Quantitative expression analysis of four low-temperature-tolerance-associated genes during cold acclimation in wheat (*Triticum aestivum* L.)

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ABSTRACT

Winter wheat (*Triticum aestivum* L.), seeded in the fall, cold acclimates when exposed to low fall temperatures. Growth resumes in spring, culminating in early summer harvest. Winter wheat yield is generally 20-25% higher than spring wheat. However, winter damage/kill can reduce its yield. A better understanding of the cold acclimation/tolerance process could help in the development of improved breeding strategies for winter wheat hardiness. Transcriptional activators and specific cold regulated (COR) genes are induced as a result of exposure to low temperatures. Thus, the objective of this study was to determine the quantitative expression of three COR genes (*Wcs120, Wcor410* and *Wcor14b*) and one transcriptional activator (*WCBF1*) in field-grown wheat using real-time PCR and to establish any association with LT_{50} (temperature at which 50% of plants are killed). Winter Norstar (*vrn-A1/vrn-A1)*, spring Manitou (*Vrn-A1/Vrn-A1)* and two near-isogenic lines (Spring Norstar (*Vrn-A1/vrn-A1)* and Winter Manitou (*vrn-A1/vrn-A1)*, respectively) were used in these studies. Plants were sampled on three dates (Sept. 29, Oct. 12 and Oct. 26) in the fall of 2004. Accumulation of *WCBF1* transcripts was highest in Norstar, but in all four genotypes there was an increase in transcripts by the second sampling date, followed by a decline on the third sampling date. *Wcs120* transcripts increased from the first to the third sampling date in Norstar, Spring Norstar and Winter Manitou, but increased to the second sampling date and decreased by the third in Manitou. For *Wcor14b*, generally there was an increase to the second sampling date, followed by a decrease or steady levels on the third. *Wcor410* showed a similar pattern, except for Spring Norstar wherein transcript levels increased by the third sampling date. With the exception of *Wcor410* in Manitou, the *Vrn-A1* locus affected gene expression in all genotypes.

However, only *Wcs120* expression followed the low-temperature tolerance pattern in these genotypes.

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LIST OF ABBREVIATIONS

Act	*Actin*
ANOVA	Analysis of Variance
bp	base pair(s)
CBF	C-repeat-Binding Factor
cDNA	complementary DNA
cDNA-AFLP	cDNA - Amplified Fragment-Length Polymorphism
cM	centiMorgan
COR gene	Cold Regulated gene
Ct	Threshold cycle
DNA	Deoxyribonucleic Acid
DREB	Dehydration-Responsive Element Binding factor
FLN	Final Leaf Number
FRET	Fluorescence Resonance Energy Transfer
Fr	Frost resistance
ICE1	Inducer of CBF Expression 1
kb	Kilobase pair
kDa	kilodalton
LB	Luria-Bertani
LT	Low-temperature
LT_{50}	Lethal temperature at which 50% of plants are killed
mRNA	messenger RNA
NILs	Near-isogenic lines
PCR	Polymerase Chain Reaction

qrRT-PCR	quantitative real-time Reverse Transcriptase PCR
Rcg	Regulator of *cor14b* gene
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic Acid
RPA	Ribonuclease protection assay
RT-PCR	Reverse Transcriptase PCR
SNP	Single Nucleotide Polymorphisms
TaVRT-1	*Triticum aestivum* Vegetative to Reproductive Transition-1
TaVRT-2	*Triticum aestivum* Vegetative to Reproductive Transition-2
UBI	Ubiquitin
Vrn-1	Vernalization loci
WAP1	Wheat APETALA1
Wcs	Wheat cold-specific
Wcor	Wheat Cold Regulated

1.0 INTRODUCTION

There are advantages to growing winter wheat over spring wheat (a higher yield potential, better utilization of moisture, earlier harvest date resulting in less frost damage late in the season, decrease in soil erosion, better weed competition that reduces the need for herbicides, longer growth period and reduced problems with wet, late springs (Fowler, 2002; Peterson, 1965)) but a major disadvantage is the lack of a high level of low-temperature tolerance. As a result, the cold tolerance of winter wheat is not high enough to survive the winter in western Canada without special management practices. Therefore, in order to increase winter wheat acceptance in this region, the low-temperature tolerance has to be improved. To improve winter wheat survival, a better understanding of the molecular basis of low temperature tolerance is required. Once the low temperature tolerance pathways and the roles of important cold tolerance genes have been elucidated, directed breeding strategies can be developed to increase the low-temperature tolerance in winter wheat.

Winter wheat requires exposure to a period of low non-freezing temperatures before switching from the vegetative phase to reproductive phase. This requirement for exposure to a period of low temperatures before flowering is known as vernalization. In wheat, the vernalization locus has been implicated in this low temperature requirement and the *Vrn-A1* gene on chromosome 5A has been suggested to be a stronger spring/winter habit determinant (Dubcovsky et al., 1998) than the homoeologues *Vrn-B1* (on chromosome 5B) and *Vrn-D1* (on chromosome 5D) (Loukoianov et al., 2005).

Spring-habit is due to the dominant *Vrn-A1* gene (*Vrn-A1*/*Vrn-A1* and *Vrn-A1*/*vrn-A1*). Winter habit homozygous recessive (*vrn-A1*/*vrn-A1*) genotypes will require a vernalization period before flowering can occur (Limin and Fowler, 2002).

LT_{50} values (temperature at which 50% of the plants are killed (Limin and Fowler, 1988)) determined from artificial freeze-tests are generally used as measures of low temperature tolerance. It would be of great practical value if a strong correlation could be demonstrated between LT_{50} measurements and biochemical or genetic determinants, since artificial freeze-tests are laborious and time-consuming. In this regard, the study of expression patterns of genes involved in low-temperature responses can contribute valuable information to the understanding of low-temperature survival. There are several methods available for expression analysis studies, including northern blotting, ribonuclease protection assays and *in situ* hybridization. These methods are generally time-consuming and laborious. More recently, the quantitative real-time reverse transcriptase PCR (qrRT-PCR) approach has provided a more rapid and sensitive system for the analysis of gene expression.

Most of the low-temperature tolerance studies in wheat have been conducted under controlled environments, with exposure to constant, low non-freezing temperatures and LT_{50} values determined using freeze-tests. These studies need to be supported by low temperature tolerance data and an understanding of the expression patterns of low-temperature-induced genes in field-grown wheat plants. Thus, the objectives of this study were to:

1. Compare quantitative real-time reverse transcriptase PCR (qrRT-PCR) and northern blot analysis for the determination of expression of low temperature-

induced genes (*WCBF1*, *Wcs120*, *Wcor14b* and *Wcor410*) in field-grown wheat plants in the fall of 2004.

2. Compare the expression patterns among four specially developed wheat genotypes.
3. Relate the level of gene expression to the cold tolerance (LT_{50}) of each genotype and identify candidate cold tolerance markers.

The hypothesis is that the *Vrn-A1* locus will affect the expression of the cold acclimation genes in wheat and that the expression levels of the cold acclimation genes could be used as a marker for cold tolerance. The wheat genotypes were Norstar (winter habit) and Manitou (spring habit) and their near-isogenic lines Spring Norstar and Winter Manitou, respectively. The near-isogenic lines (Spring Norstar and Winter Manitou) were developed by reciprocal crosses between winter Norstar and spring Manitou (Limin and Fowler, 2002), such that the winter habit of Norstar was transferred to spring Manitou and the spring habit of Manitou was transferred to winter Norstar. This inter-change of the vernalization locus should establish if differences in the expression of the low temperature-induced genes *WCBF1*, *Wcs120*, *Wcor14b* and *Wcor410,* which have previously been shown to be induced upon exposure to low temperature (Jaglo et al., 2001; Limin et al., 1997; Danyluk et al., 1998; Vágújfalvi et al., 2000), is influenced by vernalization requirement.

.2.0 LITERATURE REVIEW

2.1 Economic importance of wheat

Wheat, which is a member of the grass family (*Poaceae*), belongs to the tribe *Triticeae* and the genus *Triticum*. Among the wild and cultivated varieties of wheat, there are diploid (14 chromosomes), tetraploid (28 chromosomes) and hexaploid (42 chromosomes) genomes (Peterson, 1965). Bread wheat (*Triticum aestivum* L.) has a hexaploid genome. The hexaploid genome has three chromosome sets each with seven paired chromosomes (AABBDD) (Peterson, 1965). The three chromosome sets come from *Triticum urartu* (AA) (Dvorak et al., 1993), a B genome donor (Sarkar and Stebbins, 1956), and *Aegilops tauschii* (DD) (Zohary and Feldman, 1962). It is thought that a species closely related to *Aegilops speltoides* contributed the B genome (Sarkar and Stebbins, 1956).

Wheat is grown in many different countries in variable climatic conditions. The world production of wheat in 2003 was 550,000,000 metric tonnes (Anonymous, 2003). Canada produced 4.3% of the world wheat (24,000,000 metric tonnes). China had the highest production of wheat in the world followed by India, United States of America, Russian Federation, France, Australia and Canada (Anonymous, 2003). In Canada, 27,000,000 metric tonnes of wheat was produced in 2005 (Statistics Canada, 2006). Of this, spring wheat production was 70.2%, winter wheat 7.7% and durum wheat 22.1%. In Saskatchewan, 14,000,000 metric tonnes of wheat was produced in 2005, accounting for 51.3% of the wheat produced in Canada (Saskatchewan Agriculture and Food, 2006).

Saskatchewan has a low proportion of winter wheat production (1.3%) compared to spring (63.2%) and durum wheat (35.5%).

Wheat in Canada is divided into eight market classes: Canadian Western Red Spring (CWRS) wheat, Canadian Western Amber Durum (CWAD) wheat, Canadian Prairie Spring (CPS) wheat, Canadian Western Extra Strong red spring (CWES) wheat, Canadian Western Red Winter (CWRW) wheat, Canadian Western Soft White Spring (CWSWS) wheat, Canadian Eastern White Winter (CEWW) wheat and Hard White Spring (HWS) wheat (DePauw and Hunt, 2001). Wheat classes are based on the hardness, color of the seed and the growth habit of the wheat variety (winter or spring). Wheat classes are used to identify the characteristics of the different types of wheat. Hard wheat is used for the production of white pan breads, white specialty breads and wheat breads (Gooding and Davies, 1997) with 11-16% protein (Ranhotra et al., 1994). Soft wheat is used for the production of crackers, cookies, cakes, pie crusts, pretzels, puff pastries, doughnuts, and refrigerated dough (Gooding and Davies, 1997) with 8-11.5% protein (Ranhotra et al., 1994). Durum wheat is used to make pasta (macaroni, spaghetti) products (Gooding and Davies, 1997) with 12-15% protein (Ranhotra et al., 1994). A mix of soft and hard wheat is used to make noodles with 9-15% protein (Ranhotra et al., 1994). Oriental noodles have lower protein and egg noodles have higher protein.

2.2 Differences between winter and spring wheat

There are two main growth habits of wheat, winter and spring. Winter wheat is seeded in the fall, survives the winter and grows in the spring. Spring wheat is seeded in

the spring and grows when the temperature and photoperiod are optimal. Spring wheat is more commonly grown in western Canada than winter wheat.

The difference between winter and spring wheat is primarily due to the length of the vegetative stage. Spring plants will become reproductive soon after germination if the temperature and the day length are optimal. The transition from the vegetative stage to the reproductive stage occurs when the shoot apex becomes double-ridged (McMaster et al., 1997). The double ridge formation occurs before internode elongation and forms the spikelet. However, some spring plants are day length sensitive and when the day length is less than optimal, i.e., short day lengths, they will not enter the reproductive stage and will remain vegetative until the day length becomes longer. In regions with mild winters, some cold tolerant, day length sensitive spring wheat genotypes can be planted in the fall.

The increased length of the vegetative stage in winter habit plants is due to the vernalization requirement which is absent in spring plants. A vernalization requirement is the need for exposure to a period of low non-freezing temperatures (Fowler et al., 1999) before the plant will switch from the vegetative stage to the reproductive stage and flower normally.

2.3 Advantages and disadvantages of winter wheat

There are advantages to the production of winter cereals compared to spring cereals in western Canada. These advantages include: a higher yield potential, better utilization of moisture, earlier harvest date (less frost damage late in the season), decrease in soil erosion, better weed competition that reduces the need for herbicides, longer growth period and reduced problems with wet, late springs (Fowler, 2002;

Peterson, 1965). Winter cereals also provide a good fit for sustainable production systems. For example, a study by Ducks Unlimited Inc. showed that winter crops help to increase the duck population because they provide an undisturbed nesting area in early spring (Ducks Unlimited, 2005). Tilling of the land to plant spring crops will destroy nests in the field, but fall planting means that the nests are left undisturbed. These many advantages over spring crops provide an impetus for increased research into better understanding of winter cereal production.

One disadvantage of winter crops is that they can be killed by freezing temperatures if not properly managed and this is an especially important concern with winter wheat. In order to prevent winter kill of the winter wheat crop, it should be seeded into standing stubble from a previous crop (Fowler, 2002). This will trap snow on top of the winter wheat which will provide insulation for the plants over the winter. Consequently, an increase in understanding of cold tolerance will assist in the design of improved plant breeding strategies to reduce winter damage, which in turn will help in the production of cultivars with improved yield potential.

2.4 Photoperiod and cold acclimation

Cold acclimation results in the plant gaining the ability to withstand freezing temperatures by the accumulation of protein products from the cold acclimation genes (Thomashow, 1999). This process starts when the temperature drops lower than approximately 10°C and results in several physiological and biochemical changes in the plant (Fowler et al., 1999). Because significant levels of cold tolerance can be accumulated only when the plant is in the vegetative growth phase, the time to vegetative/reproductive transition is important in determining the duration of the cold

tolerance (Mahfoozi et al., 2001). Once the plant switches to the reproductive growth phase it will lose cold tolerance.

Photoperiod is also involved in allowing a plant to cold acclimate. As observed by Limin and Fowler (2006), some photoperiod sensitive spring habit cultivars grown under short day lengths are able to cold acclimate to a level that approaches winter habit plants. These spring habit genotypes do not have the vernalization requirement (Limin and Fowler, 2002), but the photoperiod requirement delays the transition from vegetative to reproductive growth, and allows the plants to gain cold tolerance (Limin and Fowler, 2006).

2.5 Final leaf number and cold tolerance assessment

Final leaf number (FLN) is often used to establish when a plant switches from the vegetative to the reproductive growth phase (Robertson et al., 1996) and is determined by counting the number of leaves formed on the main stem before the flag leaf appears (Mahfoozi et al., 2001). FLN for winter plants is dependent on the length of the vernalization period and the lowest FLN occurs at the vernalization saturation point (point where the minimum leaf number is reached for winter plants during cold acclimation) (Mahfoozi et al., 2001). Furthermore, low temperature tolerance decreases after the vernalization requirement has been achieved, even under acclimation conditions (Fowler et al., 1996). Measurement of this low temperature tolerance is generally determined by the LT_{50} value which is the temperature at which 50% of the plants have been killed by low temperature stress (Limin and Fowler, 1988).

