Breeding for Winter-Hardiness in Winter Wheat (*Triticum aestivum* L.)

by

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ABSTRACT

BREEDING FOR WINTER-HARDINESS IN WINTER WHEAT (*TRITICUM AESTIVUM* L.)

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Winter survival is an essential trait for winter wheat cultivars that are grown in high-latitude growing regions such as Canada. The objectives of this study were to: 1) develop a high-throughput phenotyping tool that can be used to screen winter wheat for winter survival; 2) to identify genetic regions that are associated with winter survival and; 3) evaluate optimal candidate gene combination for better winter survival in Eastern Canada. In this study, a panel of 450 winter wheat cultivars was tested in three location-year combinations. The study demonstrated that images captured by unmanned aerial vehicle were able to be used to evaluate the winter survival of winter wheat. Genome-wide association study was conducted and resulted in identification of nine QTL that are associated with winter survival. Lastly, the combination of the *Fr-A2-T* haplotype and three copies of *Vrn-A1* was associated with better winter survival in Eastern Canada.

**KEYWORDS:** winter wheat, breeding, winter survival, high-throughput phenotyping, drone, genetic markers, QTL
DEDICATION

To Ali, a one of a kind professor, mentor, breeder and friend. Thank you for always believing in me.
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<table>
<thead>
<tr>
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<tr>
<td>CBFs</td>
<td>C-repeat binding factors</td>
</tr>
<tr>
<td>CN</td>
<td>copy number</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variation</td>
</tr>
<tr>
<td>COR genes</td>
<td>cold-responsive genes</td>
</tr>
<tr>
<td>CRT</td>
<td>C-repeat</td>
</tr>
<tr>
<td>CWWDP</td>
<td>Canadian winter wheat diversity panel</td>
</tr>
<tr>
<td>DHN</td>
<td>Dehydrin</td>
</tr>
<tr>
<td>DRE</td>
<td>Dehydration-responsive element</td>
</tr>
<tr>
<td>DREB</td>
<td>Drought responsive element binding</td>
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<tr>
<td>FGCC</td>
<td>Fractional green canopy cover</td>
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<td>Frost resistance</td>
<td>Fr</td>
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<td>Field survival index</td>
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<tr>
<td>GDD</td>
<td>Growing degree days</td>
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<td>GNDVI</td>
<td>Green Normalized difference vegetation index</td>
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<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
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<tr>
<td>H²</td>
<td>Broad-sense heritability</td>
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<tr>
<td>HOS1</td>
<td>high expression of osmotically responsive gene 1</td>
</tr>
<tr>
<td>ICE</td>
<td>Inducer of CBF expression</td>
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<tr>
<td>LD₅₀</td>
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<tr>
<td>LEA</td>
<td>Late Embryogenesis Abundant</td>
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<td>Least squares means</td>
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<td>LT₅₀</td>
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<td>MSD</td>
<td>mean squared difference</td>
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<td>NDVI</td>
<td>Normalized difference vegetation index</td>
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<td>Quantitative trait loci</td>
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<tr>
<td>RAB</td>
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<td>single nucleotide polymorphisms</td>
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<td>Unmanned aerial vehicle</td>
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<tr>
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<td>Description</td>
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<tr>
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1.1 Abstract

Winter wheat (*Triticum aestivum* L.) production in the high-latitude growing region faces many challenges including having high risk of winterkill. Winter wheat however, can provide many advantages over spring wheat include generating higher yield for other crops in the rotation, weed suppression, preventing soil erosion and avoiding stress factors that are prominent later in the summer. These advantages justify the production of winter wheat in Canada. Several genes have been identified in previous freezing tolerance studies that affect winter survival including *C-repeat binding factor (CBF)*-A12, -A14 and -A15. However, the effect of allelic differences of these genes on winter survival in Canada has yet to be investigated. There are numerous methods that can be used to screen for winter survival. The image-based phenotyping method has the advantage of being more precise, high-throughput and can potentially be applied in a breeding program. These image-based methods are also quantitative which can be useful to identify genomic regions that are associated with winter survival.

1.2 Introduction

Common wheat (*Triticum aestivum* L.) is a cereal crop that is used to make a variety of products including bread, pastry and noodles. Wheat has an enormous contribution to the economy of Canada as the top agricultural commodity produced in the country with 31 million tonnes produced in 2017 (Statistic Canada 2017). In general, wheat has been classified into two groups, winter and spring, based on their growth habit. Planting of spring wheat in Canada typically occurs from April to May and the crop is harvested around mid-August to mid-September (OMAFRA Field Crop Team 2017). In comparison, winter wheat requires exposure to vernalization temperature (~4°C) to transition into flowering Therefore, winter wheat in Canada is seeded around late August to October and will need to withstand the winter prior to being harvested around June to July the next year. The majority of the wheat produced in Canada is spring wheat which accounted for 70-75% of the wheat produced while winter wheat accounted for less than 10% (the remaining comes from durum wheat) (Statistic Canada 2017). The difference is caused by major challenges for winter wheat production in Canada including harsh winter conditions that can reduce winter survival and the difficulties of fitting winter wheat in
the rotation due to overlapping of harvest with optimal dates for seeding winter wheat (Fowler 2012).

Winter wheat, however, offers several benefits that justify its production in Canada. Current winter wheat cultivars have demonstrated significantly higher yield than the spring wheat cultivars with the yield of winter wheat cultivars ranging from 76 to 87 bushels/acre while spring wheat have yield ranging from 61 to 72 bushels/acre (Alberta Regional Variety Advisory Committee 2017). In addition, winter wheat has a demonstrated weed suppressing ability. Winter wheat is planted in the fall, so it has vigorous spring growth that can suppress weeds through crop light interception, which can lead to a reduced herbicide application (Drews et al. 2009; Beres et al. 2010). Winter wheat can concurrently serve as an overwintering cover crop to prevent soil erosion providing that they have established a reasonable amount of growth over the fall (Clark 2012). Winter wheat is also harvested earlier than spring wheat. This allows winter wheat to avoid stress factors that occur later in the season including drought and *Fusarium* head blight infection period (Fowler 2012). Lastly, winter wheat has a demonstrated ability to increase the plant-available nitrogen in the soil. Through this mechanism, including winter wheat in crop rotation has been shown to increase yield of maize and soybean crops that follow it, while potentially reducing the amount of necessary nitrogen input (Gaudin et al. 2015). In summary, winter wheat can provide many benefits to farming production, which justify producers’ decision of growing winter wheat despite the challenges that winter wheat production currently face in Canada.

### 1.3 Stress factors involved in winterkill

Winter in high latitude countries such as Canada is characterized by extreme freezing temperature and in some regions such as Western Canada the temperatures can go as low as -35°C. This introduces the risk of winterkill and deters the farmers from planting winter wheat. Depending on the province, farmers lose to winterkill on average 10% of the area planted based on a farm survey with maritime provinces losing as high as 20% of the area planted (Statistic Canada 2017). This forces the producers to replant with another crop in the spring and can create a large economic burden for them. Therefore, 70% of the winter wheat is produced in Southern
Ontario where the winter temperature is more moderate and more suitable for winter wheat production (Statistic Canada 2017). There has been, however, a lack of improvement in the winter survival of Canadian winter wheat, with the most winter hardy cultivar being Norstar, which was released in Alberta 40 years ago (Limin and Fowler 1991; Fowler 2012). This emphasizes the need for the development of winter wheat cultivars that have improved winter survival in order to ensure stable winter wheat production and potentially expand the feasible production region.

The severity and the nature of the winter stress can vary from region to region and from year to year. Rather than a single stress, winterkill is the results of several interacting abiotic and biotic factors that lower the fitness of the wheat plants during winter. There is a variety of stress factors in Ontario that can contribute to winterkill including freezing soil/air temperature, prolonged winter, freeze-thaw cycles, ice encasement, and snow mold.

1.3.1 Freezing temperatures

Ontario has milder winter temperature in comparison to other growing regions in Canada such as Western Canada, which often experience extreme temperature of as low as -35°C (Chen et al. 1983). However, a study by Andrews et al. (1997) has shown that, depending on the year, tolerance to freezing temperature is also important for winter survival in Ontario. When exposed to freezing temperatures, ice forms outside of the plant cell. Since water potential of ice is lower than that of water, the extracellular ice can draw water out of the cell. This can lead to freezing-induced cellular dehydration that can destabilize the cell membrane structure and contribute to winterkill of wheat (Pearce 2001).

1.3.2 Prolonged winter

The winter in Canada is prolonged and the temperature is usual below freezing from November to early April the following year. Gusta and Fowler (1977) had shown in an in-door study that the duration of freezing temperature could affect the survival of winter wheat. This likely happens because prolonged exposure to freezing temperature leads to a decrease in freezing tolerance of wheat making it more vulnerable to winterkill (Mahfoozi et al. 2001a).
1.3.3 Freeze-thaw cycles

Freeze-thaw cycles usually happen around early spring in Ontario when the temperature fluctuates between freezing and non-freezing. It reduces the freezing tolerance of winter wheat making them more susceptible to winterkill (Gusta and Fowler 1977). The fluctuation of temperature can induce cycle of plasmolysis and deplasmolysis that caused expansion-induced lysis of the plasma membrane and led to irreversible loss of cellular membrane (Steponkus 1984). In addition, freeze-thaw cycles can also lead to frost heaving, the expanding and contraction of soil that pushes the plants out of the ground and killing it in the process. Frost heaving is more likely to happen in heavy clay soil area such as that found in Essex and Lambton counties in Ontario (OMAFRA Field Crop Team 2017).

1.3.4 Ice encasement

Ice encasement can contribute to winterkill in a region with high winter precipitation or dramatic fluctuation of temperature in the winter (short period of above freezing temperature follows by freezing temperature). During ice encasement, the rate of gas exchange is significantly reduced for the wheat plant, which can have a damaging effect. For example, accumulation of high level of ethanol and carbon dioxide can lead to cytoplasmic acidosis and eventually plant death (Andrews 1996). Ice encasement happens more frequently in the Ottawa Valley (OMAFRA Field Crop Team 2017) and ice tolerance of winter wheat correlates strongly with winter survival in this region (Andrews et al. 1997).

1.3.5 Snow mold

Snow mold is a type of fungal disease that can destroy the leaves and crown tissue of wheat and cause the death of wheat plants in the region with persistent snow cover. The two frequently occurring snow mold pathogens in Ontario were Microdochium nivale var. nivale and Typhula incarnata (Schneider and Seaman 1987). Snow mold was shown to kill on average 12 to 15% of winter wheat, however, the disease was only detected near the northern limit of production along the Lake Huron snow belt and in Ottawa (Schneider and Seaman 1987).
In summary, winter survival is a complex trait with diverse set of stress factors that can negatively influence the fitness of the wheat plant over the winter. Breeding of cultivars with tolerance to specific winter-related stress will be essential to addressing winterkill concerns for each specific growing area in Ontario.

1.4 Management practices to improve winter survival

Many stress factors can contribute to winterkill of winter wheat, however there are various management practices that the farmers can incorporate to reduce winterkill.

1.4.1 No-till management

No-till management practices to build snow cover has been shown to be instrumental for improving winter survival of winter wheat in western Canada (Fowler 2012). Snow cover can provide warmer temperatures for the meristematic crown tissue of winter wheat and can reduce the fluctuation of temperature. This limits the amount of time that a winter wheat crop is exposed to detrimental freezing temperature and ensure better winter survival (Cox et al. 1986; Larsen et al. 1987). Higher stubble can establish a thicker layer of snow cover that can provide better insulation for wheat from the freezing temperature (Larsen et al. 1987). However, presence of high stubble is highly dependent on crop rotation practices. If the previous crop did not have high stem, furrow seeding can also improve winter survival by trapping the snow and insulating the crown tissue from the freezing temperature (Alessi and Power 1971).

