2: Head emerged/pre anthesis/anthers yellow (Z55)

Stage 3: Head emerged/post anthesis/pre grainfill (Z59 to Z69)

(Zadoks *et al.*, 1974)

Two experiments were completed on two different sets of parents and lines from the TD DH population in the AGRF frost chamber. Experiment 1 was performed on the 17/09/2007 and experiment 2 on the 28/09/2007. Just prior to the frost treatment, plants were sprayed with

water to stimulate ice nucleation. The set temperature profile for both experiments is shown in Figure 1.

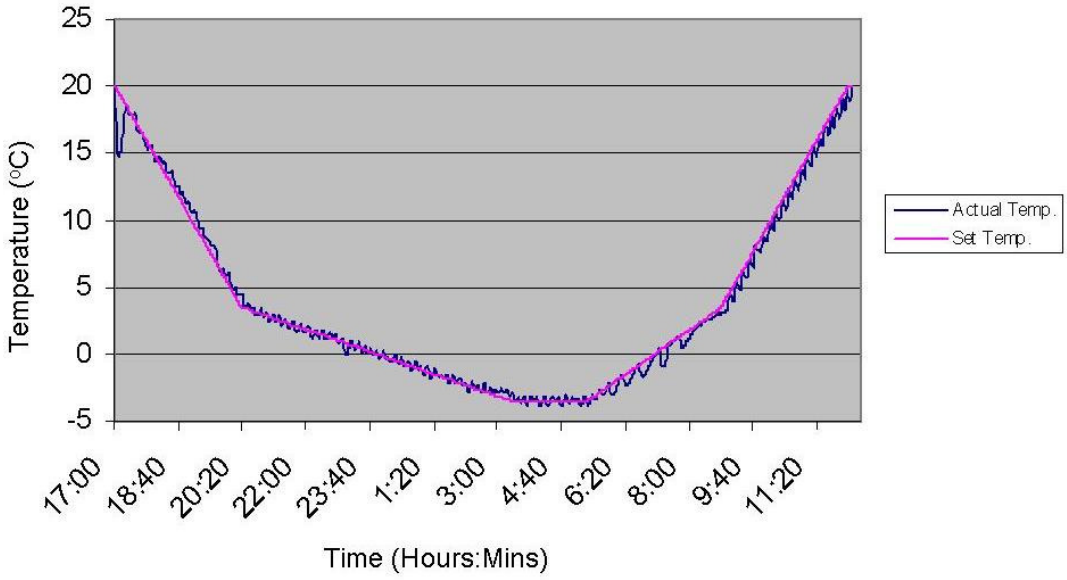


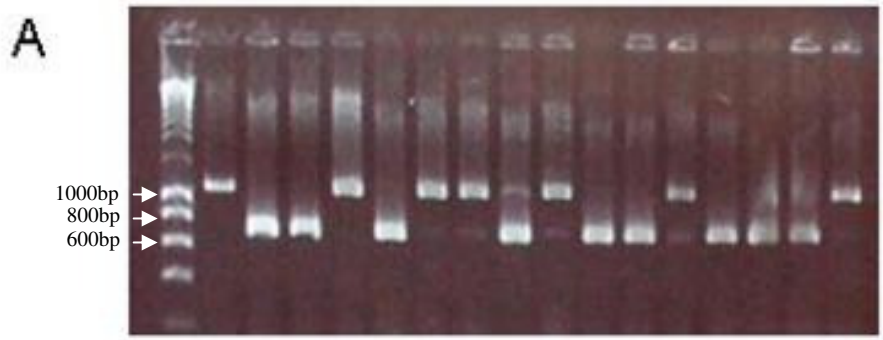
Figure 1. Temperature profile recorded in the AGRF frost chamber for experiment 1 to determine the frost tolerance of Triple Dirk DH lines. The minimum temperature was set at -3.5 ^{o}C for 2 hours. Actual temperature (Actual Temp.) was that recorded at a central point in the chamber at average spike height. Set temperatures (Set Temp.) were the same for experiment 2. Temperature profile was selected to simulate most closely the conditions experienced in the field during a frost event.

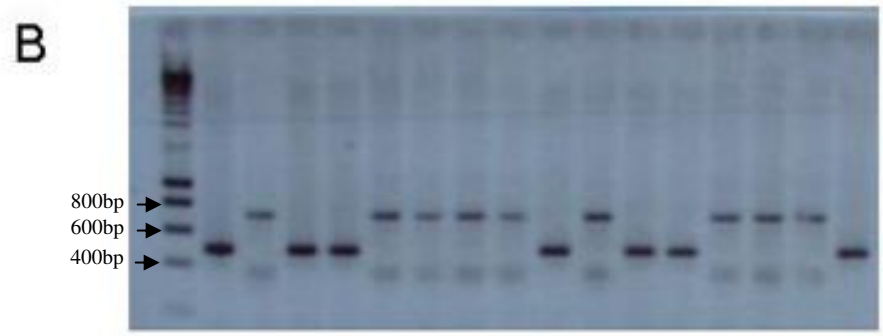
After the frost treatment, pots were returned to the field where they were grown to physiological maturity. Heads were assessed for FIS by measuring the number of sterile florets as a percentage of the total. Data collected were analysed by REML (Genstat© Version 8) fitting spatial terms to take into account any spatial temperature variation that may have occurred in the frost chamber.