2.6 Vernalization

Vrn-A1 is a stronger spring/winter habit determining gene in *Triticum aestivum* (Dubcovsky et al., 1998) than *Vrn-B1* and *Vrn-D1* (Loukoianov et al., 2005). The dominant *Vrn-A1* gene is present in spring-habit genotypes (*Vrn-A1*/*Vrn-A1* and *Vrn-A1*/*vrn-A1*) and the gene is homozygous recessive (*vrn-A1*/*vrn-A1*) in winter-habit genotypes with a vernalization requirement (Limin and Fowler, 2002). *Vrn-A1* is located on chromosome 5A in wheat (Nelson et al., 1995) and chromosomes 5A and 5D have been identified as important regions for cold tolerance in wheat (Limin et al., 1997). Similar genes to wheat *Vrn-A1* have been found in barley (*Vrn-H1*) on the 5H chromosome and in rye (*Vrn-R1*) on the 5R chromosome (Dubcovsky et al., 1998).

The promoter and the first intron are regulatory regions for the *Vrn-1* genes (Fu et al., 2005). The dominant *Vrn-A1* allele in the spring habit genotype is caused by a 1.4 kb deletion in the first intron of the *Vrn-A1* gene and without the deletion; winter habit genotypes are responsive to low temperatures (Fu et al., 2005). In spring wheat, the dominant *Vrn-A1* locus is not suppressed and a vernalization treatment is unnecessary for the plants to enter the reproductive stage (Loukoianov et al., 2005). After the vernalization requirement has been met in winter habit genotypes, the recessive *vrn-A1* locus is no longer suppressed and the plants enter the reproductive stage.

The dominant and recessive *Vrn-B1* and *Vrn-D1* alleles are regulated similar to the *Vrn-A1* allele (Fu et al., 2005). *Vrn-B1* has a 6.8 kb deletion in the first intron and *Vrn-D1* has a 4.2 kb deletion in the first intron. In barley, a deletion in the first intron of *Vrn-H1* is also the determining factor between a dominant or recessive allele (Fu et al., 2005). Loukoianov et al. (2005) proposed that *Vrn-2* is a repressor of the recessive *vrn-*

1 gene and a decrease in *Vrn-2* expression after vernalization correlates with an increase in the expression of the recessive *vrn-1* genes.

Besides its significant influence on vernalization, wheat chromosome 5A has been implicated in frost resistance in studies dating back as far as 1968 (Goujon et al., 1968, cited in Sutka, 1981). Numerous studies since then have suggested that 10 of the 21 pairs of wheat chromosomes are involved in frost resistance (cited in Sutka and Snape, 1989). A close genetic linkage between the vernalization locus and frost resistance locus, *Fr1*, on chromosome 5A was demonstrated by Sutka and Snape (1989). Subsequent RFLP mapping studies also confirmed the linkage of the *Vrn1* and *Fr1* loci on the distal long arm of chromosome 5A (5AL) (Galiba et al., 1995). Near-isogenic (Storlie et al., 1998) and substitution (Vágújfalvi et al., 2000) lines have been used to further elucidate the role of the *Vrn1-Fr1* 5AL chromosomal region on winter hardiness levels in wheat. More recently, a new locus, *Fr-A2,* was mapped 30 cM proximal to *Fr-A1* on chromosome 5A using *Triticum monococcum* RILs (Vágújfalvi et al., 2003).

Several studies have employed reciprocal NILs with interchanged *Vrn-A1/vrn-A1* loci to establish the influence of phenological development on the degree and duration of LT tolerance gene expression (Limin and Fowler, 2002, 2006; Fowler and Limin, 2004) in hardy (Norstar) and tender (Manitou) winter and spring wheat genetic backgrounds. These studies indicated that the LT_{50} difference (about 14ºC) between Norstar winter and Manitou spring wheat was due to the duration and rate of cold acclimation. Spring Norstar was able to cold acclimate faster than Manitou, but was unable to sustain the rate of acclimation observed for Norstar. Winter Manitou had a rate of acclimation similar to Manitou, but lower than Spring Norstar. It was, however, able to sustain the acclimation for a longer time than Spring Norstar. These studies

demonstrated that there are interacting developmental and low temperature tolerance genetic components that must be further elucidated in order to gain more comprehensive insights into the LT tolerance mechanisms (Fowler and Limin, 2004).

2.7 Low temperature perception and induction of cold acclimation genes

Cold acclimation requires recognition of low temperatures by cells and a signaling process to activate responsive genes that allow the plant to survive low temperatures. Sangwan et al. (2002) reviewed the initial steps of cold acclimation from cell recognition to activation of low temperature genes. Low temperatures will affect the membrane fluidity and cause the membrane to become rigid (Sangwan et al., 2002). As the membrane becomes rigid, the phospholipids become more unsaturated (Sangwan et al., 2002; Nishida and Murata, 1996). Rigid membranes will protect cells during cold acclimation by helping to maintain the cellular shape and prevent cellular components from being lost due to the loss of water during cold acclimation (Peterson, 1965). The cytoskeleton rearranges as the membrane becomes rigid and the microtubules become disassembled (Sangwan et al., 2002; Abdrakhamanova et al., 2003). It is thought that the cytoskeleton rearrangement allows the ion channels to open, which will cause an influx of ions (Sangwan et al., 2002).

Ions, such as calcium (Ca^{2+}), are released into the cell where they initiate the response to low temperatures by affecting the protein phosphorylation in cells (Sangwan et al., 2001). External and internal calcium are used by the cell during the cold response and increased availability of calcium enhances cold tolerance (Sangwan et al., 2002). Disruption of the calcium influx into the cell results in decreased cold tolerance. Calcium dependent protein kinases are activated by the increased cellular calcium levels

and activate a signal transduction cascade that results in expression of cold acclimation genes (Sangwan et al., 2002). The signal transduction cascade activated by cold is the stress activated MAP (mitogen-activated protein) kinase (SAMK) cascade (Sangwan et al., 2002).

Antifreeze proteins have been shown to be involved in protecting the cells from damage during freezing temperatures in rye (Griffith et al., 2005). During cold acclimation, water is removed from the cells and freezes in the intercellular spaces (Peterson, 1965). The antifreeze proteins are secreted by the cell into the intercellular space where they bind to ice crystals to inhibit ice growth and prevent ice recrystallization (Griffith et al., 2005). The reduction of ice growth reduces the chance of injury in plant tissues. The antifreeze proteins are also able to lower the freezing temperature of the leaves.

Glucanases were identified as antifreeze proteins that affect ice growth in winter rye (Yaish et al., 2006). β-1,3-glucanases and β-1,3;1,4-glucanases both have antifreeze activity, but β-1,3;1,4-glucanases have less activity than β-1,3-glucanases. The glucanases also have hydrolytic activity at -4ºC and another function, in addition to reducing the growth of ice crystals, is to protect the plant from pathogens during cold acclimation.

2.8 Genes activated during cold acclimation

2.8.1 *ICE1* and *CBF* family

The cold acclimation process requires a variety of genes to be activated. Transcriptional activators, which activate the cold tolerance structural genes, are triggered by low temperatures. In *Arabidopsis,* a transcriptional factor *ICE1* (Inducer of

CBF Expression) is activated by the low temperature signaling pathway and subsequently activates the CBF (C-repeat-Binding Factor) protein family (Chinnusamy et al., 2003). The CBF protein family is present in many plants including *Arabidopsis* (Chinnusamy et al., 2003), barley (Skinner et al., 2005) and wheat (Jaglo et al., 2001). Three transcriptional activators, *CBF1*, *CBF2* and *CBF3*, have been reported (Gilmour et al., 2000) to activate cold tolerance genes. *CBF2* is constitutively expressed and suppresses *CBF1* and *CBF3* expression (Novillo et al., 2004). Increased expression of *CBF1* and *CBF3* occurs early during cold acclimation. *ICE1* induces *CBF3* expression (Chinnusamy et al., 2003) and will cause an increase in *CBF1* expression. The high amount of *CBF1* and *CBF3* expression represses *CBF2* (Novillo et al., 2004). With *CBF2* repressed *CBF1* and *CBF3* activate transcription of the cold regulated (COR) genes. In time, the levels of *CBF3* and *CBF1* genes decrease allowing *CBF2* to be expressed. This slows the activation of cold acclimation genes and the cold tolerance response.

The expression of *WCBF1* from wheat is similar to the expression of *CBF1* from *Arabidopsis* with a short initial increase followed by a decrease during cold acclimation (Jaglo et al., 2001; Novillo et al., 2004). The expression of *WCBF1* increases during the first hour of cold treatment and then decreases over the next several hours of cold treatment (Jaglo et al., 2001). After 24 hours, there are low levels of *WCBF1* expression present. *Wcs120* and *Wcor410* are examples of cold-regulated (COR) genes (Danyluk et al., 1994; Kane et al., 2005) that are activated by the CBF protein family. Miller et al. (2006) have recently reported that there are 13 different *CBF* genes in wheat, 11 of which are present in the same region of chromosome 5 as the *Fr-A2* locus. *CBF1* from wheat and *Arabidopsis* were up regulated by low temperatures and dehydration (Jaglo et

al., 2001; Stockinger et al., 1997). CBF1 from *Arabidopsis* was not affected by increased levels of ABA (Stockinger et al., 1997).

2.8.2 *Wcs120*

The WCS protein family includes WCS40, WCS120, WCS66, WCS180 and WCS200 (Houde et al., 1995). WCS120 is a 50 kDa dehydrin that accumulates in the cytoplasm and nucleus of cells (Houde et al., 1992; 1995). *Wcs120* is located on the long arm of chromosome 6D in hexaploid wheat (Limin et al., 1997). Other members of the *Wcs120* gene family are present on the long arms of chromosomes 6A (*Wcs200)* and 6B (*Wcs66)*. *Wcs120* is up regulated by low temperatures, but is not up regulated by dehydration, heat shock, and ABA treatment (Houde et al., 1992). The level of WCS120 protein accumulation has been shown to be inversely related to the LT_{50} values where the highest amount of protein accumulation is at the point of maximum low-temperature tolerance (Fowler et al., 1996).

2.8.3 *Wcor410*

WCOR410 is a 28 kDa acidic dehydrin that accumulates around the plasma membrane and is present in lesser amounts in the intercellular space (Danyluk et al., 1994; Danyluk et al., 1998). This protein is absent from the cytoplasm and cellular organelles. *Wcor410* is located on the long arm of chromosomes 6A, 6B and 6D in hexaploid wheat (Danyluk et al., 1998). *Wcor410* is up regulated by low temperatures, dehydration, ABA and polyethylene glycol (Danyluk et al., 1998). It is also up regulated in lower amounts by salt and wounding stress.

In growth chamber experiments with Glenlea, Fredrick and Norstar, the highest level of expression of *Wcor410* was after 24 hours of cold acclimation, and then the transcript level decreased until the end of the cold treatment (Danyluk et al., 1994). Danyluk et al. (1998) observed that the WCOR410 protein follows the same pattern of accumulation as the *Wcor410* transcript, which increased to the highest amount by the fifth day of cold acclimation and then gradually decreased until the end of the cold treatment. Norstar and Fredrick winter wheat had a higher level of expression than Glenlea spring wheat during cold acclimation.

2.8.4 *Wcor14b*

The WCOR14b protein is located in the chloroplast of cells (Tsvetanov et al., 2000) and the amount of protein present in Cheyenne winter wheat increased over the first 28 days of cold acclimation (Vágújfalvi et al., 2000). *Wcor14b* is located on the long arm of chromosome 2A in wheat and expression is controlled by *Rcg1* (Regulator for cor14b gene) and *Rcg2* genes on chromosome 5A (Vágújfalvi et al., 2000). The expression of *Wcor14b* starts three to six hours after cold acclimation and increases until three days (Tsvetanov et al., 2000). *Wcor14b* expression then remains steady until day 20. The expression of *Wcor14b* is only affected by low temperatures and is not affected by darkness, NaCl, ABA (Abscisic Acid) or dehydration (Tsvetanov et al., 2000).

2.8.5 Other genes involved in low-temperature response

ZAT12 induces COR genes directly and is involved in down regulation of the *CBF1*, *CBF2*, and *CBF3* genes (Vogel et al., 2005) in the *Arabidopsis* low-temperature response pathway. This indicates that another pathway is involved in cold acclimation,

in addition to the CBF pathway. An understanding of these pathways will help in efforts to design strategies to increase the cold tolerance of plants.

Frost resistance (Fr) genes play a direct role in the plants response to low-temperatures (Vágújfalvi et al., 2003). The *Fr-A1* gene is located on chromosome 5A and is linked to the *Vrn-A1* gene (Galiba et al., 1995). The apparent linkage of *Fr-A1* to *Vrn-A1* has led to difficulties in determining its function due to the cosegregation of the vernalization requirement with the *Fr-A1* gene (Vágújfalvi et al., 2003). *Fr-2A* is located on chromosome 5A and is believed to be involved in the regulation of *Wcor14b* and a transcriptional activator of COR genes in wheat. *Fr-2A* has been mapped to the same region of chromosome 5A as Rcg1 and is thought to be the same gene (Miller et al., 2006).

2.9 Genes activated after cold acclimation

2.9.1 *TaVRT-1/WAP1*

TaVRT-1 (*Triticum aestivum* vegetative to reproductive transition-1) is a MADS-box transcription factor that is located on the long arm of chromosomes 5A and 5D and is involved in the transition from vegetative growth to reproductive growth in wheat (Danyluk et al., 2003). *TaVRT-1* is located on the same region of chromosome 5 as *Vrn-1* and the expression of *TaVRT-1* is controlled by vernalization, in winter wheat, and photoperiod. *TaVRT-1* is continuously expressed in spring habit plants and is only expressed in winter habit plants after vernalization saturation when they start losing cold tolerance and switch from the vegetative to the reproductive growth phase. Danyluk et al. (2003) observed that Spring Norstar has the constitutive expression of *TaVRT-1* and

Winter Manitou only expresses *TaVRT-1* after vernalization saturation, which indicates that *TaVRT-1* is possibly linked to the *Vrn-A1* locus.

WAP1 (Wheat APETALA1) is a MADS-box gene that is involved in the transition from the vegetative growth phase to the reproductive growth phase in wheat and is expressed during long days and warm temperatures (Murai et al., 2003). *WAP1* is thought to be the same gene as *TaVRT-1* because of a 98.8% similarity in the sequence. *WAP1* was isolated from spring wheat and *TaVRT-1* was isolated from winter wheat, which could explain the difference in the sequences.

2.9.2 *TaVRT-2*

Kane et al. (2005) speculate that *TaVRT-2* is a possible repressor of *TaVRT-1* during vernalization. *TaVRT-2* is a MADS-box transcription factor that is located on the short arms of chromosomes 7A, 7B and 7D. *TaVRT-2* is able to bind to *TaVRT-1* and other proteins involved in the flowering pathway. The level of expression of *TaVRT-2* is lower in spring than winter habit genotypes and there is a high level of expression of *TaVRT-2* during vernalization. Its expression then decreases after vernalization saturation in winter wheat.