1.4.2 Planting date

Studies have demonstrated correlation between planting date and winter survival of winter wheat (Fowler 1982; Andrews et al. 1997). Optimal planting date varies from early-September in central and eastern Ontario to mid-October in the southwestern region (OMAFRA Field Crop Team 2017). The consensus is that winter wheat is more susceptible to winterkill if planted later than the optimal planting date. Andrews et al. (1997) has suggested that this is likely due to the limited crown development and the reduced period of cold acclimation that comes with delayed planting. Planting date is dependent on the harvest time of the previous crop which is often soybean in Canada (Fowler 2012; OMAFRA Field Crop Team 2017), therefore, it can be
challenging for the producer in some years to plant on the optimal date to ensure better winter survival. On the other hand, if winter wheat was planted significantly earlier than the optimal planting date then it can also reduce the winter survival of winter wheat. In a study by Fowler (1982), six planting date were tested between August 1st to October 15th. Winter wheat planted in the earliest planting date (August 1st) had reduced winter survival similar to the later planting dates (October 1st). One of the possible explanations is that earlier planting can lead to additional growth of the subcrown internode, therefore, placing the crown tissue closer to the surface soil (Loeppky et al. 1989). Planting early can also increase the risk of snow mold, Hessian fly and barley yellow dwarf virus infection (OMAFRA Field Crop Team 2017).

1.4.3 Fertilization practices

Fertilization practices in the fall also can affect the winter survival of winter wheat. Excessive application of nitrogen in the fall seems to reduce winter survival and is likely attributed to the excessive fall growth (Grant et al. 1984). The benefit of phosphorous application was not consistently shown in previous work, however phosphorous application does counter the negative effect of excessive nitrogen application on winter survival (Pittman and Tipples 1978; Grant et al. 1984). Further work needs to be done to identify optimal fertilization program to ensure optimal winter survival of winter wheat.

1.4.4 Seeding depth

Lastly, seeding depth can also influence the winter survival of winter wheat. Historically, deeper seeding depth were shown to help winter wheat survive winter by providing thicker layer of insulation to the cold (Webb 1936). This was however shown not to be true in combination with no-till management (Loeppky et al. 1989). The authors compared the winter survival of shallow seeding (10-25mm) and deep seeding (25-50mm) of winter wheat (Loeppky et al. 1989) and found that in the testing year with differential winterkill, shallow seeding often resulted in better winter survival with the no-till management practice. This is probably because the snow cover that comes with no-till management provides adequate insulation to protect winter wheat in shallow planting. On the other hand, deeper planting depth resulted in slower growth stage progression that can make winter wheat more vulnerable to cold injury (Loeppky et al. 1989).
The importance of management practices on winter survival is highlighted in this section as they all contribute to the final winter survival of winter wheat. However, it is important to understand that depending on the producers’ economic situation and rotation practice, a certain management practice can be difficult to apply. In addition, management practice must be supported with good genetic tolerance towards winter stresses to ensure consistent winter survival of winter wheat.

1.5 Cold acclimation in wheat

Cold acclimation describes a process whereas wheat is exposed to non-freezing cold temperature (~4°C) to develop increasingly better freezing tolerance. Increasing the length of cold acclimation period can increase wheat’s freezing tolerance to a certain point. For the most freezing tolerant cultivar, Norstar, it survived temperature as low as -23°C after cold-acclimation (Laudencia-Chingcuancu et al. 2011). Without the cold acclimation period, the freezing tolerant genotype exhibits similar freezing tolerance to the freezing sensitize genotype (Limin and Fowler 2006). Prolonged exposure to freezing temperature, however, can have the opposite effect and can lead to the decrease of freezing tolerance (Gusta et al. 1997). In addition, when wheat is exposed to warm temperatures (~10°C), the plants can de-acclimate and loses freezing tolerance, however, they can re-acclimate when exposed to freezing temperature again (Mahfoozi et al. 2001a). These properties of cold acclimation indicate that the length of cold acclimation period late in the fall and the fluctuation of temperature in the field during the winter and early spring are important factors that affect freezing tolerance and ultimately winter survival.

Many changes occur in plants during the process of cold acclimation such as change in plasma membrane rigidity, sugar content (Winfield et al. 2010) and the upregulation of freezing tolerance genes (Thomashow 1999). In combination, these processes stabilize the membrane against freezing injury, protect protein denaturation and reduce the rate of cellular dehydration to help the plants survive the freezing temperature (Winfield et al. 2010), as described below.

First, in response to the change of temperature, there is a significant change to membrane fluidity and rigidity (Alonso et al. 1997). This change of membrane fluidity serves as a way for plant to perceive freezing tolerance. In Örvar et al. 2000, the rigidification of the plasma member
caused by chemical agent was shown to induce the expression of \textit{COR} (\textit{cold-responsive}) genes under room temperature. Jaglo et al. (2001) have reported that member fluidizer could prevent transcription of \textit{COR} genes even under cold temperature. These results supported the notion that the plasma membrane is involved in the perception of cold.

Next, soluble sugar content also changes during cold acclimation. Genes that are responsible for the synthesis of these sugar molecules were upregulated in response to cold (Winfield et al. 2010) which resulted in higher level of these sugars in the cell. Specifically, increase level of fructan, raffinose and sucrose were associated with enhanced freezing tolerance in rapeseed (Waalen et al. 2014) and wheat (Yokota et al. 2015). For example, Yokota et al. (2015) showed that a freezing-tolerant wheat cultivar accumulated higher level of fructan in comparison freezing-sensitive wheat cultivar during cold acclimation. These sugars in the cytoplasm can contribute to the development of better freezing tolerance by reducing the rate and extent of cellular dehydration (Livingston et al. 2006).

In response to cold temperature, gene expression in plant changes to initiate the cold acclimation process to ensure that the plants can survive freezing temperature. The well characterized cold signaling pathway is the \textit{C-repeat binding factors (CBFs)/drought responsive element binding (DREB)} dependent pathway which was initially characterized in the model specie \textit{Arabidopsis thaliana} (Chinnusamy et al. 2003). This pathway is controlled by \textit{inducer of CBF expression (ICE1)} which is a MYC-like basic helix-loop-helix upstream transcription factor (Chinnusamy et al. 2003). \textit{ICE1} functions by binding to the MYC \textit{cis}-elements at the promoter region of \textit{CBF3/DREB1A} to induce its expression (Chinnusamy et al. 2003). The \textit{ice1} mutant in \textit{Arabidopsis} was unable to induce the expression of \textit{CBF3/DREB1A} and could not cold acclimate to develop better freezing tolerance (Chinnusamy et al. 2003). Two \textit{ICE1}-like genes were identified in wheat, \textit{TaICE41} and \textit{TaICE87}, based on homology to \textit{ICE1}-like genes in rice (Badawi et al. 2008). Overexpressing \textit{TaICE41} and \textit{TaICE87} \textit{Arabidopsis} lines exhibited better freezing tolerance than control after cold acclimation and resulted in higher expression of cold-responsive genes such as \textit{AtCBF2} and \textit{AtCBF3} (Badawi et al. 2008). This evidence suggests that these two genes are involved in cold acclimation in wheat. However, the two \textit{ICE1-like} gene in wheat did not show temperature-dependent expression pattern which suggest that they are
regulated by other mechanism. A study in *Arabidopsis* suggests that *ICE1* is regulated by post-translational mechanism (Miura and Furumoto 2013). *HOS1* (*high expression of osmotically responsive gene 1*) is a ubiquitin E3 ligase in *Arabidopsis* and serves as a negative control on freezing tolerance by degrading *ICE1* (Dong et al. 2006). Substitution of the serine 403 of ICE1 to alanine to inhibit ubiquitylation of ICE1 by *HOS1* had shown to increase freezing tolerance (Miura et al. 2011). These evidences suggest that *TaICE41* and *TaICE87* are essential for freezing tolerance development in wheat but are likely regulated by post-translation mechanism.

However, no direct evidence has identified their respective negative regulator. Downstream of *ICE1* are the *CBFs* protein. *CBFs* are transcription factors that are part of the AP2/EREBP family of DNA-binding protein and they recognize DNA regulatory element designated the C-repeat/dehydration-responsive element (CRT/DRE) that is upstream of *COR* genes (Stockinger 2009). During cold acclimation, there is a rapid induction of *CBFs* expression in winter wheat (Winfield et al. 2010) and freezing tolerant wheat cultivars had higher expression of certain *CBFs* (Todorovska et al. 2014). The higher expression in *CBF* resulted in the upregulation of *COR* genes expression and its respective protein level. The degree of freezing tolerance had been associated with the level of *COR* genes expression and higher transcript level of *COR* genes were observed in freezing tolerant cultivars of wheat (Vítámvás and Prášil 2008; Dhillon et al. 2010; Yokota et al. 2015).

In addition to the role of the *CBF/DREB1* dependent pathway, expression studies have identified additional cold-responsive genes including *late embryogenesis abundant* (*LEA*), *dehydrin* (*DHN*) (Christov et al. 2007), and *responsive to abscisic acid* (*RAB*) (Winfield et al. 2010).

### 1.6 Regulation of freezing tolerance and winter survival

#### 1.6.1 CBF genes and their effect on freezing tolerance

*CBFs* belong to AP2 of DNA binding family superfamily, which all contain one AP2 DNA-binding motif. Within the family, *CBFs* are distinguished based on a conserved set of amino acid that is known as CMIII-3 (Nakano et al. 2006). CMIII-3 flanks the AP2 DNA binding domain and is unique to AP family subgroup IIIc. *CBFs* have other motifs such as CMIII-1, CMIII-2 and
CMIII-4 that they share with the other subgroups of AP2 superfamily (Skinner et al. 2005; Badawi et al. 2007).

Seventeen different CBFs were identified in hexaploid wheat on chromosome 5A in proximity with one another and this region is known as the Frost resistance (Fr)-A2 locus (Appels et al. 2018). These CBFs had low-temperature inducible expression and most of them demonstrated higher expression level in the more freezing tolerant cultivar of wheat (Badawi et al. 2007). These variations in the regulation and expression of CBFs can potentially be exploited to improve the freezing tolerance and winter survival of winter wheat.

Out of the 17 CBFs, CBF12, 14 and 15 are the most well-characterized and had demonstrated a significant contribution to freezing tolerance of wheat (Vágújfalvi et al. 2005; Sutton et al. 2009). CBF14 and 15 of winter wheat were transformed into barley and the transgenic barley exhibited an increase in freezing tolerance (Soltész et al. 2013). Also in recombinant substitution line, transcript levels of CBF14 and 15 were higher when the Fr-A2 allele from the frost tolerant wheat cultivar was present in comparison to the frost-sensitive allele (Pearce et al. 2013). To further understand the polymorphism within the three CBF genes, Zhu et al. 2014 sequenced the coding region and small section of 5’ untranslated region (UTR) and 3’UTR of the three respective homeologs of CBF12, CBF14, and CBF15 of 146 hexaploid wheat cultivars in the Fr2 locus. They found no sequence diversity within the B and D genome for CBF12, CBF14, and CBF15. However, on the A genome, they observed several single nucleotide polymorphisms (SNPs) and indel on CBF-A12 and CBF-A15. The polymorphism identified were linked for CBF-A12 and CBF-A15 and were, therefore, grouped into two haplotype FR-A2-T and FR-A2-S. The genotypes that carried the FR-A2-T haplotype had consistently shown better freezing tolerance and in-field winter survival in comparison to genotypes with FR-A2-S (Zhu et al. 2014; Würschum et al. 2017; Babben et al. 2018). Although, the exact cause for the differences is unknown, it may be the result of the polymorphism that distinguishes the two haplotypes or other genes that are linked to the haplotype. Another study confirms that there is no additional polymorphism in addition to the two haplotypes after sequencing CBFA12, CBFA14, and CBFA15 of 407 European wheat cultivars (Würschum et al. 2017). Interestingly, the two haplotypes were also observed within the different accessions of
wild donors of hexaploid wheat’s A genome including *T. dicoccoides* (n=25) and *T. Urartu* (n=2) (Zhu et al. 2014). From the current literature, there is a limited amount of allele variation for *CBF12, CBF14, and CBF15* that can be exploited for improving freezing tolerance of wheat. Gene editing approaches might provide opportunity for generation of new alleles of *CBFs*.