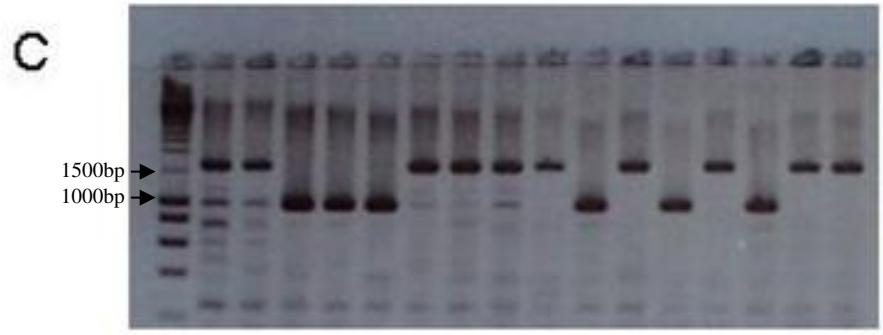
Results

Genotyping Triple Dirk DH lines with vrn1 markers

150 DH lines were screened with the 3 diagnostic *vrn*1 markers (Yan *et al.*, 2004a, Fu *et al.*, 2005). The number of lines carrying each *vrn*1 combination ranged from 13 to 22. 4 lines of each combination were randomly selected for frost tolerance screening. Some of the PCR reactions failed, and DH lines with missing data were not considered for selection.







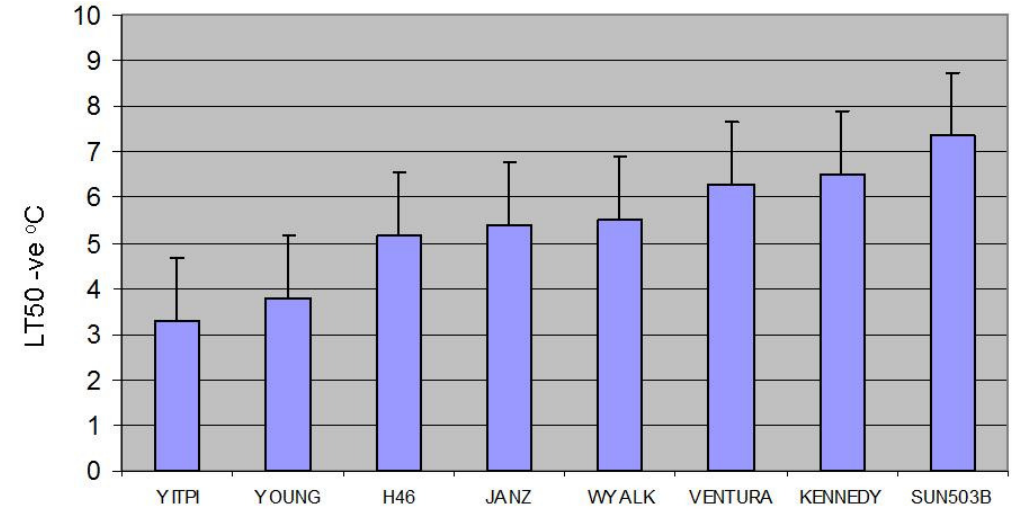
D

	vrn-A1	*vrn*-B1	*vrn*-D1
S allele	58	78	71
W allele	78	67	75

Figure 2. Analysis of *vrn*1 alleles in the F_1 derived DH lines from the cross between Triple Dirk 'A' and Triple Dirk 'E'. (A) spring (upper band) and winter (lower band) alleles at *vrn*-A1. (B) spring (strong lower band) and winter (upper band) alleles at *vrn*-B1. (C) spring (upper band) and winter (lower band) alleles at *vrn*-D1. (D) Number of individuals from TD DH population having either spring (S) or winter (W) alleles at the *vrn*1 loci. Only lines for which scores were obtained for all three loci are represented in figure 2D.

Vegetative frost tolerance of Australian germplasm

There was a significant (P<0.01) variety effect on LT50 among the 8 advanced Australian bread wheat lines. The mean LT50 was related to the number of *vrn*1 winter alleles carried (Figure 3). The effect of each allele at each *vrn*1 locus on LT50 was determined by an analysis of variance. *vrn*-A1 and *vrn*-B1 had a significant (P<0.001) additive effect on LT50 (Figure 4). Alleles at *vrn*-D1 had no significant effect on LT50 (P>0.05). There were no significant inter-locus interaction effects detected (at P<0.05) indicating a lack of epistasis between loci.



Vrn-A1	S	S	S	S	W	W	W	W
Vrn-B1	S	S	W	W	S	S	W	W
Vrn-D1	W	S	S	W	W	S	S	W

Figure 3. LT50 of Australian bread wheat varieties and their genotypes at the *vrn*1 loci. Error bars indicate the L.S.D. (P<0.05). 'S'=Spring allele and 'W'=Winter allele.