2.10 Analysis of gene expression

Northern blot is a method used for gene expression analysis. Radioactive probes can be used with northern blots and the probe binds to mRNA corresponding to the gene of interest and the intensity of the radioactive signal will determine the amount of mRNA present (Ream and Field, 1999). Ribonuclease protection assay (RPA) is

another radioactivity-based method used for gene expression analysis (Ruteshouser et al., 1991).

There are disadvantages to northern blots and RPAs. A major disadvantage is that RNA is easily degraded, which makes northern blots and RPAs more difficult to perform. There can be some loss of RNA or degradation during gel electrophoresis and the transfer of RNA from the gel to the membrane. The lack of sensitivity of visualizing the radioactive signal will make small differences in expression difficult to determine.

The low sensitivity of the northern blots and RPA has led to the development of other methods for quantifying mRNA that are more sensitive. Quantitative and real-time PCR are more sensitive methods that are quicker and easier to perform. The use of PCR for expression analysis is a more sensitive method for determining expression levels. Using real-time PCR, the amount of transcripts present for a specific gene in any particular cell, tissue or experimental sample can be determined.

2.11 Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qrRT-PCR)

2.11.1 Theory

PCR is a method used to amplify a fragment of DNA. A specific sequence of DNA is amplified with oligonucleotide primers, usually 20-24 bases long (Hayashi, 1994). With PCR, billions of copies can be made from a single piece of DNA due to the exponential growth (doubling of fragments during each cycle) and PCR generally proceeds for 30 cycles after which the reaction becomes less efficient. After PCR, an agarose gel is used to determine the size of the amplified DNA fragment.

To study gene expression with PCR, the amount of PCR product has to be related to the amount of starting template. Due to inefficient amplification in the later cycles of the PCR reaction, the amount of PCR product, after 30 cycles, will not accurately relate to the amount of starting material (Bustin, 2004). Real-time PCR was developed as a more accurate method to determine the amount of starting template in a PCR reaction by measuring the increase of PCR products during each cycle (Higuchi et al., 1993). Fluorescent probes or intercalating dyes are used to measure the increase in PCR products during each cycle (Mackay et al., 2003; Wittwer et al., 1997; Tyagi and Kramer, 1996; Hart et al., 2001; Ponchel et al., 2003).

The different qrRT-PCR chemistries used include hydrolysis probes (Taqman probes) (Mackay et al., 2003), hybridization FRET probes (Wittwer et al., 1997), molecular beacons (Tyagi and Kramer, 1996) and scorpions™ (Hart et al., 2001). A hydrolysis probe has a quencher and a reporter dye on each end and attaches to single stranded DNA during strand separation (Mackay et al., 2003). As DNA polymerase synthesizes a new strand, it will cleave the probe and separate the reporter dye from the quencher dye to emit fluorescence. Hybridization probes consist of a donor dye that is bound to one probe and a receptor dye that is bound to the second probe (Wittwer et al., 1997). When the DNA is single stranded the two probes will bind in a head to tail orientation. The donor dye activates the receptor dye, which will cause fluorescence to be emitted.

Molecular beacons have a stem and loop configuration with fluorescent and quencher dyes on either end so there is no fluorescence emitted (Tyagi and Kramer, 1996). The loop structure has a DNA binding site. When the stem separates, the sequence in the loop binds to single stranded DNA present. With binding of the probe to

single stranded DNA fluorescence is emitted because the quencher and fluorescent dyes have separated. Scorpion probes (Hart et al., 2001) contain a primer connected to a stem and loop structure with a fluorescent dye on the 5` end and a quencher dye on the 3` end. After the primer synthesizes the new strand of DNA, the loop folds over and binds to a complementary sequence on the newly synthesized strand. This separates the fluorescent dye from the quencher dye and allows fluorescence to be emitted.

The different dyes that are used for qrRT-PCR are either bound to a probe or are intercalating dyes. The most widely used dye is SYBR Green I (Ponchel et al., 2003). This is an intercalating dye that will bind to any piece of double stranded DNA and emit fluorescence. Thiazole Orange (Kohler et al., 2005) is another intercalating dye that is used with real-time PCR. Dyes that are bound to probes include FAM, CY3, CY5 and TET (Chen et al., 2005; Moreira et al., 2005). These dyes can be multiplexed for a reaction because of the different excitation and emission wavelengths, which ensures that each signal is distinct. There are quencher dyes that absorb the signal from the fluorescent dyes to stop the signal from being emitted. TAMRA is a quencher and is used with fluorescent dyes such as FAM, which have emission spectrums similar to the excitation spectrum of TAMRA (Chen et al., 2005, van Rheenen et al., 2004). Fluorescence resonance energy transfer (FRET) allows the quencher dye to stop fluorescence from being emitted from the fluorescent dye by absorbing energy from the fluorescent dye (van Rheenen et al., 2004). FRET only occurs when the fluorescent dye and quencher dye are located close together. Black hole quenchers, which are dark quenchers (non-fluorescent chromophores), absorb fluorescence and convert it into heat (Moreira et al., 2005) that will reduce the extra fluorescence present from the fluorescence quencher dyes.

The exponential amplification phase of the amplification curve from qrRT-PCR is used to determine the amount of starting material. The cycle that the exponential growth phase crosses a pre-determined threshold is used to determine the Ct (threshold cycle) value (Livak and Schmittgen, 2001). The Ct values are used to quantify differences in expression between samples by determining the ΔΔCt values (Livak and Schmittgen, 2001).

Relative quantification determines the amount of gene product relative to a calibrator sample and comparisons can be made between different samples if the same calibrator is used for all samples (Livak and Schmittgen, 2001; Lee et al., 2006). The expression is determined as fold increase over the calibrator sample, so the calibrator is usually an untreated control sample or, for an analysis that measures differences in expression over a time frame, a sample that is at time zero (Livak and Schmittgen, 2001). For relative quantification, a housekeeping gene (a gene that's expression is not affected by the experimental treatment) is included for normalization (Livak and Schmittgen, 2001). Absolute quantification is used to determine the exact number of copies present (Lee et al., 2006). Absolute quantification requires the use of a standard curve derived from amplification of fold-dilutions of a target of interest. For example, the dilutions can be ten-fold in nanogram or picogram amounts of a gene cloned into a plasmid, a gene fragment or cDNA. Fold-dilutions can also be based on copy number calculations.

2.11.2 Applications of qrRT-PCR

Single nucleotide polymorphisms (SNP) can be detected with real-time PCR (Best et al., 2005). Probes can be designed that will discriminate a single base

difference between samples and will only bind to the identical sequence. Allele discrimination can also be performed with real-time PCR (Gilmour et al., 2006). Allele discrimination is dependent on which probe binds and fluoresces since probes can be designed for different alleles and multiplexed together in the same reaction. This method can be used to determine the presence of homozygous or heterozygous alleles. Real-time PCR can be used to detect pathogens in plants and follow the build up of inoculum within the plant before visual signs can be observed (Guo et al., 2006). For example, if a fungal pathogen infects plants in the field and if detected early, fungicides can be applied to prevent huge yield reductions.

Real-time PCR can be used to determine copy numbers of genes (Hernandez et al., 2004) and can be applied to transgenics to determine transgene copy number in the plant genome. Real-time PCR gives more accurate results than Southern blots with respect to transgene copy number. In outcrossing crops such as maize, real-time PCR can be used to detect gene-flow between transgenic crops and non-transgenic crops in the field (Pla et al., 2006). This method will allow for the determination of the percentage of transgenic (GMO - genetically modified organism) contamination in harvested seed and determines if the contamination is at acceptable levels (<0.9%) for food processing. In Europe, food that contains greater than 0.9% GMO contamination has to be labeled (Pla et al., 2006).

3.0 MATERIALS AND METHODS

3.1 Plant Material

The wheat (*Triticum aestivum* L.) genotypes used in this study included Norstar, Manitou, Spring Norstar (near-isogenic line of Norstar) and Winter Manitou (near-isogenic line of Manitou) (Limin and Fowler, 2002). Norstar and Winter Manitou are winter habit and Manitou and Spring Norstar are spring habit. Spring Norstar has the complete genetic background of Norstar, but the vernalization requirement is absent (*Vrn-A1/Vrn-A1* from Manitou replaces *vrn-A1/vrn-A1*). It will therefore have a spring habit when grown under warm temperatures and long days. Winter Manitou has the complete genetic background of Manitou, but has gained the vernalization requirement (*vrn-A1/vrn-A1*) from Norstar. These near-isogenic lines were developed by crossing Manitou and Norstar and the resultant progeny were backcrossed with each parent until the Spring Norstar and Winter Manitou lines were developed (Limin and Fowler, 2002).

3.2 Field procedures

Plants were grown in the field in a randomized complete block design with two replicates. Low-temperature tolerance was measured at pre-determined intervals using artificial freeze tests (Limin and Fowler, 1988). Two replicates were seeded on September 7th, 2004 and 20-30 plants of each genotype were removed from the field on each of three sampling dates: September 29, 2004, October 12, 2004 and October 26,

2004 for the first replicate and the second replicate was removed one day later. The crowns of the plants were used for the freeze tests and the leaves were stored at -80°C for RNA extractions. Soil temperature was measured using two probes located at crown depth (Fowler et al., 1999) in the field trials. Control plants of each genotype were grown in a growth chamber (20°C/16 h days) with no stresses applied.

3.3 Freeze tests

Five crowns of each replicate of each genotype were grouped together for each of five predetermined test temperatures, i.e., -7°C, -9°C, -11°C, -13°C, -15°C, and placed inside moist sand in an aluminum container for the freeze test. These samples were then placed inside a freezer at -3°C for 12 hours and the temperature was dropped 2°C every hour after the initial 12 hours. The samples were removed at the predetermined temperatures. After each sample was removed from the freezer it was placed into a refrigerator (4°C) and left overnight to thaw. After the samples were thawed, they were planted in soil and allowed to grow in a controlled environment at 20°C/16 h days for three weeks before survival was determined. Plants were considered to have survived if roots were present and the appearance of green leaves was observed. LT_{50} was then determined as the temperature at which 50% of the plants were killed by low temperature stress.

3.4 Total RNA extraction

All molecular biology techniques were performed according to Sambrook and Russell (2001) unless otherwise indicated. A modified Trizol™ (Invitrogen, Burlington, ON) reagent method was used to extract total RNA from leaves. Leaves were ground in

liquid nitrogen, mixed with Trizol and one milliliter of the mixture was transferred to a two milliliter tube. Chloroform (one milliliter) was added to each tube and centrifuged. The top aqueous layer was separated from the chloroform and ground material. The chloroform step was repeated. Isopropanol (500 µl) was added to the aqueous layer to precipitate the RNA and centrifuged to pellet the precipitate. The RNA was then washed with one milliliter of 75% (v/v) ethanol, dried and re-suspended in 100 µl of RNase-free water. The concentration of total RNA was determined with a spectrophotometer (Beckman Coulter DU800 series) by measuring absorbance at a wavelength of 260 nm (A_{260}) and purity was assessed by the ratio of the absorbance values at 260 and 280 nm, wherein a ratio of about 2.0 was considered a good indication of purity (Sambrook and Russell, 2001). Degradation of the RNA was checked by running a denaturing formaldehyde agarose RNA gel (1% w/v) according to Sambrook and Russell (2001). The presence of ribosomal RNA on the gel after ethidium bromide staining was an indication that the RNA was not degraded. Total RNA was cleaned to remove contaminants from the total RNA extraction that could interfere with downstream applications using the RNeasy Mini Kit (Qiagen, Mississauga, ON) following the manufacturer's protocol.

3.5 cDNA synthesis

Gene specific first strand cDNA was synthesized by reverse transcription from cleaned total RNA. Five micrograms of total RNA was used for DNase treatment with the Turbo™ DNase kit (Ambion, Austin, Tx) following the manufacturer's protocol. One microgram of DNase treated total RNA was used for cDNA synthesis with Superscript III™ (Invitrogen, Burlington, ON) reverse transcriptase.

3.6 Real-Time PCR

cDNA from the field samples and from non-stressed (cold) growth chamber-grown plants were used in the real-time PCR experiment. Real-time PCR was performed on a MX3000P® real-time PCR machine (Stratagene, La Jolla, Ca). The PCR reaction consisted of 2X Brilliant® SYBR Green I QPCR Master Mix (Stratagene, La Jolla, Ca), primers, reference dye (ROX, 30 nM) and one microliter of a 1/5 dilution of cDNA as template in a final reaction volume of 25 µl. For *Wcs120* (GenBank Accession no. AF031235) and *Wcor14b* (GenBank Accession no. AF207546) the PCR conditions were: 95ºC for 10 min and 40 cycles of 95ºC for 30 seconds, 60ºC for one minute and 72ºC for one minute each. For *Wcor410* (GenBank Accession no. L29152) the annealing temperature was 60ºC for one min with no extension. For *WCBF1* (GenBank Accession no. AF376136) annealing temperature was 65ºC for one minute and extension at 72ºC for 1.5 min. *Ubiquitin (UBI)* was used as the reference gene for *Wcs120, Wcor14b* and *Wcor410* and *Actin (ACT)* was used as the reference gene for *WCBF1*. The reactions were optimized by adjusting the primer concentrations, amount of template and reaction conditions until a single PCR product was amplified and the lowest Ct was determined for each gene. Appendix A shows an optimized amplification plot and dissociation curve for each gene.

The primer concentrations for *WCBF1, Wcs120, Wcor14b*, and *Wcor410* were 600 nM for the forward primer and 600 nM for the reverse primer. The primer concentration for *UBI* was 300 nM for the forward primer and 300 nM for the reverse primer. The primer concentration for *ACT* was 600 nM for the forward primer and 400

nM for the reverse primer. The primers used for each gene and the length of the fragment amplified are shown in Table 3.1.

Table 3.1: Primers and length of the amplified product in the real-time PCR experiments. *WCBF1, Wcs120, Wcor14b* and *Wcor410* are cold acclimation genes and *Ubiquitin* and *Actin* are reference genes

Gene	Primer Name	Primer Sequence 5' 3'	Primer Length (bp)	Product Size (bp)
Ubiquitn	F/UBI	CCTTGGCGGACTACAACATC	20	164
	R/UBI	GCAACGACAGACACAGACC	19	
Actin	F/ACT	CCAGCAATGTATGTCGCAATC	21	159
	R/ACT	GCCAGCAAGGTCCAAACG	18	
Wcor14b	F/14ba	CGACCACCAGACCCAGACC	19	124
	R/14ba	CGAGCGGCGAGGAAACAC	18	
Wcs120	F/w120b	TTCACGGACAACAGTGTG	18	108
	R/w120b	CTGCGTCTGTCTCTTGGATAAG	22	
Wcor410	F/410	CCTCCTCGGCAACCTCCTC	19	110
	R/410	TCTTGACCTCGGGCTCTTCC	20	
WCBF1	F/CB1a	ACTTGTTGGACGAGCACTGGTT	22	124
	R/CB1a	TTAGTTCCAAAGCGGCGTGTAG	22	

The threshold was determined as the first cycle, above the background fluorescence that all samples were in the exponential amplification phase. This was manually set within the exponential phase of the amplification plots for all the samples (Adams, 2006). After the real-time PCR experiment was completed, a dissociation curve was conducted in order to determine if only one product was amplified. A single peak in the dissociation curve implied that only the gene of interest was amplified. If more than one peak was present then the fluorescence values for the gene of interest

would not be accurate since SYBR Green I binds to all double stranded DNA. To determine if the peak corresponds to the gene of interest an agarose gel (1.5% w/v) was run to confirm that the expected size was detected. The amplified DNA fragment was also sequenced. A standard curve was conducted with a dilution series to determine the PCR efficiency. Efficiencies close to one allow for the use of the ΔΔCt method (Livak and Schmittgen, 2001) for determination of relative expression.