### 1.6.2 Vernalization genes

Many studies present evidences that suggest freezing tolerance of wheat is linked to the developmental stages (Galiba et al. 2009; Laudencia-Chingcuanco et al. 2011). Once the shoot apex transition from vegetative to reproductive meristem, wheat experiences a decline in its ability to induce expression level of freezing tolerance genes (Fowler et al. 1996; Sarhan et al. 1997; Dhillon et al. 2010). This is reflected in the decrease in freezing tolerance of wheat that has undergo vegetative to reproductive transition (VRT) (Laudencia-Chingcuanco et al. 2011). One of the main processes that regulate the timing of VRT is vernalization.

Vernalization is the acceleration of plant’s flowering process by exposing the plants to cold temperature. Winter wheat requires vernalization in order to flower and different genotypes of wheat have different vernalization requirement. Natural variation of vernalization requirement in wheat is mainly dependent on the allelic variation in the *Vernalization-1* (*VRN1*) gene on chromosome 5 (Yan et al. 2004; Fu et al. 2005). *VRN1* is a MADS-box transcription factor and an ortholog to the meristem identity genes *AP1/CAL/FUL* in *Arabidopsis thaliana* that regulates flowering time genes (Ferrándiz et al. 2000; Galiba et al. 2009). In wheat, there are three homoeologs of *VRN1* located on chromosome 5A, 5B and 5D (Nowak et al. 2014). The promoter and the first intron of *VRN-A1* was shown to be critical for the vernalization response. Deletion at the first intron (Fu et al. 2005) and insertion at the promoter region *VRN-A1* (Yan et al. 2004) correlate with the dominant allele, *Vrn-A1*, and confer spring growth habit to wheat. Dominant allele at any of the three homeologs of *VRN1* is sufficient to confer spring growth type (Golovnina et al. 2010; Kamran et al. 2014).

*VRN1* expression is induced during cold exposure and continue to increase throughout vernalization. There are mechanisms in place that control *VRN1* expression to regulate the timing for VRT (Xu and Chong 2018). For example, RNA-binding protein *GRP2* represses the
accumulation of VRN1 mRNA by binding to the first intron of VRN1 (Xiao et al. 2014). During vernalization however, VER2, a jacalin (subfamily of lectin-like proteins), increases in transcription (Yong et al. 2003) and inhibits the function of GRP2 which results in higher mRNA accumulation of VRN1 (Xiao et al. 2014). Once VRN1 reaches a certain level, it is then able to repress the expression of VRN2 which is a zinc-finger-CCT domain transcription factor that represses flowering (Loukoianov et al. 2005; Trevaskis et al. 2007; Chen and Dubcovsky 2012). VRN2 represses flowering by down-regulating the transcript level of VRN3 which is an RAF kinase inhibitor-like protein that promotes flowering (Distelfeld and Dubcovsky 2010). Thus, the increase in expression of VRN1 represses VRN2 which enables the transcription of VRN3 under long-day condition that can occur in the spring (Shimada et al. 2009). VRN3 can then further induce expression level of VRN1 to form a positive feedback loop (Muterko et al. 2015; Xu and Chong 2018). Once VRN1 reaches the threshold level, VRT is then initiated (Muterko et al. 2015).

VRN1 has been known to be important for the process of vernalization, however, recent work provided insights into its function in freezing tolerance. Historically, winter wheat has better freezing tolerance than spring wheat. Near-isogenic line for VRN1 was developed for the cultivar Norstar (winter wheat) and Manitou (spring wheat) to make Norstar cultivar that carry the dominant Vrn1 allele and Manitou cultivar with the recessive vrn1 allele (Limin and Fowler 2006). When comparing the freezing tolerance of the near isogenic lines (eg: Norstar with vrn1 and Norstar with Vrn1), they noticed that the near-isogenic line that carry the recessive vrn1 allele has significantly better freezing tolerance (Limin and Fowler 2006; Laudencia-Chingcuanco et al. 2011). This provides evidence that VRN1 has pleotropic effect in freezing tolerance. This argument is supported by the negative correlation observed between the transcript level of VRN1 and COR genes (Fowler et al. 1996; Kobayashi et al. 2005). Furthermore, Dhillon et al. (2010) demonstrated in diploid wheat (Triticum monococcum) mutants that the mutant plant with one copy of VRN1 showed reduced freezing tolerance and had lower transcript level of multiple CBF and COR genes than the mutant plant without VRN1. In barley, higher VRN1 expression also correlated with decrease in CBF expression and it was shown that VRN1 can bind directly to the promoter region of CBF2, 4, and 9 (Deng et al. 2015a). These evidences
support the notion that, in addition to vernalization, VRN1 also plays a role in regulating freezing tolerance through direct binding of CBFs. Further work is needed to identify the direct targets of VRN1 in wheat.

### 1.6.3 Copy number variation

Copy number variation (CNV) is defined as the variation in copy number of DNA segment that is 1kb or larger in comparison to the reference genome (Zmieńko et al. 2014). Some examples relevant to wheat include the effect of CNV of genes VRN-A1 and PPD-B1 on the flowering time and \( RHT-D1b \) on the dwarf phenotype (Díaz et al. 2012; Li et al. 2012). CNV of \( CBF \) genes at \( Fr-A2 \) locus and VRN-A1 have also shown to impact winter hardiness in wheat (Zhu et al. 2014; Sieber et al. 2016; Würschum et al. 2017). CNV of \( CBFs \) at \( Fr-A2 \) locus and VRN-A1 were shown to impact winter survival in wheat (Zhu et al. 2014; Sieber et al. 2016; Würschum et al. 2017). In hexaploid wheat, CNV variation were observed for \( CBF-A12, CBF-A14 \) and \( CBF-A15 \) (Zhu et al. 2014; Würschum et al. 2017). The level of influence of CNV of \( CBFs \) on freezing tolerance and winter survival differ between studies. Zhu et al. 2014 observed significant correlation (r=0.56) between copy number (CN) of \( CBF-A12 \) and \( CBF-A14 \) with freezing tolerance in a winter wheat diversity panel (n=65). At the same time, they observed that the \( FR-A2-T \) haplotype was associated with higher copy number of \( CBF-A12 \) and \( CBF-A14 \), therefore it cannot be determined whether the difference in freezing tolerance was due to the CN of \( CBFs \) or the \( FR-A2 \) haplotype. However, Wurschscum et al. 2017 demonstrated that CNV at \( CBF-A14 \) accounted for 24.3% of the genotypic variance for winter survival in a European winter wheat diversity panel while \( Fr-A2 \) haplotype only accounted for 0.7% therefore highlighting the importance of CNV of \( CBF-A14 \) on winter survival. The importance of CN of \( CBF-A14 \) was further supported by a study done in European durum wheat. \( CBF-A14 \) CNV was correlated with freezing tolerance and explained 91.6 % of genotypic variance in freezing tolerance of European durum wheat (Sieber 2016). Out of the 184 durum wheat line within the diversity panel, 179 genotypes carry the \( Fr-A2-T \) haplotype, therefore the study was able to separate the effect of \( CBF-A14 \) CNV from \( Fr-A2 \) haplotype. In short, the relative importance of \( CBF-A14 \) CNV on winter survival varies depending on the study design (germplasm, environment), but in general
the literature suggests that higher CBF-A14 copy number is associated with better freezing tolerance and in-field winter survival.

VRN-A1 CNV were also shown to influence freezing tolerance of winter wheat and had an interaction with Fr-A2 haplotype (Zhu et al. 2014). It was demonstrated in a bi-parental population that in the background of FR-A2-T, genotypes carrying 3 copies of VRN-A1 had almost double amount of freezing tolerance in comparison to those carrying 2 copies of VRN-A1. The effect of CNV at VRN-A1 on frost tolerance was not apparent in the population carrying the Fr-A2-S haplotype. Similar effect of CNV of VRN-A1 on the winter survival of winter wheat was also observed for the European diversity panel, but the interaction effect between CNV of VRN-A1 and Fr-A2 haplotype was not observed (Wurshcum et al 2017). In short, higher CN of VRN-A1 is associated with better freezing tolerance which can potentially translate into better in-field winter survival.

No study so far has characterized the CNV of VRN-A1 and CBF-A14 in Canadian winter wheat germplasm and its relative importance for winter survival of Canadian winter wheat is still unknown.

1.6.4 Quantitative trait loci (QTL) for freezing tolerance

There have been numerous genetic studies for freezing tolerance that have been conducted on diploid (Triticum monococcum), hexaploid wheat (Triticum aestivum) and also on other members of grass family such as barley (Hordeum vulgare) (Francia et al. 2004; Båga et al. 2007; Sofalian et al. 2008; Tumino et al. 2016). Two QTLs regions on chromosome 5A have been consistently identified by various studies as explaining a large proportion of variation observed for in-door freezing tolerance study and for in-field winter survival trials (Båga et al. 2007; Würschum et al. 2017). The two frost tolerance loci are named as Frost resistance-1 (Fr-1) and Fr-2 and have been mapped within a distance of approximately 30 cM (Stockinger 2009). Fr-1 was mapped close to the Vernalization1 (VRN1) gene and many current studies have supported the notion that Fr-1 is VRN1 exhibiting pleiotropic effect (Dhillon et al. 2010; Laudencia-Chingcuanco et al. 2011). The Fr-2 locus, on the other hand, consists of a cluster of C-Repeat Binding Factor (CBF). In Triticum monococcum 11 CBF genes were shown to cluster closely on the frost
tolerance locus of Fr-Am 2 (Miller et al. 2006), 7 CBF genes were identified within the Fr-H2 locus in *Hordeum vulgare* (Francia et al. 2007) while 17 CBF genes were identified in Fr-2 of wheat (Appels et al. 2018).

1.7 Screening methods for winter survival and freezing tolerance

There are numerous methods that are used to screen winter survival ability and freezing tolerance in wheat. These screening methods fall into three main categories: field survival estimations, controlled stress test (indoor), and indirect estimation of winter survival using physiological or genetic change that occur post cold exposure. This section provides a brief overview of the advantages and disadvantages of the methods in each category and their applicability in a breeding program, which depending on program size may screen between 2,000 and 10,000 genetically unique lines in each cycle.

1.7.1 In-field winter survival estimation

The breeding materials are planted in the field and exposed to the type and level of winter-stresses that is associated with the location in a given year. In the spring, different ways of observation and statistical methods are used to estimate the winter survival of the breeding material. This method allows for characterization of breeding materials against multitude of stresses associated with winter at the testing location in a non-destructive manner. However, there are some challenges that are associated with the field test. First, the efficiency of the test is heavily dependent on the severity of the winter. If the winter is mild, the results produced may not be informative as most lines tested would have perfect survival (non-differential winter) (Limin and Fowler 1991). This limitation can be resolved by having multiple location/years of winter survival data. It ensures that the breeding material is exposed to the winter stress factors that are associated with the growing region, which can help better differentiate winter survival ability of the breeding lines (Săulescu and Braun 2001). Second, variation of the level of winter stresses within each field trial can also introduce error and make it difficult to distinguish lines with similar winter survival. Fowler 1979 illustrates that certain part of the field is exposed to more severe winter stress and that the level of winter stress can vary within the distance of a few meters. Potential solutions for this problem include experimental design such as the alpha lattice
design (Patterson and Williams 1976) and statistical method such as radial smoothing analyses which can account for spatial variability during the data analysis step to reduce error introduced by spatial variability. The alpha lattice design differs from the commonly used randomized complete block design as it divides each replication (block) of the experiment into smaller incomplete blocks. This allows for the separation of incomplete block effect during data analysis which can take local spatial variation into account (Kashif et al. 2011). Radial smoothing generates a response surface of the error distribution and can define the spatial distribution of residuals in the field (Bowley 2015). This can allow for the adjustment of final winter survival based on the physical location of the genotype within the field, which have been shown to have a better goodness-of-fit statistic in comparison to other models that correct for spatial error (Bowley 2015). In summary, field testing of winter survival has several challenges including non-differential winter and spatial variation of winter stress level, however proper experimental design and the application of spatial variability model can improve the quality of in-field winter survival results.