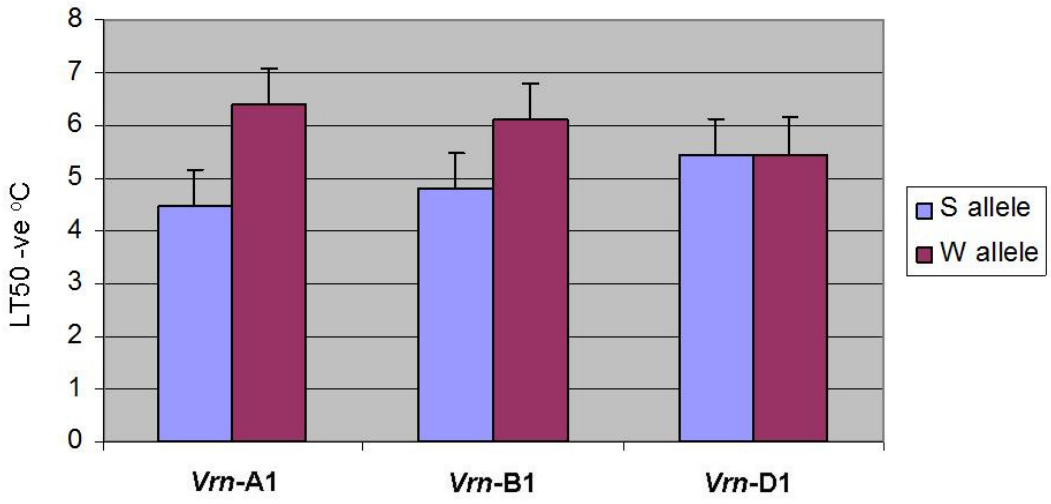


Figure 4. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on LT50 based on the Australian bread wheat varieties in Table 1. Error bars indicate the L.S.D (P<0.05).

Vegetative frost tolerance of Triple Dirk DH lines

There was a significant DH line effect (P<0.001) on LT50 for the Triple Dirk DH lines. Figure 5 shows the mean and LSD (P<0.05) for each line and the *vrn*1 locus genotypes. The overall effect of each *vrn*1 locus (Figure 6) was analysed. *vrn*-A1 had a significant effect on LT50 (P<0.001) with the winter allele providing a lower LT50. Lines with winter alleles at *vrn*-B1 and *vrn*-D1 had a lower mean LT50, but these differences were not statistically significant (P>0.05).

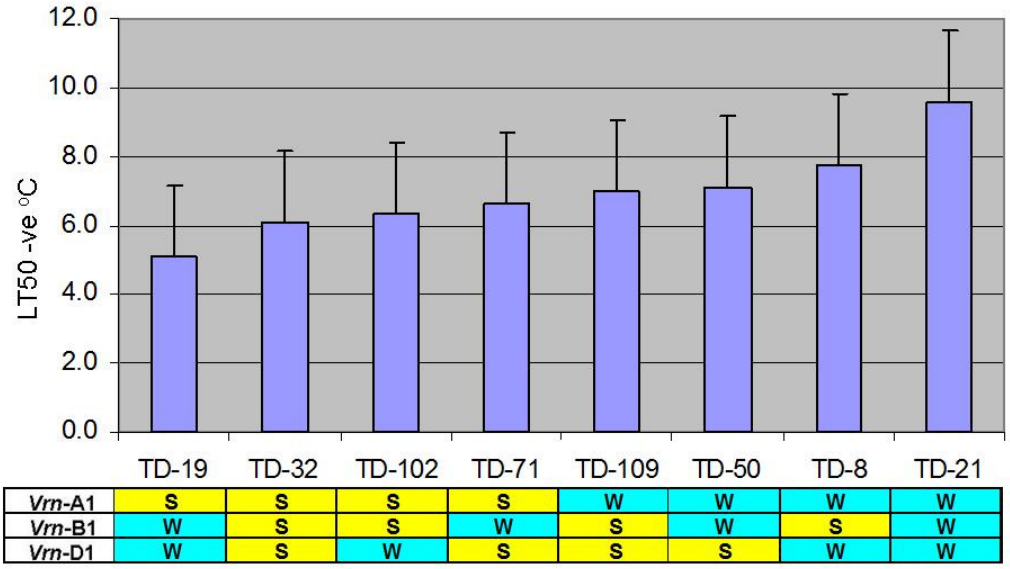


Figure 5. LT50 of Triple Dirk DH lines, and their *vrn*1 genotypes. Error bars indicate the L.S.D (P<0.05). 'S'=Spring allele and 'W'=Winter allele.