3.7 Real-Time PCR Data Analysis

The ΔΔCt method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) was used to determine the relative expression of the gene of interest. The expression rate of the gene of interest from the field samples was determined relative to the expression in a control plant grown in a growth chamber. This control sample is referred to as a calibrator.

$2^{-\Delta\Delta Ct}$ – relative expression (ΔΔCt)

ΔCt = Ct (gene of interest) – Ct (reference gene)

ΔΔCt = ΔCt (field sample) – ΔCt (calibrator)

After determining the ΔΔCt values the mean and standard error were calculated for each sampling date. The experimental design utilized in these studies was a 4 (genotype) x 3 (sampling dates) factorial in a two field replicate randomized complete block. There were two real-time PCR technical replicates for each biological replicate. Minitab statistical software was used to determine the means, standard errors and analysis of variance (ANOVA) for relative expression.

3.8 Sub-cloning of amplicons

Real-time PCR products were sequenced to determine if the amplicon corresponded to the gene of interest. The fragment amplified from real-time PCR was separated on a 1% (w/v) agarose gel, excised and purified from the gel with the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). The fragment isolated was cloned using the GeneJET™ PCR Cloning kit (Fermentas, Burlington, ON) following the manufacturer's protocols. The plasmid carrying the insert was transformed into *E. coli* (strain DH5α) and plated on LB medium. Plates were incubated overnight at 37°C. Colonies from the plates were re-cultured in LB broth. Plasmids were isolated using the QIAprep spin miniprep kit (Qiagen, Mississauga, ON), cut with restriction enzymes (XhoI and XbaI) and run on an agarose gel (1% w/v) to determine if the proper size fragment was present. Once the plasmids were confirmed to have the correct insert, they were submitted for sequencing (Plant Biotechnology Institute, National Research Council Canada, Saskatoon, SK).

3.9 Northern Blotting

Northern blots were performed according to standard protocols (Sambrook and Russell, 2001) to confirm the real-time PCR results. Total RNA extracted from the leaves was cleaned and treated with Turbo™ DNase kit (Ambion, Austin, Tx) to remove any genomic DNA contamination. Five micrograms of total RNA was loaded on a 1% (w/v) denaturing formaldehyde agarose RNA gel and electrophoresed for 1.5 – 2 hours at 100V. Ribosomal RNA present on the gel was visualized with ethidium bromide to check for equal loading of the RNA samples. Capillary transfer (Sambrook and Russell, 2001) was used to transfer the total RNA from the gel to a Hybond-N^+ membrane and the transfer proceeded for 2 hours. After the transfer, methylene blue stain (0.02% (w/v)

in 0.3M Sodium Acetate, pH 5.5) was used to visualize the total RNA on the membrane to determine if the RNA was properly transferred from the gel to the membrane.

The amplicon, from real-time PCR, for each gene of interest was run on an agarose gel (1% w/v), excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen, Mississauga, ON). The eluted fragment was quantified and used as a probe. Probe labeling was done by random priming using the Random Primer DNA labeling kit (Invitrogen, Burlington, ON) with 50 μCi of ^{32}P-dCTP for each probe. The probe was mixed with Church buffer (1M Na_2HPO_4; 0.5M EDTA; 20% SDS; ddH_2O) (Church and Gilbert, 1984) and incubated with the membrane at 65ºC overnight. The membrane was washed with 5X SSPE/0.1% SDS and 1X SSPE/0.1% SDS until the background radioactivity was removed. The membrane was then exposed to autoradiography film (Kodak BioMax MR). Each northern blot was repeated two times.

4.0 RESULTS

4.1 Soil temperatures

The mean soil temperatures during the duration of the experiment in the field are shown in Figure 4.1. The soil temperatures give an indication of when the plants experienced low temperatures in the field. Variation in temperature is expected in the field due to the unpredictability of the weather, but a gradual decline in temperatures over the three sampling dates occurred. With the decline in temperatures, an increase in expression of low-temperature tolerance genes was expected for the sampling dates considered in this study.

4.2 Freeze tests

Large differences were observed in the patterns of low-temperature acclimation of the genotypes considered in this study. Analysis of variance (Table B-1) indicated significant differences in low-temperature tolerance due to genotypes ($P<0.001$), sampling date ($P<0.05$) and their interaction ($P<0.001$). Differences due to replicates and their interactions with sampling date and genotypes were nonsignificant ($P>0.05$).

The cold acclimation curves show the LT_{50} values for each of the genotypes used in this study (Figure 4.2). The low temperature tolerance for Manitou increased to the second sampling date and then decreased by the third sampling date. The low temperature tolerance for Norstar, Spring Norstar and Winter Manitou increased over the three sampling dates. Manitou reached its maximum cold tolerance around the

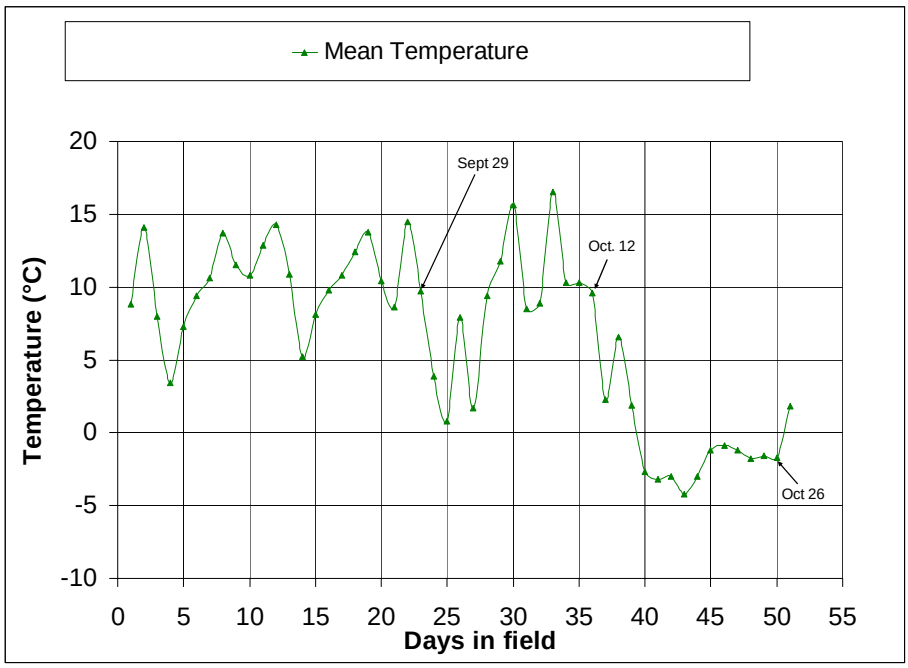

Figure 4.1: Mean soil temperature during plant growth in the field. 1st sampling date (September 29) is at 23 days, 2nd sampling date (October 12) is at 36 days and 3rd sampling date (October 26) is at 50 days in feild. The second field replicate was sampled a day later. The arrows indicate the sampling dates and when the freeze-tests were performed.

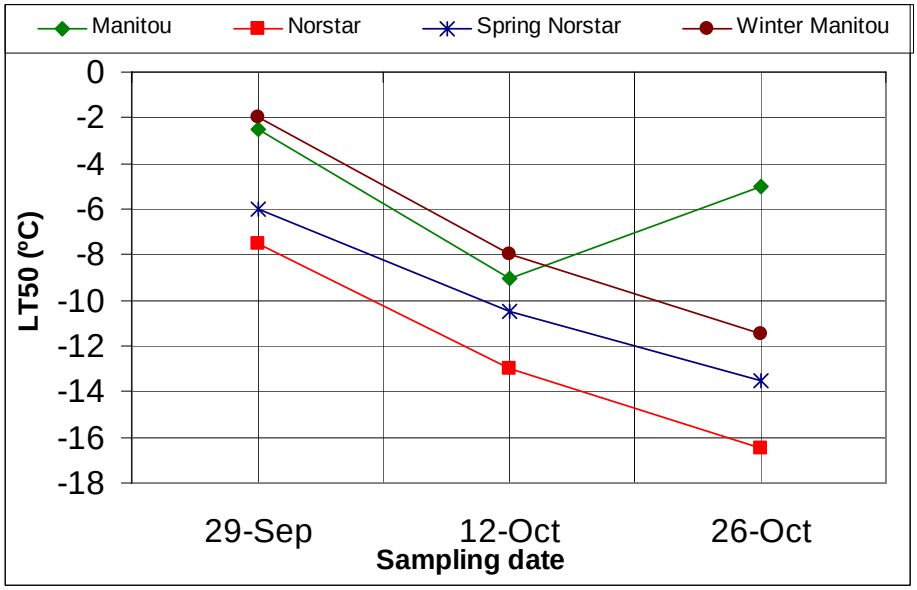

Figure 4.2: The low-temperature tolerance of wheat genotypes planted on September 7 and sampled on September 29, October 12 and October 26, 2004. SE of data points = 0.63.

second sampling date and Norstar, Spring Norstar and Winter Manitou reached their maximum cold tolerance on or after the third sampling date. Spring Norstar had a reduced level of cold tolerance compared to Norstar and Winter Manitou had an increased level of cold tolerance compared to Manitou.

4.3 Analyses of Variance (ANOVA) for gene expression studies

Large differences were observed in expression of the genes considered in this study. Analyses of variance indicated differences in expression levels due to genotypes were significant ($P<0.05$) for *WCBF1* (Table B-2), *Wcs120* (Table B-3), *Wcor14b* (Table B-4) and *Wcor410* (Table B-5). Differences due to sampling date were significant ($P<0.05$) for *WCBF1* and the genotype x sampling date interaction was significant ($P<0.05$) for all four genes. A significant genotype x sampling date interaction indicates that the pattern of expression of these genes was not consistent for the different genotypes as they prepared for winter. Differences due to field replicates and technical (laboratory) replicates within field replicates and their interactions with sampling date and genotypes were nonsignificant ($P>0.05$).

4.4 Amplification profiles and dissociation curves

An example of a typical amplification curve is shown in Figure A-3. The Ct for the gene of interest, *Wcs120* (blue), is about 23 compared to 20 for the reference gene, *Ubiquitin* (red). The difference in Ct between *Wcs120* and *Ubiquitin* is within an acceptable range (generally within 10 Ct of each other) for normalizing. A dissociation curve is shown in Figure A-4. The melting temperature of *Wcs120* (blue) is about 75ºC and for *Ubiquitin* is about 83ºC. Furthermore, as indicated by nonsignificant ($P>0.05$)

differences among technical (laboratory) replicates in the ANOVA, the amplification curve shows that the technical replicates for both genes were similar, indicating minimal pipetting errors during reaction setup.

4.5 Relative gene expression

4.5.1 Relative expression of *WCBF1*

WCBF1 expression (Figure 4.3) for Norstar, Spring Norstar, Manitou and Winter Manitou increased 118 fold, 15 fold, 4 fold and 15 fold, respectively, by the second sampling date and then decreased 54 fold, 13 fold, 3.5 fold and 12 fold, respectively, by the third sampling date. Norstar had the highest level of expression and Manitou had the lowest level of expression among all the genotypes. The expression of *WCBF1* in Spring Norstar decreased 103 fold compared to Norstar. Winter Manitou showed an 11 fold increase in *WCBF1* expression compared to Manitou. The northern blot for *WCBF1* showed expression for all sampling dates and controls except for the Norstar control sample (Figure 4.4).

4.5.2 *Wcs120*

The expression of *Wcs120* (Figure 4.5) for Norstar, Spring Norstar and Winter Manitou increased 138 fold, 55 fold and 104 fold, respectively, over the three sampling dates, which corresponds to an increase in cold tolerance of the three genotypes (Figure 4.2). For Manitou, the expression of *Wcs120* increased 77 fold to the second sampling date and then decreased 23 fold by the third sampling date. This corresponded to an

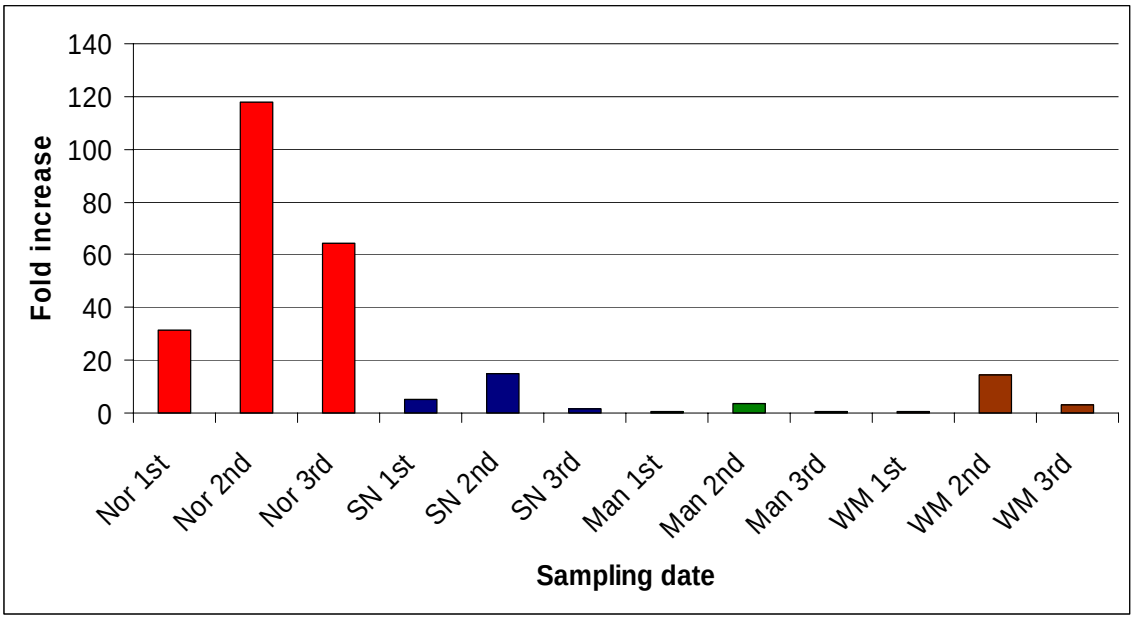

Figure 4.3: The fold increase of *WCBF1* for all genotypes over the three sampling dates [1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004.]. The expression is relative to the same calibrator (Control sample) for all samples. (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou). SE of the data points = 3.27.