1.7.1.1 Visual estimation

The most common method to evaluate winter survival in the field is the visual estimation of spring stand after the winter. The spring stand represents the plants that have survived over the winter, and each line is given a score out of 100% based on how many plants are left and sometimes leaf damage/yellowing is taken into consideration (Sâulescu and Braun 2001). This method is inexpensive and simple to implement in a breeding program. However, human observation bias can potentially introduce error as the method produces semi-quantitative result. These factors hinder the effort of detecting minor but significant differences between elite breeding lines. In short, visual estimation might not be optimal for a breeding program that strives to further improve winter survival, but it is sufficient for a program that aims to maintain certain level of winter survival.

1.7.1.2 Field survival index

Field survival index (FSI) is based on visual estimation data of over 40 field-trials within the province of Saskatchewan in Canada (Fowler and Gusta 1979). This index only uses plots that
have partial winterkill and uses percent winterkill difference between the lines in the same replication to estimate winter survival of each line. This method also uses the neighboring plot value to correct for the final FSI value (usually a line already with FSI value), which can reduce spatial error due to variation of winter stress in the field (Fowler 1979). Generating robust FSI requires multi-year and multi-location test data. This makes it difficult to incorporate FSI into an early stage of breeding pipeline or the pre-breeding evaluation, which usually involves a large number of lines to make decision based on data from one growing season. However, FSI can be applied in the later stage of the breeding process (advanced yield trial) which test smaller number of lines over many locations and years.

1.7.1.3 Image-based methods

Image-based methods have been used to characterize winter survival. The images are either captured close to the ground (Grieder et al. 2015) or in the air (Sankaran et al. 2015). Different methods are then used to quantify the amount of vegetation in the image to estimate the winter survival of each line.

1.7.1.4 Ground-based methods

Ground-based imagery usually involves a modified vehicle that is equipped with sensors and are typically referred to as phenomobiles. Ground-level imagery capture allow for data to be captured at the plot level with great definition and requires minimum amount of post-processing (Araus and Cairns 2014). Grieder et al. (2015) has demonstrated that the application of ground image-based approach is a suitable tool for estimating the vegetation cover of wheat. However, ground-level approach requires an extensive amount of time for the data collection process since the phenomobiles need to pass through all the plots and wet soil can possibly prevent the timely collection of data.

1.7.1.5 Remote sensing

Remote sensing involve sensors mounted onto an unmanned aerial vehicle (UAV). The imagery can be captured by various sensors; from inexpensive and simple consumer grade cameras to high resolution multi-spectral sensors (Torres-Sánchez et al. 2014; Grieder et al. 2015; Potgieter
et al. 2017). Inexpensive consumer-grade camera such as the Canon S100 have produced results consistent with multi-spectral sensors when attached to an UAV and can serve as an option to reduce the cost (Haghighattalab et al. 2016). Remote sensing in comparison to ground imagery, can significantly reduce phenotyping time (Araus and Cairns 2014), reduce labor cost for phenotyping and be freed from restriction that is associated with field access such as wet soil and pesticide application (Tattaris et al. 2016). However, the post-processing steps for remote sensing is complicated and includes processes such as image alignment, radiometric calibration, and automatic mosaicking (Berni et al. 2009; Haghighattalab et al. 2016).

The post processing steps are essential for the quantification of vegetation in a given plot which can help measure winter survival. The widely used approach is through color index-based approaches, which depends on the color to separate soil from the plants. There are many vegetation indices that can help highlight the vegetation in the field (as reviewed extensively in Hamuda et al. 2016). The two main indices that have been shown to accurately reflect the amount of photosynthetically active biomass on the ground for wheat are normalized difference vegetation index (NDVI) (Tucker 1979a) and green-NDVI (Gitelson et al. 1996). Both vegetation indices have demonstrated strong correlation with visually estimated data in characterizing wheat vegetation (Sankaran et al. 2015; Haghighattalab et al. 2016). However, Barati et al. 2011 had suggested that NDVI is more sensitive when used to characterize vegetation. The current limitation of vegetation indices is that it cannot distinguish vegetation of wheat from other plants. Therefore, proper management of weed and volunteer plants are needed to ensure that they do not confound with winter survival estimation.

The image-based methods can improve the overall data quality and data collection efficiency. It removes the error and bias introduced by human observation, it can be high-throughput and has the added benefit of being quantitative. This has helped detect small but significant differences in spring stands between advanced breeding materials (Sankaran et al. 2015; Khot et al. 2016). However, this type of phenotyping requires strong knowledge of imaging processing and geographic information system which are outside the expertise of typical plant breeders. Breeding programs must overcome this knowledge gap to reap the potential benefits from remote sensing methods.
1.7.2 Controlled-environment methods

A method for estimation of freezing tolerance is the controlled-environment testing. This can be later translated to winter survival ability for a given genotype. The plant materials are usually grown in the greenhouse and exposed to artificial winter-related stress such as freezing temperature. This method brings control over the environment factors that can take place which can eliminate error introduced by the heterogeneity of the field condition (Araus and Cairns 2014). This method can potentially have promising results for growing regions that have one-dimensional or well-known stress factors for winter survival such as Western Canada (Fowler et al. 1981a) and Lithuania (Gorash et al. 2017), where freezing temperature has been identified as the primary determinant for winter survival. Therefore, the results from controlled environment testing can translate well into the field.

However, several limitations have been noted in controlled environment testing (Araus et al. 2008). One would be that the complexity of stress condition in the field is hard to be mimicked in a controlled environment. For example, in the field, the plants are exposed to a multitude of stress factors that can affect final winter survival (see previous section above) and different environmental factors such as cold acclimation period and photoperiod contribute to the intensity of these stresses.

1.7.2.1 Lethal temperature 50 ($LT_{50}$)

One method that has been frequently used in controlled-environment testing is lethal temperature 50 ($LT_{50}$) (Pomeroy and Fowler 1973). It is an artificial freeze test, where the lines are exposed to increasing cold temperatures after a period of cold acclimation at ~ 4 °C. Plants from each test lines are removed at each temperature interval and allowed to regrow under optimum temperature condition. Temperature at which 50% of the plants are killed is the $LT_{50}$ and lower $LT_{50}$ represents better freezing tolerance. The result from $LT_{50}$ showed strong correlation with in-field winter survival result in studies conducted in Saskatchewan, Canada and $LT_{50}$ had demonstrated potential to differentiate cultivars with very similar freezing tolerance (Fowler et al. 1981a). However, it has been reported, that the correlation between $LT_{50}$ and in-field winter survival was only significant for cold-hardy cultivar but not for semi-cold-hardy cultivar (Gusta
et al. 2001). They also observed that certain genotypes can cold acclimate to better freezing tolerance under controlled environment than under outdoor condition therefore LT$_{50}$ can overestimate winter survival ability. Measurement for LT$_{50}$ has also shown evidence of being inconsistent between different independent experiments and extra effort is required to control the experimental condition (Pomeroy and Fowler 1973). In addition, LT$_{50}$ is a destructive method that on average requires about 50 plants to estimate and is therefore unrealistic to be conducted for breeding materials with limited seeds. With the high differentiation power, this method is useful for genetic studies and had been used extensively in studies where only a few lines were evaluated for freezing tolerance (Mahfoozi et al. 2001a; Båga et al. 2007).

1.7.2.2 Prolonged freeze test

The second method is the prolonged freeze test. Winter wheat is exposed to cold stress for months in the field, which has been argued more accurately reflect the condition in comparison to the short-term cold exposure in LT$_{50}$ (Gusta et al. 1997, 2001; Skinner and Garland-Campbell 2008). In prolonged freeze-tests, experimental lines are exposed to a pre-determined freezing temperature for a long period of time after cold acclimation. Plants are allowed time to recover in optimal growing condition and percentage of survival is determined afterwards. Higher survival percentage represents higher winter survival ability. Prolonged freeze tests were able to differentiate two cultivars with similar LT$_{50}$ (Gusta et al. 1997) and the method also has produced statically significant correlation with LT$_{50}$ (r=0.69) (Skinner and Garland-Campbell 2008). However, there has yet to be a study that can demonstrate strong correlation between the result of prolonged freeze test and in-field winter survival. Similar with LT$_{50}$, prolonged freeze test is laborious and cannot be applied to a breeding program. In summary, both controlled-freeze test methods demonstrated strong differentiation power of freezing tolerance and could be better applied in genetic studies, where only a few lines are evaluated.

1.7.2.3 Ice encasement tests

Tolerance to ice encasement was also evaluated in the controlled environment to predict winter survival of wheat. Usually the wheat plants are encased in ice completely then the plants are thawed and allowed for regrowth to estimate survival. Tolerance to ice encasement is measured
as the number of days encased in ice where 50% of the plants survived (LD_{50}) (Andrews and Pomeroy 1975). Previous work had demonstrated genetic difference in ice encasement tolerance between wheat cultivars (Andrews and Gudleifsson 1983). For example, the cultivar Norstar had the LD_{50} of 13.5 days in comparison to the 6.7 days of the cultivar Fredrick. Studies have looked at the correlation between ice tolerance and winter survival in Eastern Ontario. Out of the two testing sites, strong and significant correlation between ice tolerance and winter survival were observed at the Douglas location (r=0.71-0.85), in three out of the four testing-years. But the relationship between the two traits were not significant in the Ottawa location in Ontario (Andrews et al. 1997). The result suggested that although ice encasement tolerance might not be essential for improving the winter survival in all growing regions but can be important to breed for in regions where ice encasement occurs frequently.

In summary, the controlled-environment testing can provide a great understanding of the mechanism or the genetic basis of the tolerance towards winter related stresses. Most of these testing methods are laborious and can be difficult to implement and their result must be complemented by field testing to confirm its fitness for winter survival.

1.7.3 Estimation of freezing tolerance using physiological and genetic changes

The last category of freezing tolerance estimating method is composed of screening methods that measure physiological or genetic changes that occur during exposure to freezing temperature to estimate the freezing tolerance of the genotypes.

1.7.3.1 Fv/Fm

The photosynthetic apparatus of plants is sensitive towards stress and low temperature stress induced photoinhibition of photosystem II (PSII) by inhibiting the repairing mechanism of PSII (Kalaji and Guo 2008). The Fv/Fm method evaluate the health of the photosynthetic machinery by measuring the maximum quantum efficiency of PSII (Murata et al. 2007). Previous studies had demonstrated a strong correlation between Fv/Fm and freezing tolerance in wheat (Clement and Van Hasselt 1996; Rizza et al. 2001; Armoniene et al. 2013). Wheat cultivar with a better freezing tolerance had higher Fv/Fm ratio post exposure to freezing temperature in comparison
to one with lower freezing tolerance. This method has the benefit of being efficient and as such having the potential to be applied in a breeding program to screen a large population for freezing tolerance.