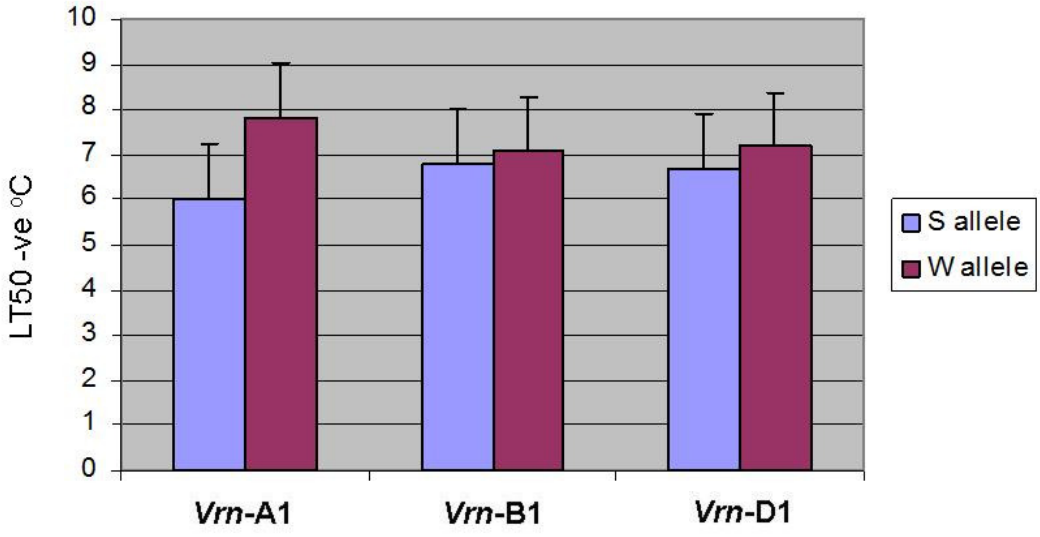


Figure 6. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on LT50 in the Triple Dirk DH lines from Figure 5. Error bars indicate the L.S.D (P<0.05)

Comparison of vegetative frost tolerance screening with previous studies

The study of Koemel *et al.* (2004) also examined effects of different *vrn*1 genes on low temperature tolerance using lines carrying a cv. Dirk genetic background. Koemel *et al.* (2004) reported significant differences between lines carrying contrasting alleles at each of

the wheat *vrn*1 loci. Although in the present study we were unable to observe significant effects of the *vrn*-B1 and *vrn*-D1 loci on LT50 in the Dirk background, the correlation between the data obtained in the two studies is very high (Figure 7).

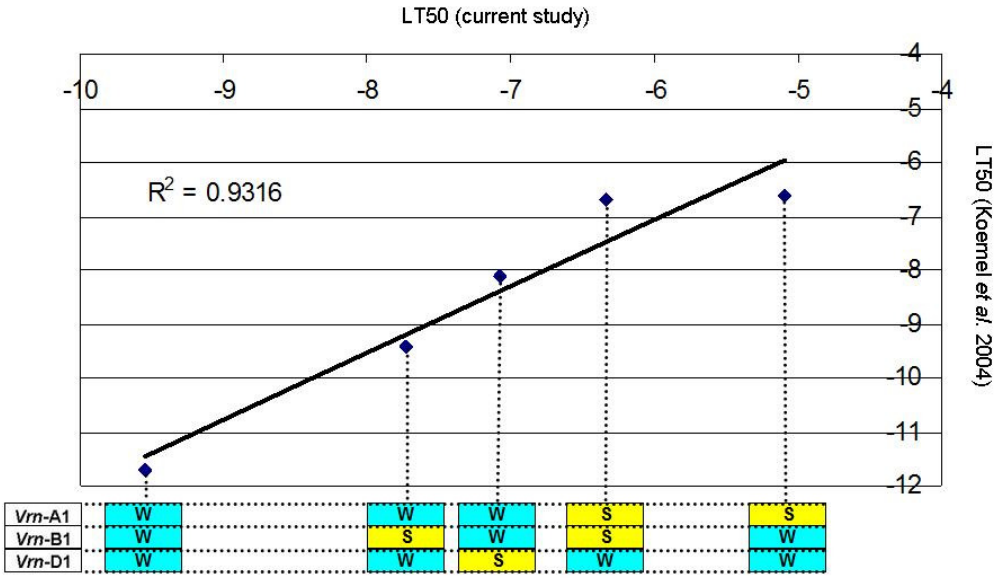


Figure 7. Correlation between the LT50 values obtained by Koemel *et al.* (2004) and that of the present study. Allele combinations at the *vrn*1 loci are shown below. 'S'=Spring allele and 'W'=Winter allele.

Reproductive frost tolerance in Australian wheat germplasm

The Australian varieties were exposed to a frost event on the 15th of August 2007 where the minimum canopy temperature was -3.3^{o}C. All lines except the winter genotype SUN503B had tillers at the post heading but pre anthesis stage of development. There was a significant variety effect on FIS ($P<0.001$, Figure 8). For each of the Australian wheat varieties the *vrn*1 alleles were fitted into the statistical model so means and effects could be determined. For each *vrn1* locus, lines with the winter allele showed lower mean FIS (Figure 9), however the differences were not significant ($P>0.05$).