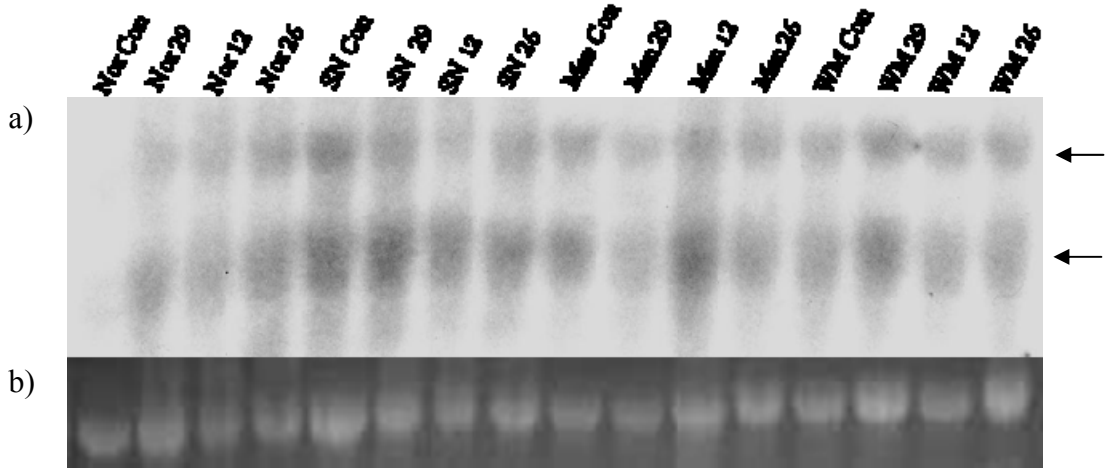

Figure 4.4: a) Northern blot of total RNA for *WCBF1* over the three sampling dates with the control sample (Con). (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou) (1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004). b) Ribosomal RNA from the RNA gel. Arrows indicate constitutively expressed transcripts.

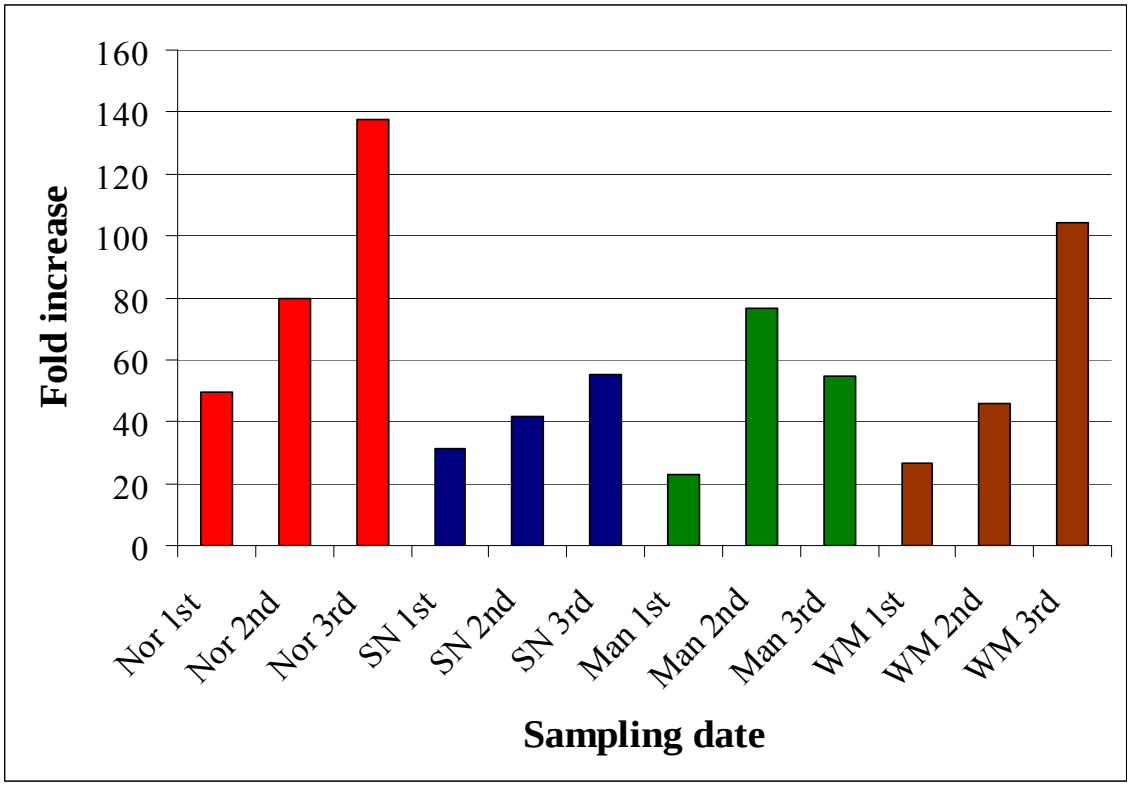

Figure 4.5: The fold increase of *Wcs120* for all genotypes over the three sampling dates [1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004.]. The expression is relative to the same calibrator (Control sample) for all samples. (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou). SE of the data points = 7.4.

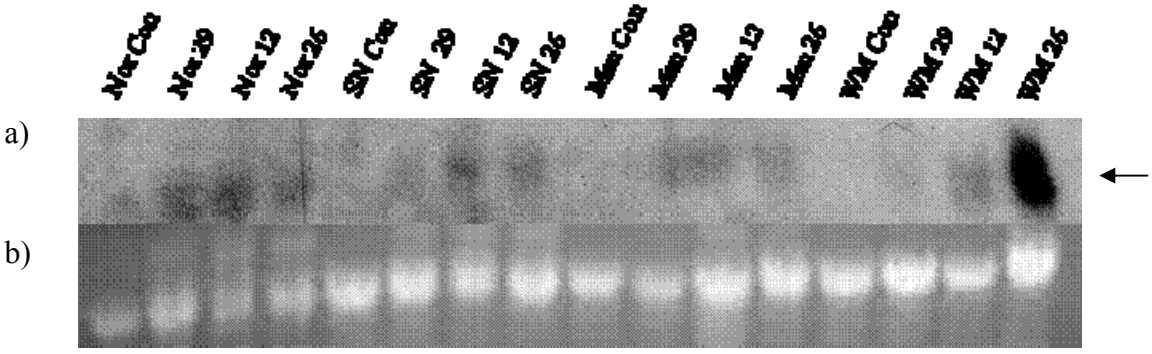

Figure 4.6: a) Northern blot of total RNA for *Wcs120* over the three sampling dates with the control sample (Con). (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou) (1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004). b) Ribosomal RNA from the RNA gel. Arrow indicates differentially expressed transcripts

increase and then a decrease in cold tolerance as observed in the cold acclimation curve (Figure 4.2). Spring Norstar had an 83 fold decrease in *Wcs120* expression compared to Norstar. Winter Manitou had a 77 fold and sustained increase in expression compared to Manitou. The northern blot for *Wcs120* (Figure 4.6) had an increase in expression to the second sampling date and then a decrease for Norstar, Spring Norstar and Manitou. Winter Manitou increased over the three sampling dates.

4.5.3 *Wcor14b*

The expression of *Wcor14b* (Figure 4.7) increased 3776 fold to the second sampling date and then decreased 2589 fold on the third sampling date for Norstar. The expression of *Wcor14b* in Spring Norstar and Winter Manitou increased 1616 fold and 2138 fold, respectively, to the second sampling date and the expression remained steady to the third sampling date. The expression of *Wcor14b* in Manitou remained steady over the first two sampling dates and then increased 361 fold by the third sampling date. Spring Norstar lost the high level of expression present in Norstar (2091 fold decrease) and Winter Manitou had a higher level of expression than Manitou (1164 fold).

Wcor14b transcripts were detected on the northern blot (Figure 4.8). Transcript levels for Norstar increased to the second sampling date and decreased by the third sampling date. *Wcor14b* transcripts in Spring Norstar and Winter Manitou increased to the second sampling date and remained steady to the third sampling date. Transcript levels in Manitou were steady during the first two sampling dates and then increased to the third sampling date. The expression of all genotypes matched the expression determined from the real-time PCR experiment.

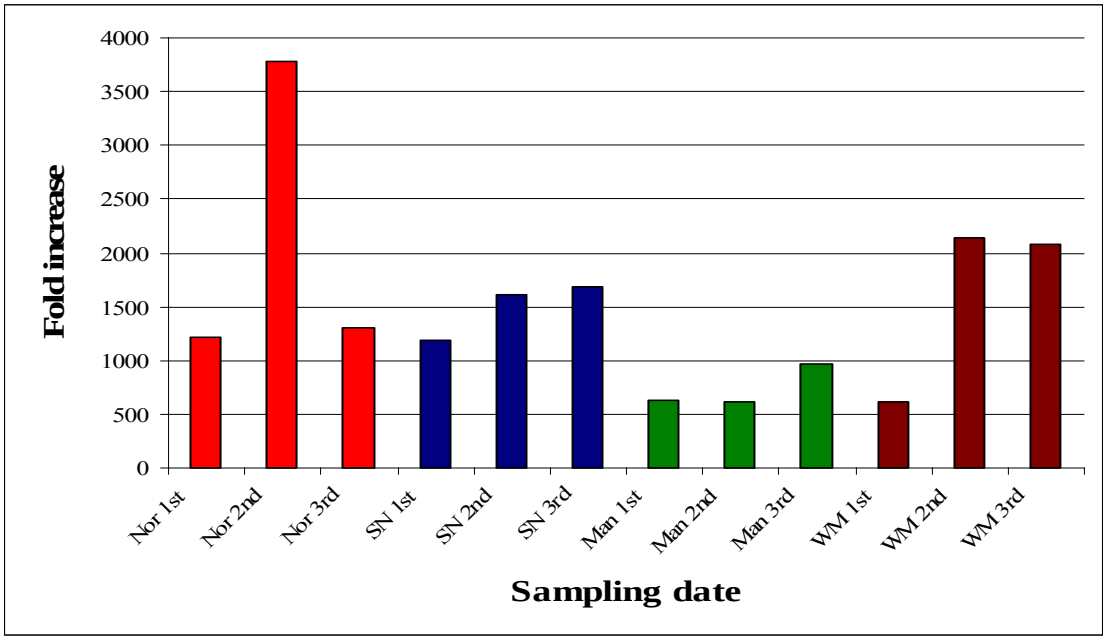

Figure 4.7: The fold increase of *Wcor14b* for all genotypes over the three sampling dates [1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004.]. The expression is relative to the same calibrator (Control sample) for all samples. (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou). SE of the data points = 312.

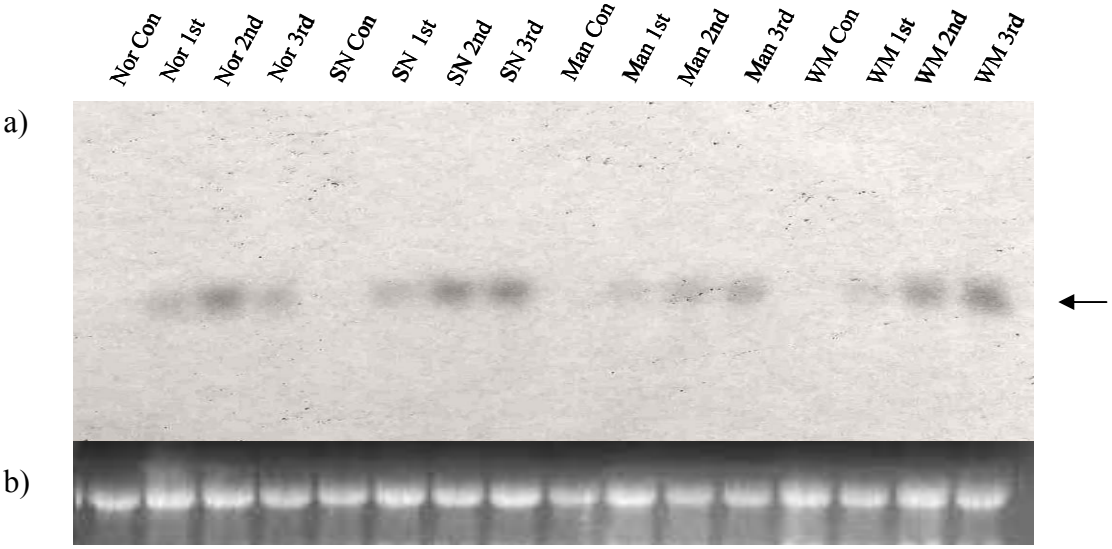

Figure 4.8: a) Northern blot of total RNA for *Wcor14b* over the three sampling dates with the control sample (Con). (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou) (1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004). b) Ribosomal RNA from the RNA gel. Arrow indicates differentially expressed transcripts

4.5.4 *Wcor410*

The expression of *Wcor410* for Norstar, Manitou and Winter Manitou increased 12 fold, 5 fold and 5 fold, respectively, to the second sampling date and decreased 5 fold, 2 fold and 2 fold, respectively, by the third sampling date (Figure 4.9). The expression of *Wcor410* for Spring Norstar decreased slightly (0.4 fold) by the second sampling date and increased 4.6 fold by the third sampling date. Spring Norstar had reduced expression on the second sampling date compared to Norstar (9 fold decrease). The level of *Wcor410* expression in Manitou and Winter Manitou was similar.

The northern blot for *Wcor410* (Figure 4.10) showed the transcript accumulation in Norstar, Spring Norstar and Manitou increased up to the second sampling date and then decreased by the third sampling date. Transcript levels in Winter Manitou increased over the three sampling dates. The transcript accumulation pattern for Norstar and Manitou was comparable to the expression pattern determined in the real-time PCR experiment.

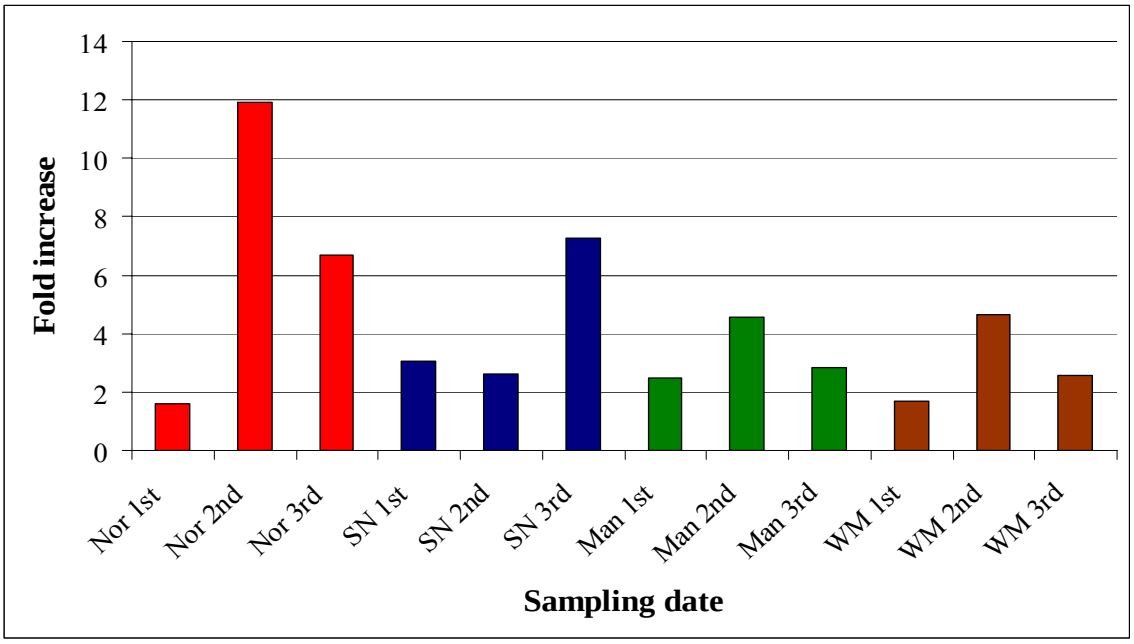

Figure 4.9: The fold increase of *Wcor410* for all genotypes over the three sampling dates [1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004.]. The expression is relative to the same calibrator (Control sample) for all samples. (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou). SE of the data points = 0.7.