### 1.7.3.2 WCS120

Work has been done to identify protein target that can be used to predict freezing tolerance and one of the well-known examples is the dehydrin protein WCS120. WCS120 protein belongs to a group of dehydrin protein regulated by cold and was first isolated from cold acclimated wheat (Houde et al. 1992a). WCS120 protein level increased during cold acclimation and accumulated to a higher level as wheat developed better freezing tolerance (Vítámvás and Prášil 2008). Houde et al. (1992b) developed an antibody using WCS120 to screen for protein family that share common antigenicity, which can quantify their level. The antibody had shown that a cultivar with better freezing tolerance accumulated more WCS120 protein after cold treatment (Vítámvás et al. 2007; Kosová et al. 2013). Interestingly, studies were able to show that the protein level of WCS120 at room temperature also correlated significantly with freezing tolerance (Vítámvás et al. 2010; Kosová et al. 2013) and proposed that the evaluation of freezing tolerance through WCS120 protein level can be done with cold acclimation. However, further studies that quantified WCS120 levels during cold acclimation in winter wheat raised doubts on the applicability of WSC120 protein level (Trischuk et al. 2014). Trischuk et al. (2014) reported that winter wheat was exposed to the process of cold acclimation, de-acclimation and re-acclimation. At the end of the treatment, the protein level returned to the same level right as that after the initial cold acclimation but the freezing tolerance was significantly lower. This suggested that WSC120 likely requires other proteins to confer freezing tolerance. Recently, Vítámvás et al. 2019 investigated whether WCS120 protein level can be used in predicting in-field winter survival or not. The protein level of WCS120 and in-field winter survival were not correlated, however, winter survival showed a strong correlation with the total amount of the most abundant wheat dehydrin (WCS200, WCS180, WCS120, WCS66, and WCS40). In summary, WCS120 has demonstrated promise as a potential tool to screen wheat cultivars for freezing tolerance. It has the potential to screen a large population without having to undergo cold acclimation process which can save considerable amount of time. Further work will need to be done to see if the
addition of quantification of other abundant wheat dehydrins can improve the freezing tolerance evaluation.

Most of these screening methods that use physiological or genetic changes to estimate freezing tolerance have the advantages of being high-throughput and non-destructive, which is rather beneficial for breeding programs. However, most of these methods measure the factors that can contribute or reflect freezing tolerance, without any clear evidence of being highly correlated with field survival (Fowler et al. 1981a). Therefore, they are a great tool for screening freezing tolerance and rather than winter survival per se.

1.8 Research hypothesis

1. Canadian winter wheat germplasm has significant variation in winter survival

2. Ground-based and UAV-based images are effective tools for phenotyping winter wheat for winter survival

3. Canadian winter wheat germplasm contains favorable alleles at the genomic regions influencing winter survival

4. Canadian winter wheat germplasm has allelic variation in freezing tolerance candidate genes, *CBF*-A12 and *CBF*-A15. The allelic variation is associated with the variation in winter survival

5. Canadian winter wheat germplasm has variation in CN for freezing tolerance candidate genes, *CBF*-A14 and *VRN*-A1. The variation in CN is associated with the variation in winter survival

1.9 Research objectives

1. To compare methods for measuring winter survival including visual estimation, ground-based imagery and UAV-based imagery (Chapter two)
2. To identify genomic regions that are associated with winter survival ability in the Canadian winter wheat germplasm and to identify candidate genes (Chapter two)

3. To genotype the Canadian winter wheat germplasm for variation in freezing tolerance candidate genes, $CBF-A12$ and $CBF-A15$ (Chapter Three)

4. To genotype the Canadian winter wheat germplasm for CNV of freezing tolerance candidate genes, $CBF-A14$ and $VRN-A1$ (Chapter Three)

5. To identify the optimal allele/CN combination for winter survival in Eastern Canada (Chapter Three)
Chapter 2: Application of Image-Based Phenotyping Tools to Identify QTLs for In-Field Winter Survival of Winter Wheat 

(*Triticum aestivum* L.)

The work described in this Chapter has been written and formatted as a manuscript submitted to Theoretical and Applied Genetics. It has been accepted.

**Contribution:**

Yi Chen and Dr. Alireza Navabi designed the study. Yi Chen collected phenotypic data and performed the analyses. Harwinder Singh Sidhu assembled the panel used in the study and performed population structure analysis. Dr. Mina Kaviani collected the DNA samples for the panel and Curtis Pozniak provided the SNP data. Dr. Michel S. McElroy tested the diversity panel in Quebec. Yi Chen prepared the first draft of the manuscript. Yi Chen, Harwinder Singh Sidhu, Dr. Mina Kaviani, Dr. Michel S. McElroy and Dr. Alireza Navabi reviewed and edited the manuscript.
2.1 Abstract

Winter survival is an essential trait of winter wheat (*Triticum aestivum* L.) grown in regions with high risk of winterkill. We characterized a diversity panel of 450 Canadian wheat cultivars that included mostly winter-growth habit wheats to identify key genetic factors that contribute to higher winter survival under field conditions. To more accurately quantify winter survival differences among cultivars, image-based phenotyping methods, captured by unmanned aerial vehicle (UAV) and on ground level, were used to estimate the winter survival of each varieties. Winter survival index (WSI) was developed to correct for emergence when evaluating winter survival. Genome-wide association studies resulted in the identification of nine quantitative trait loci (QTLs) for winter survival including *Vrn-A1* and *Fr-A2*. In addition, by using the recently released annotated sequence of the wheat genome and the available RNA-Seq data, three putative candidate genes underlying the QTLs for winter survival were identified. Collectively, our study demonstrated the feasibility of using UAV-based imagery in high-throughput phenotyping for the identification of loci associated with winter survival in wheat. This increased precision in-field phenotyping makes it a valuable complement to indoor frost tolerance studies in the identification of genetic factors not directly linked to simple cold tolerance.

2.2 Introduction:

Winter wheat (*Triticum aestivum* L.) is an autumn-sown crop that requires successful overwintering to induce flowering which enable the crop to realize its yield potential. However, in areas with severe winter, such as the high latitude growing regions of the Northern hemisphere, winterkill of the winter wheat crop can be severe. Winterkill can result in significant reduction in crop stand, and hence the final grain yield (Alessi and Power 1971; Thomas et al. 1993; Zheng et al. 2018), to the extent that it may force the farmers to replant their winter wheat field with other crops the following spring. High levels of winter survival is, therefore, an essential trait for the winter wheat cultivars targeted for the growing regions with a high risk of winterkill.

The severity and the nature of the winter stress can vary from region to region and from year to year. Rather than a single stress, winterkill is the result of a number of interacting abiotic
and biotic factors that lower the survival during winter. These factors include: prolonged exposure to sub-freezing temperatures and freezing-induced dehydration (Steponkus and Webb 1992; Steponkus et al. 1998); frost heaving, the upward swelling of soil due to ice crystal formation in the soil, which can physically push the crown and the root system out of the soil (Säulescu and Braun 2001); ice encasement, the formation of ice sheets that reduce the rate of gas exchange, leading to cytoplasmic acidosis (Andrews 1996); and snow mold, a fungal disease that is prevalent in regions with extensive snow cover (Schneider and Seaman 1987; Murray et al. 1995). Winter survival, therefore, represents the ability of winter wheat to survive a multitude of biotic and abiotic stress factors that are associated with the winter in a specific growing region.

Two principal decisions by the farmers contribute to successful winter survival of winter wheat: crop management practice and the genetics of the winter wheat cultivar grown. No-till management practice has been shown to contribute significantly to winter survival. The stubble leftover can help maintain snow cover, which insulates the wheat plants from the cold (Cox et al. 1986; Fowler 2012). Other management decisions such as planting date (Fowler 1982; Andrews et al. 1997), planting depth (Loeppky et al. 1989), and fertilization program (Pittman and Tipples 1978; Grant et al. 1984) all influence winter survival of winter wheat.

Winter wheat genotype is also an important determinant of winter survival. Most studies have focused on frost tolerance, which is a major genetically-controlled contributor to overall winter survival. Chromosomes 5A contains two frost tolerance loci known as Frost Resistance-1 (Fr-1) (Galiba et al. 1995) and Fr-2 (Vágújfalvi et al. 2003). Studies have suggested that Fr-1 is the Vernalization-1 (Vrn-1) gene exhibiting pleiotropic effects (Dhillon et al. 2010; Laudencia-Chingcuanco et al. 2011). The Vrn-1 gene influences the vernalization requirement of wheat and have been shown to be necessary to downregulate the expression of Cold Regulated (COR) genes (Dhillon et al. 2010). Copy number variation of Vrn-A1 has also been shown to influence frost tolerance with higher copy number being associated with better frost tolerance (Zhu et al. 2014). The Fr-2 locus consists of at least eleven C-Repeat Binding Factors (CBFs) (Vágújfalvi et al. 2005; Miller et al. 2006; Appels et al. 2018) and some of these CBFs demonstrated the ability to induce the expression of COR genes (Vágújfalvi et al. 2003). Zhu et al (2014) identified linked
polymorphism between CBF-A12 and CBF-A15 on the A genome and classified them into two haplotypes, Fr-A2-S and Fr-A2-T. The two haplotypes were examined in other studies and genotypes that carry Fr-A2-T consistently demonstrate better frost-tolerance (Zhu et al. 2014; Würschum et al. 2017; Babben et al. 2018).

The most common way to evaluate a genotypes in field trials for winter survival potential is through visual rating, often on a 0% (all plants dead) to 100% (all plants alive) scale (Săulescu and Braun 2001). This method is easy to apply, but the human eye can have difficulties detecting the subtle differences between plots. Visual rating is subjected to bias and human error which can both contribute to the reduction in the precision and the accuracy of the data. Studies have also used an artificial freeze evaluation to determine lethal temperature 50 (LT_{50}), the temperature at which 50% of the plants are dead, to predict the winter survival of winter wheat in the field (Pomeroy and Fowler 1973). A strong negative correlation (r = -0.95) has been reported between LT_{50} and in-field visual survival (Pomeroy and Fowler 1973; Gorash et al. 2017). However, the strong correlation was not observed across different studies and can vary among environments if winter-related stress factors other than frost tolerance influence the final survival (Bridger et al. 1996; Andrews et al. 1997; Gusta et al. 2001). To improve the data collection process, researchers have applied image-based methods and vegetation indices to improve the characterization of vegetation in the field (Grieder et al. 2014; Humplík et al. 2015; Patrignani and Ochsner 2015; Dammer et al. 2016; Hamuda et al. 2016). These images can be captured at the ground level or in the air via unmanned aerial vehicles (UAV). Ground-level images are usually captured in proximity to the experimental units by modified vehicles equipped with sensors (Busemeyer et al. 2013; Deery et al. 2014). The proximity to the experimental unit allows the collection of data with higher resolution (Araus and Cairns 2014). The data collection process usually requires extensive amount of time since the vehicle will need to pass by all the plots and the process can be limited by field access (wet soil, herbicide spray). UAV-based image can alleviate some of these limitations. UAV can perform data collection regardless of field access and can allow for rapid assessment of large number of plots. The main limitation for UAV based phenotyping is the processing time as it involved computationally intensive steps such as image alignment, radiometric calibration and automatic mosaicking (Araus and Cairns
However, paid photogrammetry software such as Pix4D (Pix4d, Lausanne, Switzerland) and Metashape (Agisoft, St. Petersburg, Russia) or pipeline proposed by recent literature (Haghhighattalab et al. 2016; Roy et al. 2017) can automate the process. Regardless of the limitations, these image-based methods produced comparable result with visual rating when evaluating crops for winter survival (Sankaran et al. 2015; Khot et al. 2016) and have the additional potential benefit of increasing precision and being high-throughput.

By collecting high quality image-based phenotypic data in the field from a genotyped diversity panel of wheat from higher latitudes of North America, our study aims to identify key genetic factors essential for winter survival via genome-wide association study (GWAS).