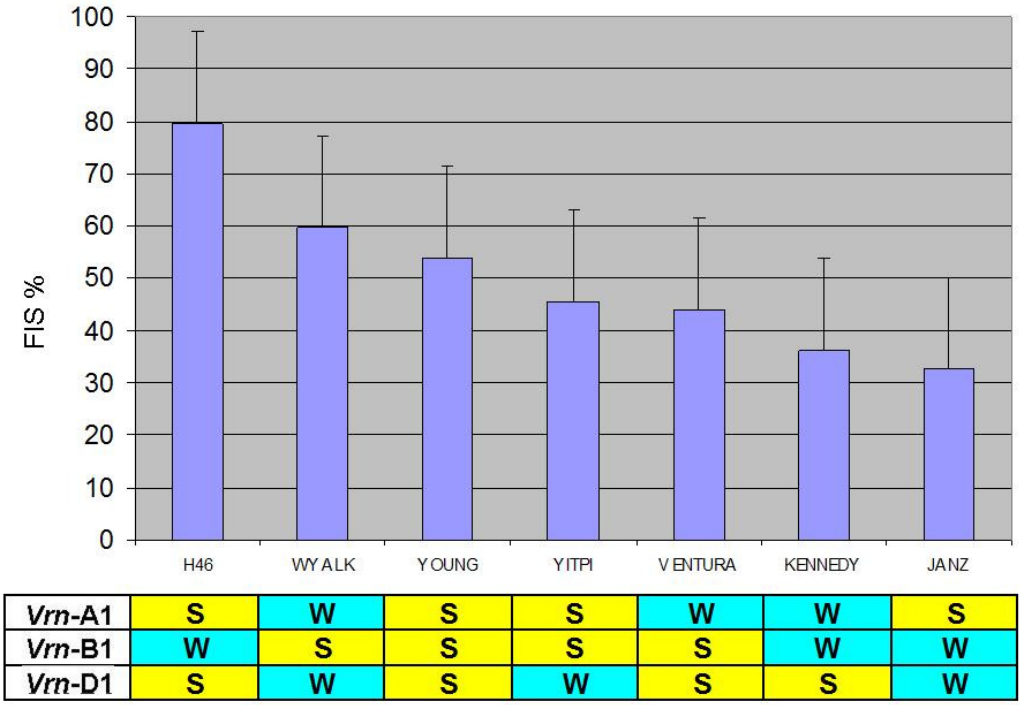


Figure 8. FIS of Australian bread wheat lines and their genotype at the *vrn*1 loci. Error bars indicate the L.S.D. (P<0.05). 'S'=Spring allele and 'W'=Winter allele.

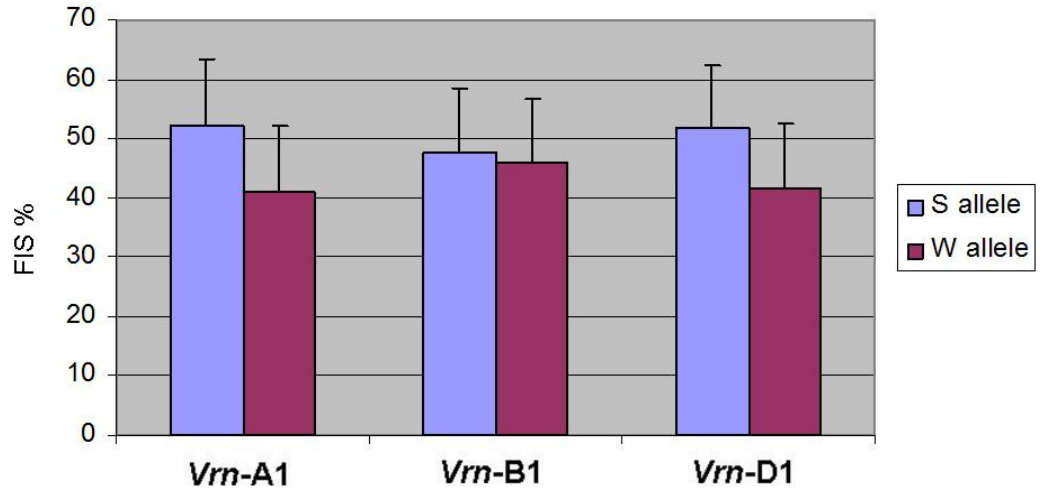


Figure 9. FIS effects of the spring (S) and winter (W) allele at each *vrn*1 locus in the advanced Australian lines in Figure 8.

Reproductive frost tolerance in Triple Dirk DH lines

Lines selected from the TD DH population were exposed to a simulated frost event in the AGRF frost chamber. Data were analysed within maturity classes as well as overall, with growth stage fitted as a fixed effect. There was no significant DH line effect in either the first or second experiment using either method of analysis. Also, none of the *vrn*1 alleles showed any significant effects with either methods of analysis. The majority of data collected was from the developmental stage 2 (head emerged/pre anthesis/anthers yellow), the stage that had been used to collect data from the field. Results from stage 2 are represented in Figures 10 and 11, while results from the other developmental stages are not shown.

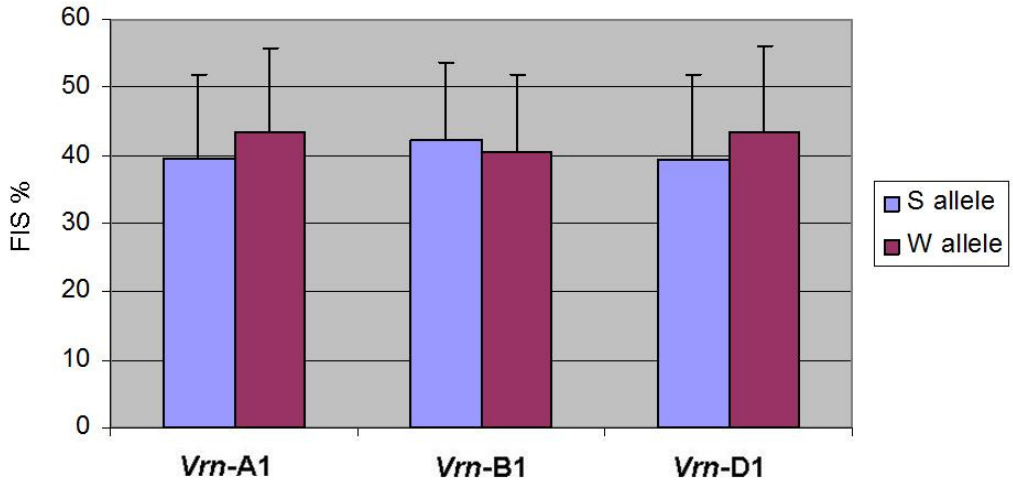


Figure 10. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on FIS, in frost chamber experiment 1, based on the Triple Dirk DH population lines in Figure 5.