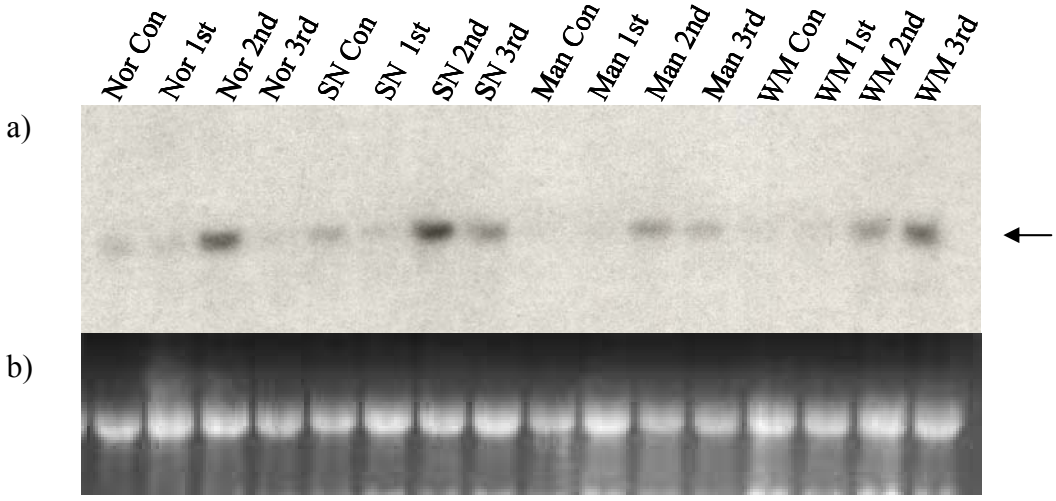

Figure 4.10: a) Northern blot of total RNA for *Wcor410* over the three sampling dates with the control sample (Con). (Nor - Norstar, SN - Spring Norstar, Man - Manitou and WM - Winter Manitou) (1st - September 29, 2004, 2nd - October 12, 2004, 3rd - October 26, 2004). b) Ribosomal RNA from the RNA gel. Arrow indicates differentially expressed transcripts

5.0 DISCUSSION

5.1 Cold acclimation

Day length sensitivity, vernalization requirements and increased final leaf numbers all interact to delay the vegetative/reproductive transition. It has been suggested that the cold tolerance genes are down regulated and cereal plants gradually lose their low-temperature tolerance once they switch to the reproductive growth phase (Mahfoozi et al., 2001). The near-isogenic lines used in this study (Limin and Fowler, 2002) were developed to verify this hypothesis and determine the effect of the *Vrn-A1* allele on cold acclimation in Manitou spring and Norstar winter wheat cultivars.

The difference in LT_{50} was 3.0ºC for Norstar compared to Spring Norstar and 5.5ºC for Manitou compared to Winter Manitou for samples taken on October 26 (Figure 4.2), indicating that the vernalization locus had an influence on the level of cold acclimation within the near-isogenic lines. Manitou had a LT_{50} that decreased until October 12 and then increased by October 26 (Figure 4.2). The improved low-temperature tolerance of Spring Norstar compared to Manitou was due to a higher final leaf number and photoperiod sensitivity (Limin and Fowler, 2002).

Manitou has a dominant *Vrn-A1* allele and does not have a vernalization requirement. Without the vernalization requirement, Manitou does not remain vegetative for as long as Winter Manitou and cannot gain a high level of cold tolerance. Winter Manitou has two recessive *vrn-A1* alleles and remains in the vegetative stage until the vernalization requirement has been met allowing for longer expression of cold

tolerance genes (Limin and Fowler, 2002) and improved low-temperature tolerance when the plant enters the winter (Figure 4.2).

The vernalization requirement of Winter Manitou allowed it to remain in the vegetative stage longer, which was reflected in increased low-temperature tolerance compared to the spring wheat cultivar Manitou. Spring Norstar does not have a vernalization requirement, but an increased final leaf number and short day sensitivity allowed it to remain vegetative (Limin and Fowler, 2002) and increase its low-temperature tolerance over the sampling period considered in this study.

5.2 Effect of *Vrn-A1* locus on gene expression

Near-isogenic lines were used in this study to determine the effect of the *Vrn-A1* locus on the expression of cold acclimation genes in Norstar and Manitou. Limin et al. (1997) have shown that the substitution of chromosome 5A from winter into spring wheat increases the expression of *Wcs120* and the accumulation of WCS120 protein during low-temperature acclimation. Winter habit plants with the dominant *Vrn-A1* locus (Spring Norstar) have been shown to lose the high level of cold tolerance (Limin and Fowler, 2002) and have a reduced level of *Wcs120* expression (Kane et al., 2005) due to a shorter vegetative stage. Conversely, substituting the recessive *vrn-A1* locus for the *Vrn-A1* locus in spring habit plants (Winter Manitou) increases the length of the vegetative phase, their ability to cold acclimate (Limin and Fowler, 2002) and elevates their level of *Wcs120* expression (Kane et al., 2005).

Spring Norstar had reduced levels of *WCBF1* (103 fold), *Wcs120* (83 fold), *Wcor14b* (2091 fold) and *Wcor410* (9 fold) expression compared to Norstar (Figures 4.3, 4.5, 4.7 and 4.9) and Winter Manitou had increased levels of *WCBF1* (11 fold),

Wcs120 (27 fold), and *Wcor14b* (1164 fold) expression compared to Manitou. The expression of *Wcor410* was similar for Manitou and Winter Manitou. These observations demonstrate that the *Vrn-A1* locus affects the expression of all the genes evaluated in this study except for *Wcor410* in Manitou. The *Vrn-A1* locus also had an effect on the pattern of expression for several of the genes.

The differences in gene expression within the near-isogenic lines are due to the presence or absence of the vernalization requirement. However, it is not known if the regulation of gene expression is directly related to the *Vrn-A1* gene and the vernalization requirement or is due to genes that are linked to the *Vrn-A1* locus, which were transferred with the *Vrn-A1* locus when the near-isogenic lines were produced. A study by Kobayashi et al. (2005) suggested that the *Vrn-1* loci are not directly involved in low temperature tolerance in wheat, but rather the *Vrn-A1/Fr-A1* interval affects the low temperature tolerance. Further studies are required to determine the exact mechanisms responsible for these differences in gene expression and the roles of genes closely linked to the *Vrn-A1* locus in low temperature tolerance.

5.3 Expression of *WCBF1*

Jaglo et al. (2001) grew Norstar winter wheat at a constant 4ºC in the growth chamber to study *WCBF1* expression during cold acclimation. Northern blot analyses revealed that *WCBF1* transcript increased during the first hour of cold treatment and then decreased during the next several hours. After 24 hours of cold treatment, there was still *WCBF1* transcript present. The field samples in the present study showed the same pattern of expression in the real-time PCR, with an increase and a decrease in expression for all genotypes (Figure 4.3). The main difference between the two

experiments was that plants were cold acclimated under a constant temperature in the growth chamber, or experienced temperature fluctuations in the field.

Norstar winter wheat had a higher level of *WCBF1* expression (114 fold) than Manitou spring wheat under field conditions (Figure 4.3). This difference in *WCBF1* expression between winter and spring wheat is thought to be responsible for the higher level of cold tolerance in winter wheat. The higher level of *WCBF1* expression in Norstar allowed for a higher accumulation of COR genes which are activated by the CBF protein family during cold acclimation (Shen et al., 2003) and provide greater protection from low-temperature damage. The CBF proteins are transcriptional activators that bind to the C-repeat sequence found in the promoter of many COR genes (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Jiang et al., 1996).

The high level of *WCBF1* expression in Norstar corresponded to a higher level of cold tolerance and the low level of *WCBF1* expression in Manitou was consistent with a lower level of cold tolerance. Spring Norstar and Winter Manitou both had higher levels of cold tolerance than Manitou, but only had slightly higher levels of *WCBF1* expression (11 fold) than Manitou indicating that the level of *WCBF1* expression does not relate directly to the level of cold tolerance in the plant.

The northern blot for *WCBF1* showed transcript accumulation for all sampling dates and the controls (Figure 4.4). The presence of transcript in all samples could be due to the constitutive expression of some specific *CBF* genes (Novillo et al., 2004). The highly conserved NLS (Nuclear localization sequence) and/or AP2 coding regions (Miller et al., 2006) are present on many *CBF* genes can affect binding of the *WCBF1* probe to other *CBF* genes. In wheat there are 13 identified *CBF* genes (Miller et al.,

2006) on chromosome 5A and the two bands identified relate to the different sizes of these *CBFs*.

5.4 Expression in COR genes

5.4.1 *Wcs120*

Fowler et al. (1996) studied the expression of *Wcs120* in growth chamber experiments where Norstar winter and Glenlea spring wheat were grown hydroponically and cold acclimated at 4ºC. *Wcs120* expression (northern blot) showed an initial increase to the second day of cold treatment and then a decline. The expression of *Wcs120* in spring wheat declined rapidly after two days while winter wheat maintained a high level of expression until 49 days and then declined. The field samples in the present study showed a different pattern of expression for all genotypes except Manitou (Figure 4.5). The pattern of expression in Norstar, Spring Norstar and Winter Manitou increased over the three sampling dates. The pattern of *Wcs120* expression in Manitou increased and then decreased.

A major difference between the growth chamber and field experiments is the fluctuation of temperatures in the field. The plants in the growth chamber were grown under a constant 4ºC and were not subjected to fluctuations in temperatures present in the field. A similarity between the growth chamber (Fowler et al., 1996) and field experiments was that the amount of *Wcs120* transcripts in Norstar was higher than in Manitou. This indicates that *Wcs120* transcripts accumulated at a higher level during cold treatment in the winter than in the spring habit genotypes.

The northern blot data for *Wcs120* correlated with the real-time PCR expression patterns discerned in Manitou and Winter Manitou (Figure 4.6). The pattern of

transcript accumulation in Norstar and Spring Norstar was, however, different. The transcript accumulation on the northern blot in the present study was similar to that of Fowler et al. (1996) in growth chamber studies, wherein there was an increase followed by a decrease in transcript for Norstar, Spring Norstar and Manitou. Houde et al. (1992) used a cDNA insert corresponding to *Wcs120* as a probe to detect mRNA transcripts on a Northern blot and found one major 1.7 kb transcript and four other transcripts ranging in size from 0.8 to 5 kb. To avoid this detection of multiple transcripts the short 108 bp fragment from the real-time PCR amplicon was used as a probe in the present study to specifically detect the *Wcs120* transcripts.

5.4.2 *Wcor14b*

The expression of *Wcor14b* was determined by northern blot in Mironovskaya 808 winter wheat cold acclimated at 4ºC in a growth chamber (Tsvetanov et al., 2000). *Wcor14b* transcripts increased during the first three days of cold treatment and remained steady until day 20. *Wcor14b* transcripts in Norstar showed an increase and decrease in the field samples from the present study (Figure 4.7). The expression of *Wcor14b* in Spring Norstar, Manitou and Winter Manitou showed an increase over the three sampling dates.

Norstar had a higher amount of *Wcor14b* transcripts than Manitou (2802 fold) (Figure 4.7) and the pattern of accumulation was different for the two genotypes. Manitou had a longer duration of transcript accumulation than Norstar. Manitou stopped cold acclimating after the second sampling date (Figure 4.7), but still had increased accumulation of *Wcor14b* transcripts over the three sampling dates, indicating that the *Wcor14b* transcript accumulation was not directly related to cold tolerance in Manitou.

The northern blot for *Wcor14b* (Figure 4.8) was in concert with the real-time PCR results (Figure 4.7) for all genotypes indicating that expression rates of *Wcor14b* were in agreement with both methods.

Northern blot analysis of *Wcor14* in a growth chamber study of Chinese Spring wheat and Mironovskaya 808 winter wheat showed similar levels of *Wcor14* expression in both these cultivars over a ten day cold acclimation period (Kobayashi et al., 2004). These results are in contradiction with the field grown plants in the present study. Kobayashi et al. (2004) however showed that protein accumulation over the 10 day cold-acclimation period was different between the spring and winter wheat, the protein accumulation being higher in the winter wheat. This indicates that the accumulation of *Wcor14b* transcripts may not be a potential marker for low temperature tolerance.

5.4.3 *Wcor410*

Danyluk et al. (1994) determined the expression of *Wcor410* in growth chamber experiments (6ºC day/2ºC night cold treatment) using Glenlea spring and Fredrick and Norstar winter wheat cultivars. The expression of *Wcor410* increased over the first 24 hours and then declined gradually over the next 35 days of acclimation. The level of expression for Fredrick and Norstar winter wheat was higher than the expression in Glenlea spring wheat at the end of the cold acclimation period. In contrast to the study of Danyluk et al. (1994), Kobayashi et al. (2004) showed that the expression of *Wcor410* was similar in Chinese Spring wheat and Mironovskaya 808 winter wheat over a 10-day cold acclimation period. In the present study, Norstar, Manitou and Winter Manitou cold-acclimated in the field and had the same pattern of *Wcor410* expression (Figure 4.9) as reported in the growth chamber experiments. Norstar winter wheat had a higher

level of *Wcor410* expression than Manitou spring wheat (7 fold) when grown under field conditions (Figure 4.9). Danyluk et al. (1994) also observed similar differences in levels of expression between spring and winter wheat in growth chamber experiments. In contrast, the pattern of expression for Spring Norstar from the field was different than that observed in the growth chamber experiments.

The similar pattern of expression between the northern blot (Figure 4.10) and real-time PCR (Figure 4.9) indicates that real-time PCR can be used to study the expression rates of *Wcor410* in Norstar and Manitou. However, the northern blot for *Wcor410* did not match the real-time PCR data for Spring Norstar and Winter Manitou. Only one replicate was used for the northern blot and two replicates were included for real-time PCR, but ANOVA for *Wcor410* showed there was no variation between the biological replicates that could contribute to the different expression rates.