2.3 Materials and Methods

2.3.1 Experimental design

The Canadian Winter Wheat Diversity Panel (CWWDP; n =450; Sidhu et al. unpublished) was evaluated for winter survival in three year and location combinations. Each year and location combination is considered as an environment. The panel includes representative winter wheat cultivars that have been grown in Canada since the 1880s, current Canadian commercial winter wheat cultivars, advanced breeding materials from a number of breeding programs, key contributing genotypes in the ancestry of the Canadian winter wheat (Robert Graf, personal communication), and a number of spring wheat cultivars that were specially bred to be frost tolerant (Robert Graf, personal communication; Whittal et al. 2018).

2.3.2 Experimental locations

The panel was grown at the University of Guelph Elora Research Station (43°38' 27.0456” N, 80°24' 18.6948” W) near Elora, ON, Canada, in 2016/2017 (planted on Oct 14th, 2016) and 2017/2018 (planted on Oct 21th, 2017) growing seasons. The panel was also grown in Centre de Recherche sur les Grains (CÉROM) (45° 34' 57.12” N, 73° 14' 10.62”W) near Montreal, QC, Canada, in the 2017/2018 growing season (planted on September 27, 2017). The experimental design in all three environments was a two-replication α-lattice design (Patterson and Williams 1976; Piepho et al. 2006). For Elora 2016/2017 and CÉROM 2017/2018, each experimental unit
was a 1 m long plot with 6 rows and 17.8 cm row-spacing. In Elora 2017/2018, each experimental unit was a 2 m plot with 6 rows, with 17.8 cm row spacing.

2.3.3 Climatic data

For Elora 2016/2017 and 2017/2018, the weather data including minimum and mean daily air temperature, minimum and mean soil temperature (5 cm below ground-level) and daily snow depth were obtained from the University of Guelph’s School of Environmental Sciences Agrometeorology group. The meteorological station was 4 km away from the experiment location. For CÉROM 2017/2018, the weather data were collected from the nearest weather station operated by Environment and Climate Change Canada (WMO identifier 71627). Data collected included minimum and mean daily air temperature and daily snow depth. Growing degree days (GDD) from planting to freeze-up was calculated. Five-day moving mean of the mean daily air temperature was calculated. The day in which the moving mean drop below zero is defined as the freeze-up day. GDD is the sum of the mean daily air temperature that is above zero degree from the day of planting to freeze-up.

2.3.4 UAV-captured data

UAV flights were performed by Deveron UAS (Toronto, Canada), using the platform DJI Matrice 100 (DJI, Shenzhen, China). Spectral reflectance was captured by RedEdge™ narrow-band multispectral camera (MicaSense, Washington, United States) at the altitudes of 30 meters. The five bands captured include blue (480 nm), green (560 nm), red (670 nm), red edge (720 nm), and near-infrared (840 nm). Fall flights to estimate emergence were operated after plants reached Zadoks stage 21 (Zadoks et al. 1974). Spring flights to estimate winter survival were performed once the snow has melted and the plants were given some time to recover. For Elora 2016/2017 and 2017/2018, flights were performed on May 8th, 2017, and May 9th, 2018, respectively. UAV flight was not performed for CÉROM 2017/2018 due to the proximity of the research station to the local airport and concern for air traffic safety.

After each flight, images from each of the wavelengths were processed using the Pix4d software (Pix4d, Lausanne, Switzerland). Default settings for multispectral camera were applied
in the software to generate five geo-referenced orthomosaic of the flight for each wavelength. Variation in light condition was adjusted for by using an image of a calibration panel with known reflectance (blue 0.70, green 0.71, red 0.71, near-infrared 0.66, and red edge 0.70), taken right before each UAV flight. Orthomosaic image of respective bands were imported into ArcGIS (Esri, California, United States) to generate the normalized difference vegetation index (NDVI) map using the Map Algebra Tool in ArcGIS by following formula 1 (Tucker 1979b).

\[
NDVI = \frac{(\text{Near infrared} - \text{Red})}{(\text{Near infrared} + \text{Red})}
\] (1)

A threshold of > 0.3 was used to exclude the reflectance generated by the soil background. Shapefile for the extraction of plot-level data were generated via the python code described by Haghighattalab et al. (2016). The shapefiles were imported into ArcGIS and were manually curated to ensure a better cover over the plot. The Zonal Statistic tool was used to extract NDVI sum for each plot following formula 2.

\[
\text{NDVI Sum} = \sum_{i=1}^{N} \text{NDVI}_i
\] (2)

, where NDVI sum was the sum of NDVI values from the pixels within each experimental unit that exceeded the 0.3 threshold value. The NDVI sum took into consideration the greenness of the vegetation within each experimental unit and was used to represent winter survival of each unit. \(\text{NDVI}_i\) refers to NDVI value of individual pixel, where N represents the number of pixels in an individual experimental unit after thresholding.

To avoid the confounding effect of poor emergence on winter survival, a winter survival index (WSI) was developed according to formula 3 to take germination into account.

\[
WSI = \frac{\text{NDVI Sum}_{\text{Spring}} - \text{NDVI Sum}_{\text{Fall}}}{\text{NDVI Sum}_{\text{Fall}}}
\] (3)

, where \(\text{NDVI Sum}_{\text{Spring}}\) and \(\text{NDVI Sum}_{\text{Fall}}\) represent the NDVI Sum of each experimental unit in the spring and fall, respectively. WSI was only calculated for Elora 2016/2017, with the fall imagery captured on November 18, 2016. In Elora 2017/2018, estimating WSI was not possible due to the short growing window in the fall resulting in plants not reaching Zadoks stage 21.
2.3.5 Ground-level and the visually-estimated data

Nadir images were taken at the center of each experimental unit by a Nikon D7100 camera (Nikon, Tokyo, Japan) on a tripod at 1.5m above the ground surface to capture the same amount of area for each experimental unit. This was done to validate the data captured by UAV and use as an alternative way to measure the amount of green vegetation remained on the ground when UAV flight was not possible i.e., CÉROM 2017/2018. The images were taken on the same day as the UAV flights. All data were recorded in JPEG format with the resolution of 6000 x 4000 pixels. The software CANOPEO (Patrignani and Ochsner 2015) was operated in Matlab (MathWorks, Massachusetts, United States) to determine fractional green canopy cover (FGCC). CANOPEO classified pixels to vegetation and non-vegetation pixel based on the ratio of red/green, ratio of blue/green, excess green index and pixel continuity. FGCC is the ratio of the number of vegetation pixels to the total number of pixels and multiplied by 100 to represent a percentage (0-100%). Noise reduction was set at 1000, while other settings were left as software default. FGCC was used to estimate how much vegetation has survived after the winter within each given plot.

For visual estimation of winter survival, plots were inspected and scored based on how much plant stand has remained (0 = no plant stand visible in the plot area, 100 = full plant stands remained after the winter). Visual Estimation was performed at the time when UAV and ground images for spring stand were captured. The summary of winter survival measurement done at each environment is summarized in table 2.1.

<table>
<thead>
<tr>
<th>Table 2.1 Environments and the methods used to measure winter survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAV Imagery</td>
</tr>
<tr>
<td>Ground Imagery</td>
</tr>
<tr>
<td>Visual Estimation</td>
</tr>
<tr>
<td>Winter Survival Index</td>
</tr>
</tbody>
</table>

2.3.6 Statistical analysis

A linear mixed model was used to analyze the winter survival data. Data was first analyzed using the PROC GLIMMIX procedure of the SAS software® version 9.4 (SAS Institute Inc 2018).
Wheat genotype, environment and their interaction were considered fixed effects, while block and incomplete block within block effects were considered random effects. A significant genotype-by-environment interaction was detected for the visual estimation of winter survival (Table 2.3). A linear mixed model was therefore used to analyze the winter survival data (UAV image, ground image, visual) for each environment separately. Wheat genotype was considered a fixed effect, while block and incomplete block within block effects were considered random effects. Residuals were computed and analyzed to validate the assumptions made by the statistical model. This included visual inspection of residual plot, studentized marginal residual by fixed effects plot, studentized conditional residuals by predicted variate plot, studentized conditional residuals by factor variate plots and cumulative probability plot of residuals (Bowley 2015). The PROC UNIVARATE procedure was then used to perform a test of normality on the residuals based on Shapiro-Wilk test (Shapiro and Wilk 1965) at a Type I Error rate of 0.01. Outliers were detected using Lund’s test (Lund 1975) and experimental units associated with studentized residual values outside of +/- 3.4 were removed. Likelihood ratio tests were used to test the significance of the random effects. Least squares means (LSmeans) were obtained for each genotype using the LSMEANS statement in PROC GLIMMIX procedure.

Pearson’s coefficients of correlation were calculated to examine the correlation among different winter survival phenotyping methods. Scatter plots and histograms depicting associations of phenotyping methods and their respective frequency distributions, were generated using the pairs function in RStudio® (R Core Team 2017).

2.3.7 Broad-sense heritability

Broad-sense heritability ($H^2$) was estimated to evaluate the precision of the winter survival parameters measured. For estimating heritability of each trait at each environment, variance components were calculated using PROC MIXED where block, incomplete block(block) and genotype were considered as random effects. For estimating heritability of each trait across the environments, variance components were calculated using PROC MIXED where environment, block(environment), incomplete block(block by environment), genotype and genotype by environment were considered as random effects. Heritability was calculated on an entry-mean
basis for each individual environment (formula 4) and across the environments (formula 5) (Holland et al. 2003).

\[
H^2 = \frac{\sigma_g^2}{\sigma_p^2} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{error}^2_{replication}}
\]

(4)

\[
H^2 = \frac{\sigma_g^2}{\sigma_p^2} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{ge}^2_{environment} + \sigma_{error}^2_{replication \times environment}}
\]

(5)

where \(\sigma_g^2\) is genotypic variance, \(\sigma_p^2\) is phenotypic variance, \(\sigma_{error}^2\) is error variance, \(\sigma_{ge}^2\) is the genotype \(\times\) environment variance, \(environment\) is the number of environments in which the traits were evaluated (three for visual estimation and FGCC and two for NDIV sum) and \(replication\) is the number of replications at each environment (two replications).

2.3.8 Genome-wide association studies:

Genomic DNA was extracted from freeze-dried tissues that were cut from seedlings grown out of 4-5 seeds per each genotype. One stainless steel bead (3.2 mm diameter) was placed in tube with the freeze dried tissues and they were ground into fine powder using the 1600 MiniG (SPEX® Sample Prep, Metuchen, NJ, USA) at the frequency of 1500 RPM for 1 min. Preparation of total DNA from each sample was performed using the Qiagen DNeasy Plant DNA Mini Kit (Qiagen Gmbh, Maryland, USA). The quality and quantity of each DNA sample was tested using a NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The 90K wheat iSelect SNP array (Illumina Inc., California, USA) (Wang et al. 2014) was used to genotype CWWDP at the University of Saskatchewan Wheat Breeding and Genetics Lab. Genotype calling was performed by Dr. Matthew Hayden at La Trobe University using proprietary script for genotype calling in unrelated samples. It assigned sample to clusters previously identified if the likelihood that the sample belonged to that cluster is higher than the confidence score of 8.0. Arbitrary allele states were assigned to each cluster (\(i.e., AA\) or \(BB\)) as the actual nucleotide variant was not determined (Matthew Hayden, personal communication). This resulted in 51 649 SNP markers being called. SNPs were filtered based on three criteria: 1)
have been physically mapped onto Chinese Spring NRGv1.0 genome assembly, 2) minor allele frequency (MAF) higher than 5% and 3) missing value less than 25%. A total of 20,645 SNP markers met these criteria. The missing genotype calls were imputed using the software package BEAGLE v.4.1 (Browning and Browning 2007) using the default setting.