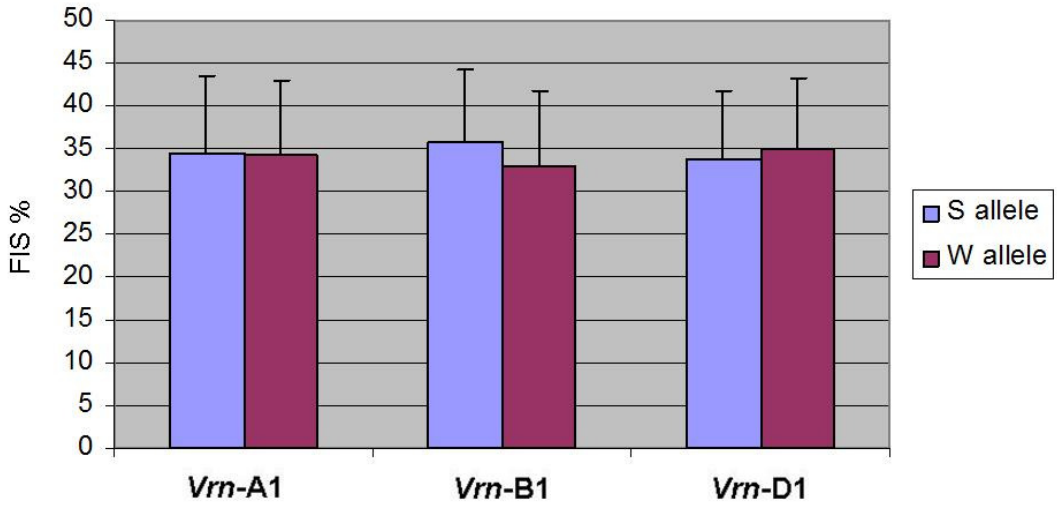


Figure 11. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on FIS, in frost chamber experiment 2, based on the Triple Dirk DH population lines in Figure 5.

RFT in the Triple Dirk doubled haploid population was assessed in the field as well as in the frost chamber. Frost events occurred on the 15th August (minimum temp -3.3 ^{o}C) and the 1st of September (minimum temp. -2.2 ^{o}C). Mean FIS from both frost events was lower than that observed in the frost chamber experiments, with ranges from 8-15% and 39-44% respectively. No significant effect of DH line ($P>0.05$) on FIS was detected in the field based screening, consistent with the frost chamber experiments. There was no significant association between *vrn*1 allele and FIS from the TD DH lines when exposed to the August 2007 frost event (Figure 12). However, there was a significant effect of *vrn*-A1 on FIS from the September frost event ($P<0.05$), with the winter allele being unexpectedly associated with increased FIS by an average of 3%. (Figure 13).

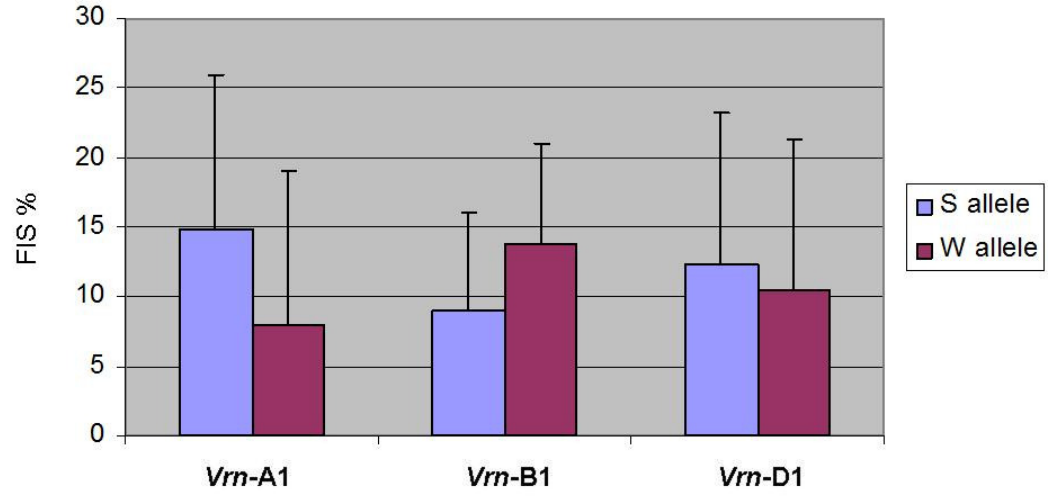


Figure 12. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on FIS, from the Loxton frost on August 2007, measured in the Triple Dirk DH lines.