5.5 Relation of *WCBF1* expression to COR gene expression

The C-repeat/dehydration-responsive element binding factor (CBF1) was first shown to be induced by low temperature and dehydration in *Arabidopsis* (Stockinger et al., 1997). Subsequently, Jaglo et al. (2001) showed that the wheat CBF1 was also induced by low temperature and was a potential activator of COR genes. A similar transcription factor (*TaDREB1*) was shown to be induced by low temperature, salinity and drought in wheat (Shen et al., 2003). The presence of the C-repeat in the promoter region of several COR genes has been described (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Jiang et al., 1996). With regards to *Wcs120*, a C-repeat sequence in its promoter region has been reported (Ouellet et al., 1998). Shen et al. (2003) were able to demonstrate the induction of *TaDREB1* by low temperature in

winter wheat after 30 min was followed by induction of *Wcs120* after one to two hours. The expression of *TaDREB1* decreased after 72 hours, while that of *Wcs120* was highest from 24-72 hours. Thus, as far as *Wcs120* is concerned there is evidence of *WCBF1* being the transcriptional activator. The expression of *Wcs120* in winter Norstar, Winter Manitou and Spring Norstar in the present field study corroborates the published results from the growth chamber studies of Shen et al. (2003). The discrepancy observed in *Wcs120* expression in Manitou spring wheat is most likely due to the very low expression of *WCBF1* in the field. Therefore transcriptional activation of the *Wcs120* could not be sustained.

The transcriptional activation of *Wcor14b* and *Wcor410* by *WCBF1* is less clearly understood. However, Crosatti et al. (2003) identified a C-repeat/DRE element in the promoter region of *Wcor14b* and therefore they suggested the involvement of CBF-like genes as transcriptional activators. Kobayashi et al. (2005) reported that wheat *CBF2* acted as a transcriptional activator of several COR genes including *Wcor14* when winter wheat was exposed to 4^{o}C temperature. They were able to demonstrate up-regulation of *WCBF2* in a winter wheat line carrying recessive *vrn-1* alleles at all three homoeologous loci and a greater suppressive effect of the *Vrn-A1* locus on *WCBF2* expression. Concurrently, with a northern blot, a steady increase in *Wcor14* expression up to 28 days of cold treatment in winter wheat was observed. In a NIL (near-isogenic line) carrying the *Vrn-A1* locus, expression of *Wcor14* was only observed after three days and had decreased to very low levels after seven days. In RT-PCR (reverse transcription PCR), *WCBF2* was expressed steadily from 3 to 28 days of cold exposure in the winter wheat, but was only expressed at low levels and in inconsistent patterns in the NIL carrying the *Vrn-A1* allele. The *WCBF2* used by Kobayashi et al. (2005) shares

89% similarity, at the nucleotide level, with the *WCBF1* gene used in the present study. The *Wcor14b* gene shares 86% and 84% similarity, at the nucleotide level, with *Wcor14c* and *Wcor14a*, respectively. The expression of *Wcor14b* in the field grown spring wheat genotypes in the present study also shows the low level of expression attributed to the presence of the *Vrn-A1* locus by Kobayashi et al. (2005). However, unlike their growth chamber experiments where expression was high on day three and decreased significantly on day seven in NIL with *VrnA-1* allele, the spring wheat in the present study showed sustained expression over the three sampling dates.

The potential activation of *Wcor410* by *WCBF1* is yet to be determined. In this study, except for Spring Norstar, the patterns of expression of the two genes were similar. Furthermore, the contradictory levels of expression for *Wcor14* and *Wcor410* in growth chamber experiments (Kobayashi et al., 2004) and the present field study indicates that there is genetic variation for these characters and the wheat regulatory mechanisms may be more complex then first indicated. Clearly, more experiments will have to be conducted to further elucidate the role of *WCBF1* in the transcriptional activation of COR genes.

5.6 Relation of gene expression to the level of cold tolerance

The expression levels (Figures 4.3, 4.5, 4.7 and 4.9) of the genes considered in this study were compared to the LT_{50} values (Figure 4.2) to determine if they reflected the level of plant cold tolerance. *Wcs120* was the only gene where the pattern of expression followed the pattern of cold tolerance for all genotypes. This indicates that the increase and decrease of *Wcs120* expression is indicative of the increase and decrease of cold tolerance in the field. The pattern of expression of the other genes

(*WCBF1, Wcor14b* and *Wcor410*) only corresponded to the level of cold tolerance for some genotypes in a few instances (*WCBF1* in Manitou, *Wcor14b* in Spring Norstar and *Wcor410* in Manitou and Spring Norstar) and there are several genotypes where the level of cold tolerance did not correspond to the level of gene expression (*WCBF1* with Spring Norstar and Winter Manitou, *Wcs120* with Spring Norstar, *Wcor14b* with Spring Norstar and *Wcor410* with Manitou and Winter Manitou). These results indicate that when only Norstar and Manitou were compared there was a close association observed between the level of cold tolerance and the level of gene expression. However, these relationships broke down when the near-isogenic lines were included in the comparisons.

There are several factors that can affect how gene expression relates to cold tolerance, and cytoplasmic mRNA levels could act as determinants for turn-over into proteins. Steady-state mRNA levels are consequent to differences in mRNA stability to a great extent (reviewed in Atwater et al., 1990; Green, 1993). Therefore, this may be a control point for the accumulation of proteins for certain cellular functions. Moreover, proteins that are only transiently needed by the cells are frequently encoded by unstable mRNAs, whose half-lives may even be less than 60 minutes (reviewed in Atwater et al., 1990; Green, 1993). Consequently, fluctuations in temperatures in the field (Figure 4.1) could influence not only the expression of cold acclimation genes, but also the stability of the message. In a recent study, Lidder et al. (2005) demonstrated that mRNA stability is controlled by the circadian clock for two transcripts, wherein regulation was due to mRNA stability at different times of the day. A similar cold-induced mRNA decay may be operating in wheat, precluding any correlation between transcript and protein accumulation.

Using simulation models, Fowler et al. (1999) demonstrated that there was a direct relationship between average daily soil temperature at crown depth and the accumulation of LT_{50} in field acclimated Norstar winter wheat. In the present field study, the mean soil temperature approached 10°C for the first two sampling dates and the lowest LT_{50} values for Norstar, Spring Norstar, Winter Manitou and Manitou were -17°C, -13.5°C, -11.5°C and -9°C, respectively (Fig. 4.2). These same genotypes had minimum LT_{50} values of -19°C, -8°C, -10°C and -5°C for Norstar, Spring Norstar, Winter Manitou and Manitou, respectively, when acclimated at 10°C in growth chamber studies (Fowler and Limin, 2004). Some interesting observations can be discerned from these data. For example, in the growth chamber study at 10°C, Manitou attained its lowest LT_{50} value (-5ºC) by 14 days (Fowler and Limin, 2004) compared to -9°C in the field at the second sampling date, which was approximately the same span of time (2 weeks) the plants were in the 10°C mean soil temperature window.

The real-time PCR data from the field samples indicated that the level of *Wcs120* transcripts in Manitou declined after the second sampling date (Fig. 4.5), corresponding to a loss in low-temperature tolerance observed in the controlled environment studies (Fowler and Limin, 2004). There was an increase in *Wcs120* transcript accumulation over the three sampling dates for the other three genotypes. In the growth chamber experiment at 10°C, winter Norstar reached its lowest LT_{50} between 42 and 49 days and after the third sampling date in the field (corresponding to 28 days from the first sampling date), winter Norstar had reached its lowest LT_{50}, -17°C, and the soil temperature dropped to the -2°C range (Fig. 4.5). Further sampling would have been required to establish if this was the lowest LT_{50} that was achieved, but there were clear similarities in responses in the controlled and field environments. The LT_{50} values were

similar for Winter Manitou and Spring Norstar under the growth chamber and field conditions with the LT_{50} values for the latter being reflected in increased *Wcs120* transcript accumulation. Even though similar trends are not apparent for the other genes under study, further quantitative real-time PCR and low temperature tolerance pattern discernment would be expected to eventually assist in the modelling of gene expression responses in both field and controlled environments.

The influence of fluctuations in soil temperature on the expression of low temperature-induced genes needs to be addressed further in future experiments. If a high level of protein is present in the cell when transcription of the mRNA is reduced, the protein is expected to remain in the cell for a longer period of time to protect the plant during rapid fluctuations in the level of cold stress (Fowler et al., 1996; Houde et al., 1995; Danyluk et al., 1998; Tsvetanov et al., 2000). As a result, the level of mRNA in the cell does not necessarily have to relate to the level of cold tolerance obtained by the plants growing in the field. Therefore, in studies of this nature, the amount of protein products of cold acclimation genes should be determined to provide a more complete picture of the plants' response to low temperature stress (Fowler et al., 1996).

6.0 CONCLUSIONS

1. The use of near-isogenic lines confirmed that the *Vrn-A1* locus had an affect on the cold tolerance of Norstar and Manitou. The dominant *Vrn-A1* locus caused a reduction of cold tolerance in Norstar (Spring Norstar), which was due to the loss of the vernalization requirement. The recessive *vrn-A1* locus caused an increase of cold tolerance in Manitou (Winter Manitou).
2. The use of near-isogenic lines in this study also showed the effect of the *Vrn-A1* locus on the expression of cold acclimation genes. In the real-time PCR experiments, the near-isogenic lines (Spring Norstar and Winter Manitou), had different expression levels than Norstar and Manitou for all genes except for *Wcor410* where Manitou and Winter Manitou had the same expression level.
3. Norstar had the highest level of expression of all genes considered in this study. The high level of cold acclimation gene expression was expected since Norstar has the highest level of cold tolerance. Similarly, the low level of gene expression was expected in Manitou since it has the lowest level of cold tolerance. The level of expression in Spring Norstar and Winter Manitou did not correspond to their level of cold tolerance indicating that the expression rates of these cold acclimation genes does not completely relate to the level or pattern of cold acclimation within the plants.

4. Wcs120 expression is a candidate marker for cold tolerance in wheat independent of vernalization requirement.
5. Real-time PCR is a valuable tool for the determination of quantitative expression of cold acclimation genes in wheat. Northern blots confirmed the *Wcor14b* transcript accumulation rate observed in real-time PCR and there was partial confirmation between real-time PCR and northern blots for *Wcor410* (Norstar and Manitou) and *Wcs120* (Manitou and Winter Manitou).
6. Real-time PCR provided a more sensitive and expedited method for studying gene expression rates compared to northern blots.

7.0 FUTURE WORK

Future work should focus on studying the expression of additional cold acclimation genes to get a better understanding of the complex gene interactions that regulate cold acclimation in the field. Besides genes already implicated in cold tolerance, transcripts identified through cDNA-AFLP (cDNA-amplified fragment-length polymorphism) transcriptome profiling (Ganeshan et al., 2006) and candidate genes identified through genetic mapping (Båga et al., 2007) can be assessed using real-time PCR for their expression rates and patterns under field acclimation conditions. The level of protein accumulation should be studied to provide a better understanding of how protein accumulation relates to the level of cold tolerance. It is expected that a combination of gene expression levels and protein accumulation measurements will provide a better overall understanding of the functions of the different components of the cold acclimation mechanisms. More frequent field sampling could also be included in future experiments to provide a more complete picture of the cold acclimation process.

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APPENDIX A

A-1 *WCBF1*

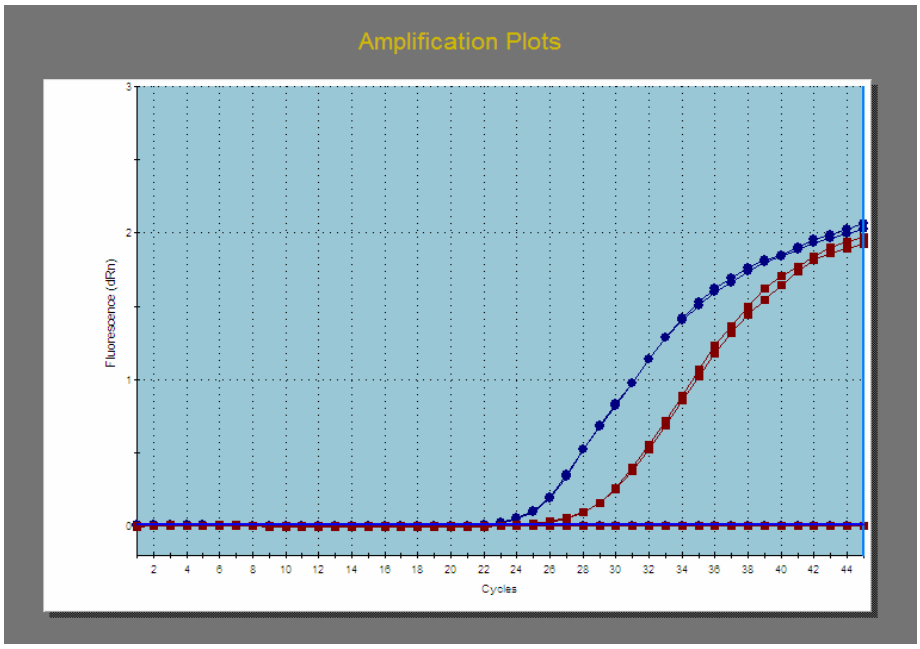

Figure A-1: The amplification curve for *WCBF1* (blue) and *ACT* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation in the PCR reaction.

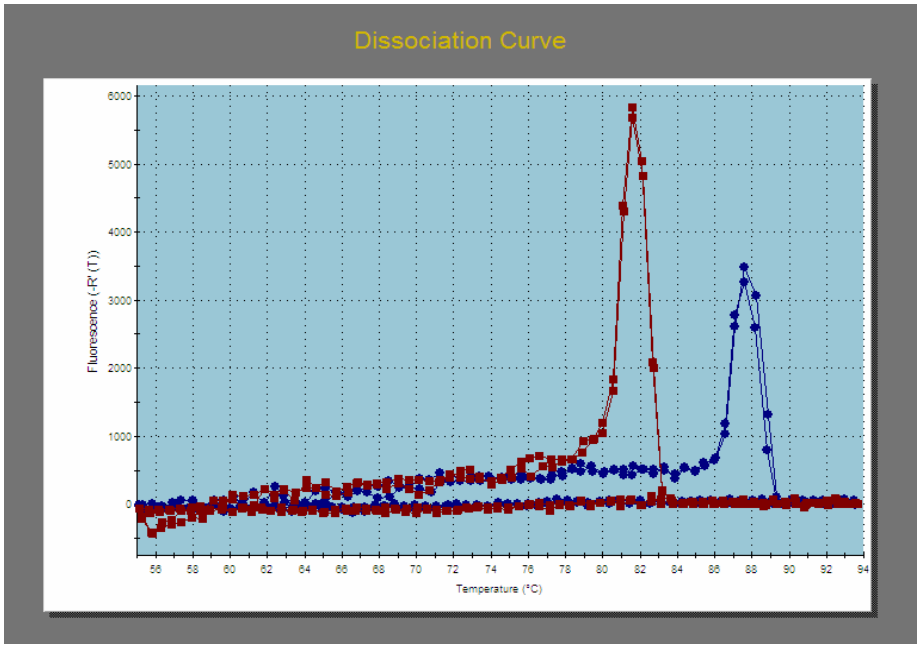

Figure A-2: The dissociation curve for *WCBF1* (blue) and *ACT* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation between the PCR replicates.