To investigate the population structure, subset of markers was selected based on linkage disequilibrium (LD). LD pruning was done using PLINK and the filtering method was “--indep” which prunes based on the variance inflation factor (VIF). The parameter was set as 100 for window size in SNPs, 5 for the number of SNPs to shift the window at each step and 2 as the VIF threshold (Purcell et al. 2007). This narrowed the number of independent markers to 1,923. Admixture model within the Structure software package v. 2.3.4 was used to determine the optimal number of subpopulations (Pritchard et al. 2000). The model parameters of 10K burn-in and 100k Markov Chain Monte Carlo (MCMC) iterations were used to run k value from 1-10 with 10 replications at each k. Visualization of the result was done using STRUCTURE HARVESTER software and the number of subpopulations was selected at k=7 based on the Evanno method (Evanno et al. 2005; Earl and VonHoldt 2012). An additional STRUCTURE run using 500,000 burn-in and MCMC iteration with 20 replications was done for k=7.

The genome-wide association study (GWAS) was conducted using the package GAPIT in R (Lipka et al. 2012). Principle component (PC) analysis was performed and kinship matrix was generated in GAPIT using default settings. Three different association models were evaluated to identify the most powerful model: compressed mixed linear model (CMLM) + kinship matrix (K); CMLM +K+ PC3; CMLM + K+Q. The model with the smallest mean-squared-difference (MSD) between the observed $P$ value and the expected $P$ value was selected.

Bonferroni correction was used to adjust for multiple testing and to reduce the chance of false positive. However, this correction is over-conservative given that not all markers are truly independent due to genetic linkage (Perneger 1998; Bush and Moore 2012). Therefore, similar to other studies (Yang et al. 2014; Parra-Londono et al. 2018), a more liberal threshold was included to capture putative genetic regions that are associated with winter survival. For significant marker-trait associations (MTA), $P < 2.42 \times 10^{-6}$ ($0.05/\text{number of markers}$) while for
suggestive MTA, $P < 4.84 \times 10^{-5}$ (1/number of markers). The Manhattan plot for each GWAS was visualized using CMplot package (Yin 2018) in RStudio® (R Core Team 2017).

To investigate known genetic factors associated with winter survival, the panel was genotyped with a kompetitive allele specific PCR (KASP) assay to identify genotypes carrying the $Vrn-A1a$ dominant mutation that confers spring growth habit. wMAS000033, described in Grogan et al. (2016) was developed from iSelect SNP marker IWA0001, which was shown to be associated with the $Vrn-A1a$ allele at $Vrn-A1$ (Cavanagh et al. 2013). After the removal of genotypes that carry the $Vrn-A1a$ allele (n=35), the panel had 415 genotypes. A second GWAS was then conducted on this subpanel following the procedure described above.

2.3.9 Candidate gene search:

Significant or suggestive MTAs on the same chromosome were analyzed for linkage disequilibrium using Haploview (Barrett et al. 2005). Haplotype block is defined by the method “Solid Spine of LD”. When a group of MTAs are found to be in linkage disequilibrium with one another, they were considered as one quantitative trait locus (QTL) and the MTA with the lowest $P$ value was presented. Literature review was conducted to identify candidate gene for the respective QTL. In addition, WheatMine® (Smith et al. 2012) was used to gain access to the International Wheat Genome Sequencing Consortium RefSeq v1.0 data that included gene annotation and marker data (Appels et al. 2018). High confidence genes within the 5Mb range of the most significant MTA of each QTL are considered as potential candidate genes and the protein functions of these genes were extracted via WheatMine® (Smith et al. 2012) (Data S2). Gene expression data from Li et al. 2015 of spring wheat (cv ‘Manitou’) under normal temperature treatment (23°C) and cold treatment (4°C) was accessed using http://www.wheat-expression.com/ (Ramírez-González et al. 2018) to identify genes with change in expression between the two treatments. These genes were further analyzed by searching for their respective orthologs in *Oryza sativa* and *Arabidopsis thaliana* using Ensembl Plants (Kersey et al. 2018). The gene with orthologs that has proven function in winter survival related traits was considered the candidate gene for the respective QTL.
2.4 Results

2.4.1 Climate data and winter survival of the CWWDP across environments

CWWDP genotypes had different amount of time to establish themselves in the fall for each environment due to the difference in planting and freeze-up date. For Elora 2016/2017, 289 growing degree days (GDD) occurred prior to the freeze-up (Table 2.2). Elora 2017/2018 had the shortest window for growth in fall with only 101 GDD prior to freeze-up date. This was due to the combination of unfavorable planting conditions and the early arrival of freezing temperature. CÉROM 2017/2018 had the longest window for fall establishment with 504 GDD due to earlier planting date.

Table 2.2 Growing degree days (GDD) from planting date to the estimated date of freeze-up across the 3 testing environments.

<table>
<thead>
<tr>
<th>Environment</th>
<th>Planting Date</th>
<th>Freeze-up Date</th>
<th>GDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elora 2016/2017</td>
<td>October 14th, 2016</td>
<td>November 21st, 2016</td>
<td>289</td>
</tr>
<tr>
<td>CÉROM 2017/2018</td>
<td>September 27th, 2017</td>
<td>November 10th, 2017</td>
<td>504</td>
</tr>
</tbody>
</table>

The three environments had very different winter conditions which resulted in the difference in winter survival observed in the spring. For Elora 2016/2017, the minimum temperature for the season was -19.5°C on January 7th, 2017 (Appendix I). There was adequate snow cover during most of the winter so that the soil temperature remained around 0°C. During March, episodes of severe freezing air temperature of -15°C occurred with no snow cover. This brought the soil temperature to the season’s lowest, -4.6°C, on March 16th, 2017. The mean winter survival of CWWDP was 75.81% with the standard deviation of 12.17% (Figure 2.1). For Elora 2017/2018, the minimum air temperature was -22°C on December 31st, 2017 (Appendix II). Snow cover was observed during most of the winter to provide insulation, therefore the soil temperature remained around 0°C. This growing season had mean winter survival of 76.60% with the standard deviation of 12.14% despite having the shortest window for fall growth. For CÉROM 2017/2018, the winter temperature was much lower than the two other environments.
The lowest temperature was -27°C on January 14, 2018. CWWDP on average had poorer survival in this location with the mean survival of 54.91% with the standard deviation of 15.34%.

![Figure 2.1](image)

**Figure 2.1** Phenotypic variation of winter survival based on visual estimation at the three environments. Center line represents the median; the box represents the 25th and 75th percentiles; whiskers represent 5th and 95th percentiles; least-square mean of each genotype is represented by black dots.

The data from the three testing environments were combined. Analysis of variance was conducted and the effects of genotype \((P<0.0001)\), environment \((P<0.0001)\) and genotype by environment interaction \((P<0.0001)\) on winter survival (estimated visually) were all significant (Table 2.3).

<p>| Table 2.3 Mixed model analysis of the effect of genotype, environment and genotype by environment on the visually estimated winter survival of winter wheat | 39 |</p>
<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Numerator DF</th>
<th>Denominator DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>444</td>
<td>1228</td>
<td>3.95</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Environment</td>
<td>2</td>
<td>1228</td>
<td>715.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Genotype × Environment</td>
<td>842</td>
<td>1228</td>
<td>1.38</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effect</th>
<th>Estimate</th>
<th>Standard error</th>
<th>Chisq</th>
<th>Pr &gt; Chisq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>12.11</td>
<td>18.11</td>
<td>11.26</td>
<td>0.0004</td>
</tr>
<tr>
<td>Incomplete block(Block)</td>
<td>15.13</td>
<td>4.33</td>
<td>57.58</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>160.51</td>
<td>6.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.2 Methods for estimating winter survival:

The relationship between the methods of estimating winter survival were evaluated using Pearson’s coefficient of correlation. UAV generated-readings (NDVI Sum) demonstrated strong correlation (r=0.95 P<0.0001) with the ground-level imagery (FGCC) at estimating spring stand after the winter (Figure 2.1). Both methods also had strong correlation with the visual estimation of winter survival (NDVI Sum r=0.85 P<0.0001; FGCC r=0.84 P<0.0001). Winter survival index (WSI) was developed to take emergence into consideration when evaluating winter survival. WSI had significant correlation with the other three methods with the correlation coefficient of 0.47 with NDVI sum, 0.41 with FGCC and 0.47 with visual estimation.
Figure 2.2 Pearson’s correlation coefficients ($r$) and their significance (above diagonal), frequency distribution (on diagonal) and scatter plot (below diagonal) of different winter survival parameters captured on May 8th, 2017 at the Elora Research Station. Aerial represents NDVI sum, ground represents the fractional green canopy cover (FGCC), visual represents visually-estimated survival data and WSI represents the winter survival index.

2.4.3 Broad sense heritability

In Elora 2016/2017, high broad sense heritability values were observed for the four different methods for estimating winter survival, visual estimation (0.87), FGCC (0.88), NDVI Sum (0.85) and WSI (0.71). The heritability for the first three methods were comparable however WSI, in comparison, had lower heritability. Similarly, in Elora 2017/2018, high broad sense heritability
was observed for the three different methods for estimating winter survival, visual estimation (0.73), FGCC (0.88) and NDVI Sum (0.85). Interestingly, visual estimation had lower heritability than the other two image-based methods. In CÉROM 2017/2018, the heritability values of the methods were medium (0.57 for visual, 0.60 for FGCC) but lower than the other two location, Elora 2016/2017 and Elora 2017/2018. In this environment, FGCC and visual estimation had similar heritability. When overall broad-sense heritability was estimated across the testing environment, we see high and comparable heritability between visual estimation and FGCC (0.69 and 0.66 respectively). NDVI sum, however, had lower heritability than the other two methods (0.41).

**Table 2.4** Broad sense heritability of different winter survival parameter at each environment and across environment

<table>
<thead>
<tr>
<th>Environment</th>
<th>Broad-sense heritability ($H^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elora 2016/2017</strong></td>
<td></td>
</tr>
<tr>
<td>Visual Estimation</td>
<td>0.87 (0.01)$^b$</td>
</tr>
<tr>
<td>FGCC (ground-level image)</td>
<td>0.88 (0.01)</td>
</tr>
<tr>
<td>NDVI Sum (UAV)</td>
<td>0.85 (0.01)</td>
</tr>
<tr>
<td>WSI (UAV)</td>
<td>0.71 (0.02)</td>
</tr>
<tr>
<td><strong>Elora 2017/2018</strong></td>
<td></td>
</tr>
<tr>
<td>Visual Estimation</td>
<td>0.73 (0.03)</td>
</tr>
<tr>
<td>FGCC (ground-level image)</td>
<td>0.80 (0.02)</td>
</tr>
<tr>
<td>NDVI Sum (UAV)</td>
<td>0.82 (0.02)</td>
</tr>
<tr>
<td><strong>CÉROM 2017/2018</strong></td>
<td></td>
</tr>
<tr>
<td>Visual Estimation</td>
<td>0.57 (0.04)</td>
</tr>
<tr>
<td>FGCC (ground-level image)</td>
<td>0.60 (0.04)</td>
</tr>
<tr>
<td><strong>Combined$^a$</strong></td>
<td></td>
</tr>
<tr>
<td>Visual Estimation</td>
<td>0.69 (0.03)</td>
</tr>
<tr>
<td>FGCC (ground-level image)</td>
<td>0.66 (0.03)</td>
</tr>
<tr>
<td>NDVI Sum (UAV)</td>
<td>0.41 (0.06)</td>
</tr>
</tbody>
</table>

$^a$Heritability estimate off each winter survival parameter based on data across the three environments except for *NDVI Sum* which was measured only in Elora 2016/2017 and Elora 2017/2018. $^b$Standard error of the heritability estimate
2.4.4 *Genome-wide association study on winter survival*

The quantile-quantile (QQ) plot of each model’s genome-wide association study was evaluated and mean squared difference (MSD) with the expected distribution was calculated (Appendix IV). The model CMLM+K+Q was selected because it had the lowest MSD.