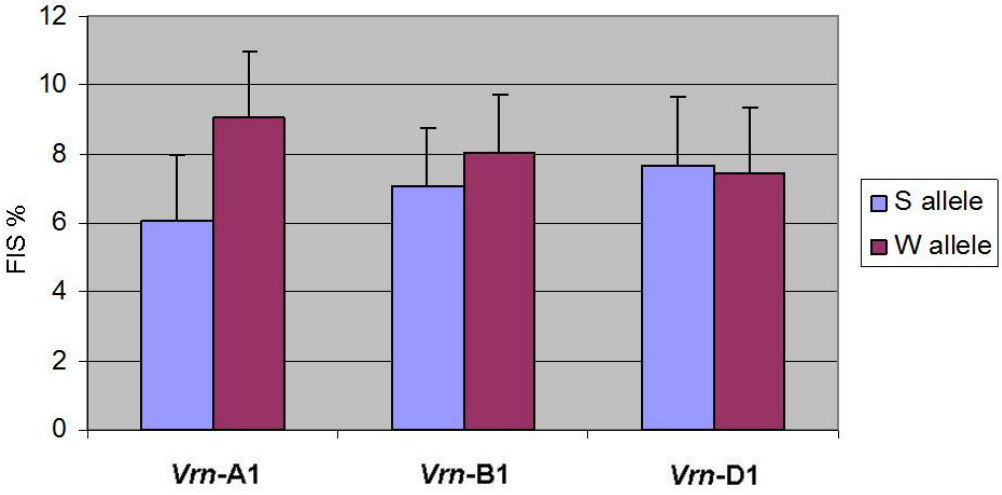


Figure 13. Effect of the spring (S) and winter (W) allele at each of the *vrn*1 loci on FIS, from the Loxton frost on September 2007, measured in the Triple Dirk DH lines.

Discussion

The eight Australian varieties included in this study are directly relevant to the major Australian wheat production environments. They also represent all 8 possible allele combinations at the 3 *vrn*1 loci. This study provided an initial investigation of associations between *vrn*1 alleles and frost tolerance at both the vegetative and reproductive

developmental stages. Results indicated that at least *vrn*-A1 and *vrn*-B1 were having an effect on VFT.

In contrast to VFT, RFT was not found to be significantly associated with *vrn*1 genotype. The error associated with field based screening most likely limited the power of the field RFT test. However, a significant variety effect on FIS was observed. Although there appeared to be a general trend towards the winter alleles providing reduced FIS, there were no significant effects detected for any of the three *vrn*1 loci. The use of genetically unrelated varieties may have confounded detection of *vrn*1 effects due to developmental differences or other variation contributed by the genetic backgrounds of these lines.

The Triple Dirk DH lines provided a more powerful genetic resource for examining *vrn*1 effects. This material reduced the probability that differences would arise from developmental loci or other confounding traits. The lines selected to be parents of the Triple Dirk DH population represented NILs made in the cv Dirk background. Based on the number of backcrosses (3-4) used in making the parent lines it can be estimated that approximately 94-97% of the genomes of the derived DH progeny would be identical. Thus, other loci that may affect frost tolerance were unlikely to be segregating in the Triple Dirk DH population, allowing a more specific focus on the effects of the group 5 vernalisation response genes.

VFT screening of the Triple Dirk DH lines revealed a significant increase in LT50 associated with the winter allele at the *vrn*-A1 locus. A significant effect was not detected for *vrn*-B1 or *vrn*-D1, although lines carrying the winter alleles at these loci did tend to have higher VFT. These results are consistent with those of Koemel *et al.* (2004) who found that *vrn*-A1 was associated with the major effect on VFT, with *vrn*-B1 and *vrn*-D1 being associated with lesser, intermediate effects on tolerance. Overall, these data suggest that the

winter alleles at the *vrn*1 loci are associated with enhanced frost tolerance at the vegetative stages of development. This was also supported by LT50 data obtained from the screening of the Australian adapted varieties with different combinations of alleles at the *vrn*1 loci.

The influence of the *vrn1* loci on seedling development was not measured in the LT50 experiment. Seedlings compared for LT50 possessed different winter alleles for vernalisation response. Different alleles at the *vrn1* loci can impact on the timing of floral initiation and their effect is influenced by vernalisation treatment. The treatment of seedlings for 28 days at 4^{o}C would not have fulfilled the complete vernalisation requirements for all genotypes carrying winter alleles at the three *vrn1* loci. It is unknown if the influence of different alleles at each of the *vrn1* loci had an effect on the developmental stage of the genotypes in this experiment even though the differences in development between seedlings at this early growth stage is likely to be small. The effect of developmental stage of the seedling may influence frost sensitivity because once the vernalisation requirement is met, the seedling will initiate the double ridge stage of development, after which the ability to acclimate and express tolerance progressively diminishes. However, Prasil *et al.* (2004) reported a delay of four weeks between the time that vernalisation becomes saturated and the time that frost sensitivity starts increasing. As seedlings were acclimated for four weeks, commencing on seedlings that were six days old, differences in observed frost sensitivity seem unlikely to have been related to differences in vernalisation requirement between the lines.

Winter alleles at *vrn*1 loci did not display any association with increased RFT. No *vrn*1/RFT association could be identified from two frost chamber experiments on the Triple Dirk DH lines. In both frost chamber experiments, DH lines containing alternate alleles at each of the 3 *vrn*1 loci showed very similar mean FIS levels. The mean level of frost damage in these trials was 41.4% and 34.5% for experiment 1 and 2, respectively. These

levels of damage are similar to those found to be most effective in detecting the RFT effect linked to *vrn*-H1 in barley (data not shown).

The field based RFT screening also revealed little association of spring and winter *vrn*1 alleles with FIS. Only the *vrn*-A1 locus showed a significant association (Figure 12) but it was the spring allele that was associated with increased tolerance. The mean FIS was much lower in the field than in the frost chamber, which may have made it more difficult to detect genetic effects due to a reduced range in FIS values.