A-2 *Wcs120*

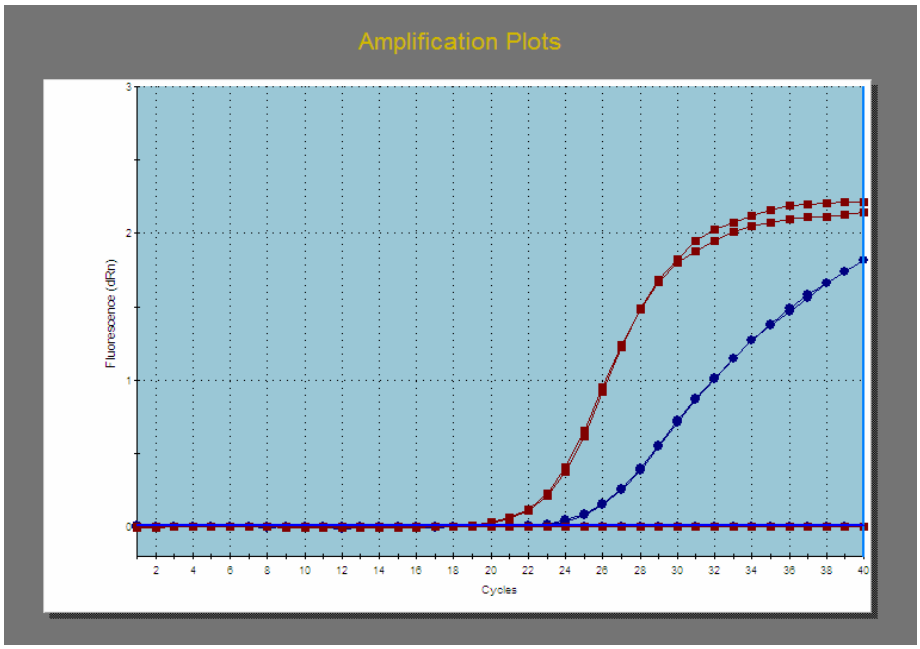

Figure A-3: The amplification curve for *Wcs120* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are present to show the variation in the PCR reaction.

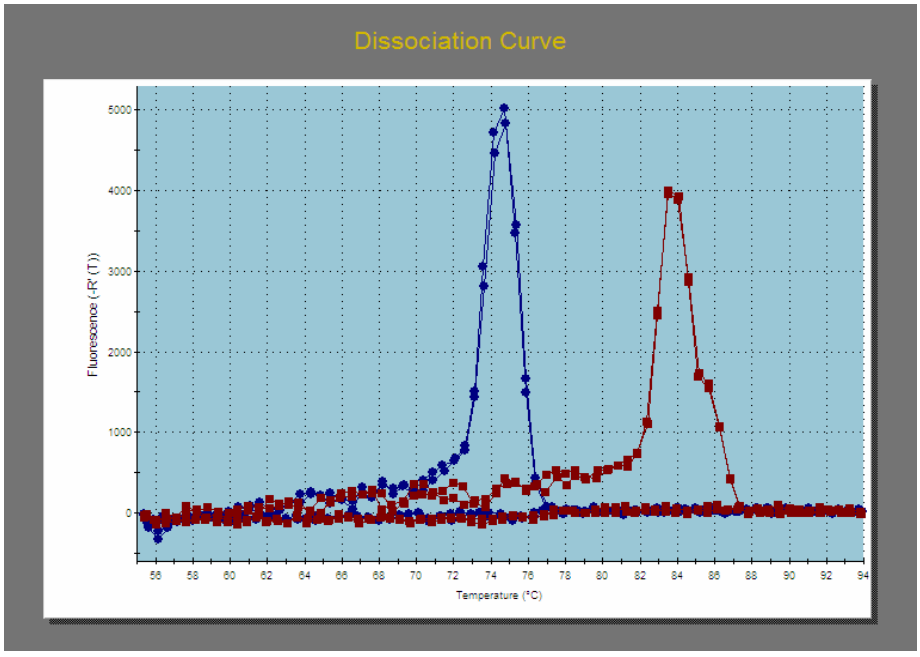

Figure A-4: The dissociation curve for *Wcs120* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are present to show the variation between the PCR replicates.

A-3 *Wcor14b*

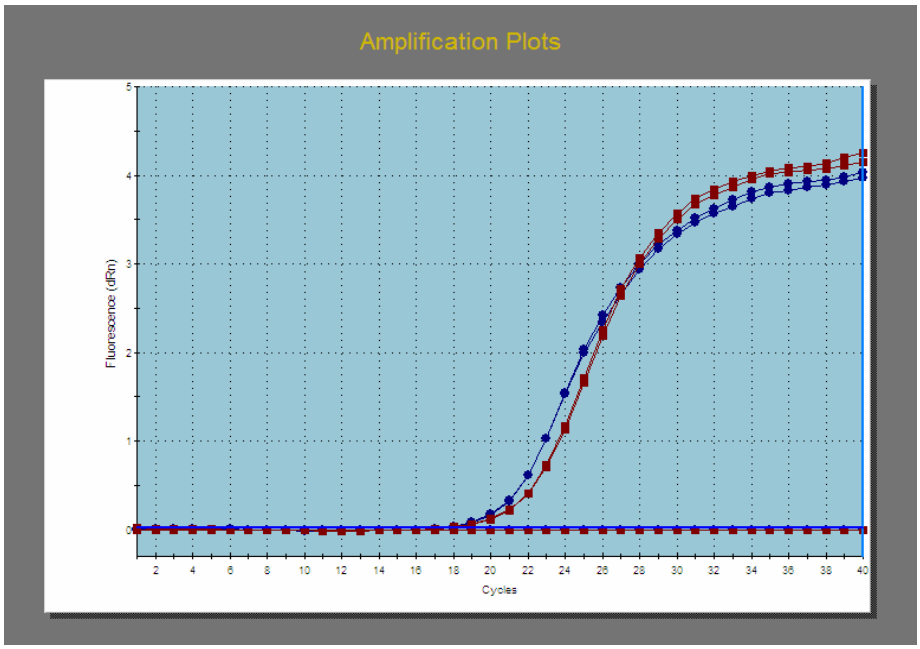

Figure A-5: The amplification curve for *Wcor14b* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation in the PCR reaction.

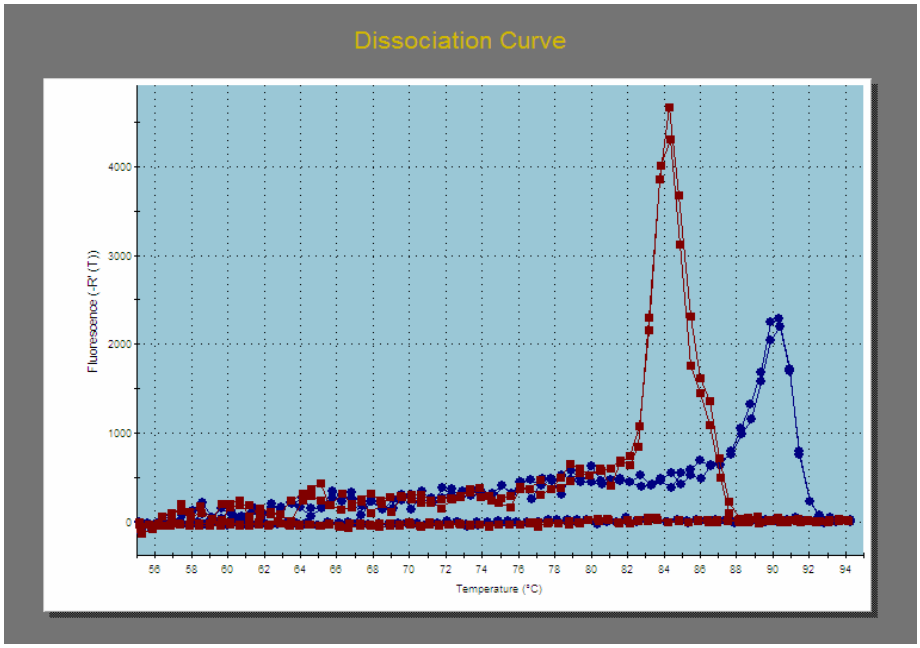

Figure A-6: The dissociation curve for *Wcor14b* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation between the PCR replicates.

A-4 *Wcor410*

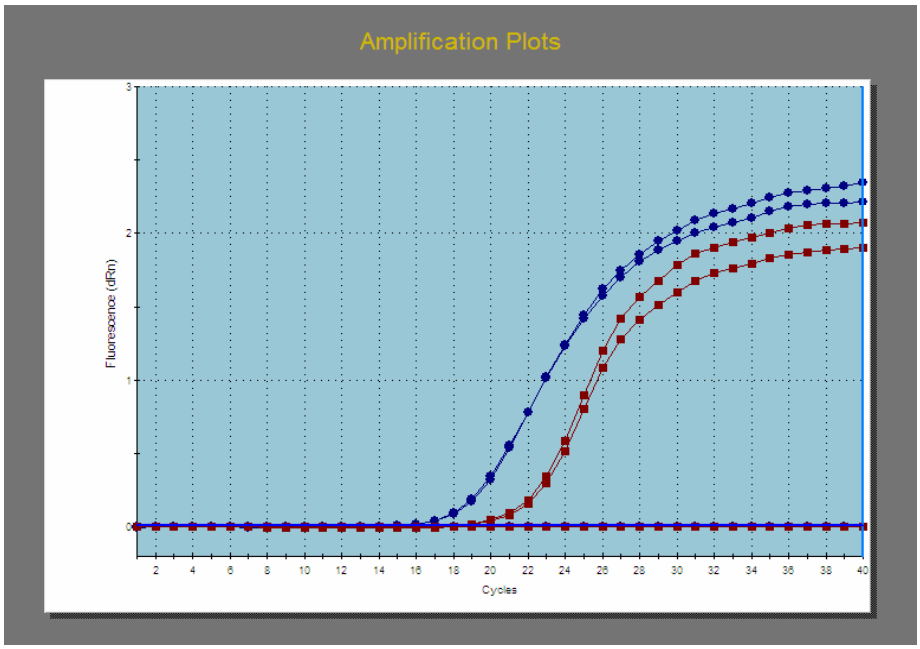

Figure A-7: The amplification curve for *Wcor410* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation in the PCR reaction.

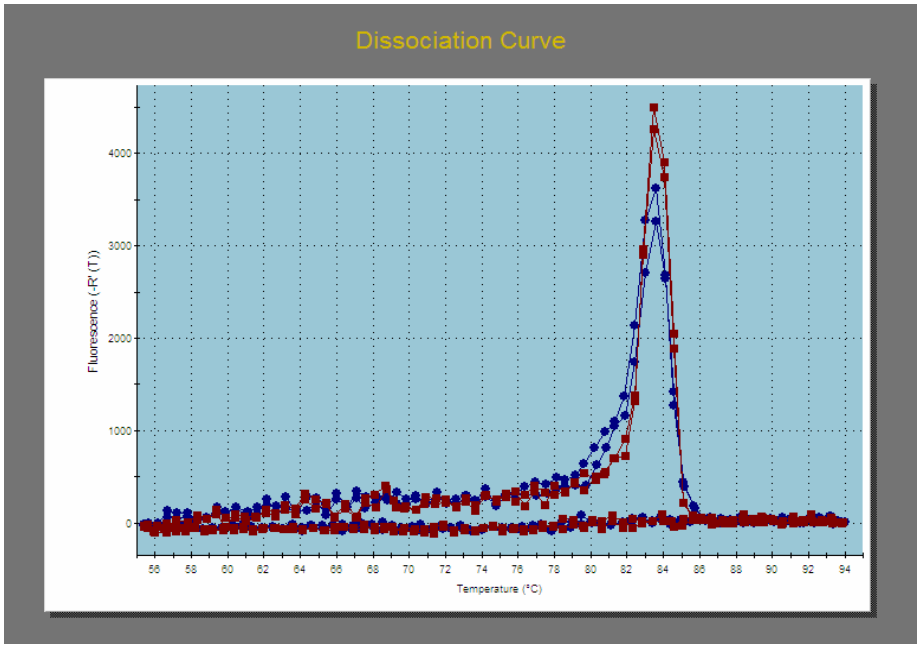

Figure A-8: The dissociation curve for *Wcor410* (blue) and *UBI* (red) for the third sampling date (October 26) of Norstar. Both PCR replicates are shown to show the variation between the PCR replicates.

Appendix B

Table B-1: Analysis of variance for the LT_{50} values of four wheat genotypes grown in the field in the fall of 2004 and sampled at three dates.

Source	DF	Adj MS	F	P
Genotypes	3	54.94	164.83	0.001
Sampling date	2	112.88	34.29	0.028
Replicates	1	0.67	0.24	0.691
Genotype* s. date	6	8.15	10.30	0.006
Genotypes*rep	3	0.33	0.42	0.745
S. date*rep	2	3.29	4.16	0.074
Error	6	0.79		
Total	23			

Table B-2: Analysis of variance for *WCBF1* of four wheat genotypes grown in the field in the fall of 2004 and sampled at three dates.

Source	DF	Adj MS	F	P
Genotype	3	13217.3	2028.89	0.000
Sampling date	2	3428.3	31.82	0.030
Genotype*Sampling date	6	1534.9	35.89	0.000
Biological replicates	1	1.1	0.02	0.924
Genotype*Biological	3	6.5	0.15	0.924
Sampling*Biological	2	107.7	2.52	0.161
Technical reps (Biological)	2	15.9	0.67	0.521
Genotype*Sampling*Biological	6	42.8	1.81	0.143
Error	22	23.6		
Total	47			

Table B-3: Analysis of variance for *Wcs120* of four wheat genotypes grown in the field in the fall of 2004 and sampled at three dates.

Source	DF	Adj MS	F	P
Genotype	3	5116.8	63.88	0.003
Sampling date	2	10641.0	6.20	0.139
Genotype*Sampling date	6	1304.0	5.89	0.024
Biological replicates	1	24.3	0.02	0.912
Genotype*Biological	3	80.1	0.36	0.783
Sampling*Biological	2	1716.2	7.75	0.022
Technical reps (Biological)	2	4.4	0.02	0.983
Genotype*Sampling*Biological	6	221.4	0.88	0.529
Error	22	252.9		
Total	47			

Table B-4: Analysis of variance for *Wcor14b* of four wheat genotypes grown in the field in the fall of 2004 and sampled at three dates.

Source	DF	Adj MS	F	P
Genotype	3	3797535	57.16	0.004
Sampling date	2	5085991	2.84	0.260
Genotype*Sampling date	6	2284195	5.88	0.024
Biological replicates	1	2276263	1.67	0.398
Genotype*Biological	3	66442	0.17	0.912
Sampling*Biological	2	1788529	4.61	0.061
Technical reps (Biological)	2	12844	0.11	0.893
Genotype*Sampling*Biological	6	388329	3.45	0.015
Error	22	112656		
Total	47			

Table B-5: Analysis of variance for *Wcor410* of four wheat genotypes grown in the field in the fall of 2004 and sampled at three dates.

Source	DF	Adj MS	F	P
Genotype	3	34.64	17.08	0.022
Sampling date	2	59.08	3.89	0.205
Genotype*Sampling date	6	29.57	16.51	0.002
Biological replicates	1	9.45	0.61	0.515
Genotype*Biological	3	2.03	1.13	0.408
Sampling*Biological	2	15.21	8.49	0.018
Technical reps (Biological)	2	0.50	1.10	0.352
Genotype*Sampling*Biological	6	1.79	3.89	0.008
Error	22	0.46		
Total	47			