There are few published investigations into possible effects of vernalisation genes on RFT. Fletcher (1988) performed RFT screens of substitution lines carrying whole chromosomes from cv. Cheyenne in a cv. Chinese Spring background. The RFT measured was based on tiller mortality after a frost event. Cheyenne carries the *vrn*-D1 winter allele on chromosome 5D, while Chinese Spring carries a *Vrn*-D1 spring allele. The 5D substitution line and Chinese Spring showed no difference in RFT during the stem elongation growth stage.

Fuller *et al.* (2007) attempted to compare reproductive and vegetative frost tolerance using one 'winter' and one 'spring' wheat cultivar. While they reported significant differences in frost tolerance between the 'winter' and 'spring' cultivars at the vegetative stages of development, no differences were detected at the reproductive stages, leading to the conclusion that the tolerance mechanisms at the two stages were unrelated. Although *vrn* locus genotypes for these two cultivars were not reported, winter/spring growth habit in wheat is most often determined by *vrn*1 loci. Therefore, the findings by Fuller *et al.* (2007) add circumstantial evidence that *vrn*1 winter alleles are not associated with increased RFT in wheat.

Fowler and Limin (2004) presented data suggesting that the ability of even the most VFT wheat genotypes to cold acclimate, and therefore develop tolerance, significantly declined once the developmental switch from vegetative to reproductive growth had been made. The rate and duration of cold acclimation, associated with the level of VFT in winter genotypes, did not continue past floral initiation. The duration of cold acclimation was later attributed to the *vrn*1 locus in a winter/spring germplasm (Limin and Fowler, 2006). The VFT that is associated with *vrn*1 winter alleles may therefore not be effective at the reproductive stages of development.

Winter/spring alleles at the *vrn*1 loci are unlikely to have a significant effect on RFT based on the current study. The few previous reports that failed to associate vegetative and reproductive frost tolerance in wheat also support this conclusion. This contrasts the situation in barley where linkage was found in two separate mapping populations between RFT and winter alleles of *vrn*-H1, the homologue of the *vrn*1 genes in barley (Chapter 2). The RFT QTL identified in Chapter 2 spanned a relatively large genetic interval (average 15cM across the two populations). While the *vrn*-H1 locus was located in this interval, the possibility that the RFT effects were arising from another closely linked locus could not be discounted.

If RFT on Triticeae group 5 chromosomes is controlled by a locus separate to *vrn*1, it is possible that such a locus may not have been segregating in the wheat Triple Dirk DH population. The Australian varieties with winter alleles at *vrn*-A1 or *vrn*-D1 showed an average 10% greater RFT than lines carrying the corresponding spring allele. Although these differences were not significant, the trend suggests that some of the Australian lines may have contained RFT genes present in coupling with winter *vrn*-A1 and *vrn*-D1 alleles. Further genetic analysis of the 5L regions may result in the identification of linked alleles conferring improved RFT.

The experimental approach was based on the assumption that the *vrn*1 genetic markers were completely diagnostic of winter/spring allele type. There was also an assumption that the *vrn*1 alleles sampled in this study was representative of those present in hexaploid wheat. In terms of allele sequence, the winter alleles in this study represent the most common *vrn1* variants in the hexaploid gene pool (B. Trevaskis, personal communication, 2009). Recent reports have revealed additional winter allele sequence variants at individual wheat *vrn1* loci (Chen *et al.*, 2009c, Pidal *et al.*, 2009). These other winter *vrn1* alleles could be assessed for an association with RFT.

Improving the RFT in wheat could be pursued through further investigations including:

1. Identification of genetic variation for RFT in wheat and mapping population development using tolerant and intolerant lines as parents. This would be a similar to the path taken in barley (Chapter 2) and could focus on identifying tolerance that is not associated with *vrn1*.
2. Fine mapping of the RFT locus on chromosome 5H in barley. This may or may not genetically separate *vrn*-H1 from a RFT locus. If they are separated, cloning the RFT gene(s) in barley and determining what expression/sequence difference distinguishes a tolerant from intolerant allele would enable a search for similar gene(s)/allele(s) in wheat. As the 5L genetic region is highly conserved in the Triticeae, it would be highly likely that a gene of similar function would be present in wheat.
3. If *vrn*-H1 was found to control RFT in barley, and the allele was different to those currently known in common hexaploid wheat, allele mining to identify similar alleles in wheat could be undertaken.

Chromosome 5L has been implicated in the control of a range of stress responsive traits in the Triticeae (Cattivelli *et al.*, 2002a). A locus called *Fr*2 has a major effect on frost tolerance at the vegetative stages of development. Within the *Fr*2 locus is a cluster of C-repeat Binding Factor (*CBF*) genes that are closely linked but separate to *vrn*1. Genes from the *CBF* family reside in this genomic region in wheat, barley and rye (Cattivelli *et al.*, 2002a, Francia *et al.*, 2007, Vagujfalvi *et al.*, 2003). *Fr*2 has yet to be assessed at the reproductive stages of development. Potentially, the 5L genomic region may still have a role to play in improving RFT in wheat. The research reported in this chapter has found that the winter alleles at the 3 *vrn*1 loci in wheat are unlikely to have a direct effect on RFT.