For Elora 2016/2017, 2 significant and 19 suggestive marker-trait associations (MTAs) were detected across chromosomes 4A, 4B, 5A and 7A using NDVI sum (Table 2.5a). When WSI was used, 30 significant and 8 suggestive MTAs were detected across chromosomes 4A, 4D, 5A and 7A. For Elora 2017/2018, 1 significant and 1 suggestive MTAs were detected on chromosome 5A using NDVI sum. For CÉROM 2017/2018, 15 significant and 14 suggestive MTAs were detected on chromosome 5A using FGCC. The MTAs explained from 3-9 % of the phenotypic variance. The minor allele frequency ranged from 6-31 %.

For chromosome 4A, the MTAs identified at Elora 2016/2017 using NDVI sum, located in a 4.8 Mb interval between 120571944 and 125404137 bp, were in linkage disequilibrium (LD). The interval was designated *QWs.ugw-4A.1* (Figure 2.3). For the same environment, WSI identified other MTAs on chromosome 4A. These suggestive markers on chromosome 4A, located in a 6.2 Mb interval from 570469586-576702853bp, were in LD and was designated *QWs.ugw-4A.2* (Figure 2.3). *QWs.ugw-4A.1* and *QWs.ugw-4A.2* were not in LD with each other.

Significant and suggestive MTAs on chromosome 5A, located in a 4.5 Mb interval from 584672946 to 589223758 bp, were in LD . The interval was designated *QWs.ugw-5A.3* (Figure 2.3). Markers in this linkage block were found to be significant in Elora 2016/2017 and CÉROM 2017/2018. The candidate gene *Vernalization-A1* (*Vrn-A1*) was mapped within this region from 587411454 to 587423416 bp on chromosome 5A. Significant and suggestive MTAs on chromosome 5A for Elora 2017/2018 were BS00024602_51 and RAC875_c232_1895 at 499660213 bp and 478894740 bp, respectively (Table 2.5a). BS00024602_51 and RAC875_c232_1895 were not in LD with one another or with *QWs.ugw-5A.3*.

*QWs.ugw-4B, QWs.ugw-4D* and *QWs.ugw-7A* were identified for Elora 2016/2017 only. *QWs.ugw-4B* was only identified using NDVI sum and *QWs.ugw-4D* was only identified using WSI, but *QWs.ugw-7A* was identified using both methods.
To investigate the genetic basis of winter survival, specifically for the winter wheat, the panel was genotyped with the KASP assay wMAS000033, described in Grogan et al. (2016), to detect the Vrn-A1a spring allele. The genotypes that were not carrying the spring Vrn-A1a spring allele were considered winter wheat (n=417) and formed the panel for the subsequent GWAS (Figure 2.4). For Elora 2016/2017, 1 significant and 4 suggestive MTAs were detected on chromosome 5A for NDVI sum (Table 2.5b). When WSI was used, 4 suggestive MTAs were detected on chromosome 5A. For CÉROM 2017/2018, 6 suggestive MTAs were detected on chromosome 5A. These MTAs were in LD with one another in a 10 Mb interval from 514092445 to 524244529 bp (Figure 2.4). This region was designated QWs.ugw-5A.4. QWs.ugw-5A.4 corresponds to Frost-Resistance 2 locus, which contained 17 C-Repeat Binding Factors that were mapped in the interval 522066500 to 523616562 bp on chromosome 5A. For Elora 2017/2018, QWs.ugw-5A.4 was not detected but QWs.ugw-5A.2 was again detected to be significant.
on the functions of its respective orthologs (Kersey et al. 2018). 

Table 2.5 Significant and suggestive marker-trait associations resulted from genome-wide association on winter survival with the Canadian Winter Wheat Diversity Panel (CWWDP; n= 450) and b) after the removal of genotypes with Vrn-A1a spring allele (n=413).

**a)**

<table>
<thead>
<tr>
<th>QTL</th>
<th>SNP Name</th>
<th>Marker-trait association</th>
<th>Environment</th>
<th>Trait</th>
<th>LOD</th>
<th>MAF</th>
<th>R²</th>
<th>Gene Candidate Gene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>QWn. nwg-4A.1</td>
<td>Tdurum_contig34920_104</td>
<td>4A 125404137</td>
<td>Elora 2017</td>
<td>NDVI Sum</td>
<td>4.66</td>
<td>0.08</td>
<td>0.04</td>
<td>TraesCS4A01G107100</td>
</tr>
<tr>
<td>QWn. nwg-4A.2</td>
<td>GENE-3043_279</td>
<td>5A 576702853</td>
<td>Elora 2017</td>
<td>WSI</td>
<td>5.91</td>
<td>0.1</td>
<td>0.05</td>
<td>TraesCS4B01G020300</td>
</tr>
<tr>
<td>QWn. nwg-4B</td>
<td>Kukri_c4078_180</td>
<td>4B 16787936</td>
<td>Elora 2017</td>
<td>NDVI Sum</td>
<td>4.75</td>
<td>0.43</td>
<td>0.04</td>
<td>TraesCSA01G286800</td>
</tr>
<tr>
<td>QWn. nwg-4D</td>
<td>IAAV1674</td>
<td>4D 1876448</td>
<td>Elora 2017</td>
<td>WSI</td>
<td>4.76</td>
<td>0.09</td>
<td>0.04</td>
<td>TraesCSA01G391700</td>
</tr>
<tr>
<td>QWn. nwg-5A.1</td>
<td>RAC875_c232_1895</td>
<td>5A 478896740</td>
<td>Elora 2018</td>
<td>NDVI Sum</td>
<td>4.78</td>
<td>0.31</td>
<td>0.03</td>
<td>TraesCS4A01G39700</td>
</tr>
<tr>
<td>QWn. nwg-5A.2</td>
<td>BS000024602_51</td>
<td>5A 499660213</td>
<td>Elora 2018</td>
<td>NDVI Sum</td>
<td>6.78</td>
<td>0.1</td>
<td>0.05</td>
<td>TraesCSA01G286800</td>
</tr>
<tr>
<td>QWn. nwg-5A.3</td>
<td>wsnp_Ex_c7729_13177883</td>
<td>5A 585409285</td>
<td>Elora 2017</td>
<td>NDVI Sum</td>
<td>5.72</td>
<td>0.06</td>
<td>0.05</td>
<td>TraesCSA01G391700</td>
</tr>
<tr>
<td>QWn. nwg-7A</td>
<td>Kukri_c22315_472</td>
<td>7A 287862831</td>
<td>Elora 2017</td>
<td>NDVI Sum</td>
<td>4.83</td>
<td>0.06</td>
<td>0.04</td>
<td>TraesCS4A01G107100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QTL</th>
<th>SNP Name</th>
<th>Marker-trait association</th>
<th>Environment</th>
<th>Trait</th>
<th>LOD</th>
<th>MAF</th>
<th>R²</th>
<th>Gene Candidate Gene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>QWn. nwg-5A.2</td>
<td>BS000024602_51</td>
<td>5A 499660213</td>
<td>Elora 2018</td>
<td>NDVI Sum</td>
<td>4.91</td>
<td>0.09</td>
<td>0.04</td>
<td>TraesCS4A01G286800</td>
</tr>
<tr>
<td>QWn. nwg-5A.4</td>
<td>Excalibur_c22783_765</td>
<td>5A 519989550</td>
<td>Elora 2017</td>
<td>NDVI Sum</td>
<td>5.64</td>
<td>0.07</td>
<td>0.05</td>
<td>TraesCSA01G286800</td>
</tr>
<tr>
<td>QWn. nwg-5A.4</td>
<td>Excalibur_c2598_2052</td>
<td>5A 519951364</td>
<td>Elora 2017</td>
<td>WSI</td>
<td>4.53</td>
<td>0.07</td>
<td>0.04</td>
<td>TraesCSA01G391700</td>
</tr>
<tr>
<td>QWn. nwg-5A.4</td>
<td>Kukri_c7933_828</td>
<td>5A 524244529</td>
<td>CÉROM 2018</td>
<td>FGCC</td>
<td>5.00</td>
<td>0.26</td>
<td>0.05</td>
<td>TraesCS4A01G286800</td>
</tr>
</tbody>
</table>

**b)**

Two significant thresholds were used for this study. The significant marker-trait association threshold, \( P < 2.42 \times 10^{-6} \) (0.05/number of markers), and a more liberal threshold to capture suggestive genetic region, \( P < 4.85 \times 10^{-6} \) (1/number of markers) (Yang et al. 2014; Parra-Londono et al. 2018). Chr., chromosome; Pos., position on the chromosome in base pair based on alignment to Chinese Spring NRGv1.0 genome assembly; MAF, minor allele frequency; LOD, \(-\log_{10}(P\text{-value})\); R², the amount of phenotypic variance explained by the marker.

* Significant marker-trait association has bolded SNP name. Only the most significant marker within a linkage block was presented in this table. *Putative candidate genes were identified based on the gene expression pattern originated from Li et al 2015 which looked at expression profile of spring wheat (cv ‘Manitou’) under room temperature (23°C) and cold temperature (4°C) condition and based on the functions of its respective orthologs (Kersey et al. 2018). * Multiple C-repeat binding factors were within this region; therefore, no specific gene identifier was reported.
Figure 2.3 Genome-wide association study (GWAS) for winter survival conducted on the Canadian winter wheat diversity panel (n=450). a) Manhattan plot of GWAS performed on different parameters representing winter survival across the three environments. Black solid and black dotted lines represent the significant marker–trait association (MTA) threshold ($P < 2.42 \times 10^{-6}$, 0.05/N) estimated after Bonferroni correction and the suggestive association threshold ($P < 4.84 \times 10^{-5}$, 1/N), respectively. Horizontal bar below the Manhattan plot represents the distribution of 20645 SNP markers (vertical color bar to the right is the respective color scale). b) Linkage disequilibrium (D') for the MTAs on the same chromosome; 4A and 5A.
Figure 2.4 Genome-wide association study (GWAS) for winter survival conducted on the Canadian winter wheat diversity panel after genotypes carrying the Vrn-A1a spring allele were removed from the panel (n = 413). a) Manhattan plot of GWAS performed on different measurement of winter survival across the three environments. Black solid and black dotted lines represent the significant marker–trait association (MTA) threshold (p < 2.42 e-6, 0.05/N) estimated after Bonferroni correction and the suggestive association threshold (p < 4.84 e-5, 1/N), respectively. Horizontal bar below the Manhattan plot represents the distribution of 20645 SNP markers (vertical color bar to the right is the respective color scale). b) Linkage disequilibrium (D') for the MTAs on chromosome 5A.
2.4.5 Candidate gene search

For *QWs.ugw-4A.1*, 29 high-confidence genes were identified around it, the gene TraesCS4A01G107100 was the only candidate gene that had an induced expression under cold treatment (Figure 2.5). This gene shares sequence homology with *OsRCI2-5* in *Oryza sativa* with 84.4% protein identity. For *QWs.ugw-4B*, 134 high-confidence genes were identified, the gene TraesCS4B01G020300 has shown change in expression under cold treatment (Figure 2.6). This gene shared sequence homology with *AtGRP7* in *Arabidopsis thaliana* with 81.5% protein identity. The orthologs for each respective candidate gene identified have demonstrated involvement in stress tolerance (Appendix V).

**Figure 2.5** Expression profile of high-confidence genes within the 5Mb of *QWs.ugw-4A.1* displayed as heat map on expVIP (Ramírez-González et al. 2018). RNA-Seq data originated from Li et al. 2015 which looked at expression profile of spring wheat (cv ‘Manitou’) under room temperature (23°C) and cold temperature (4°C) condition.

**Figure 2.6** Expression profile of high-confidence genes within the 5Mb of *QWs.ugw-4B* displayed as heat map on expVIP (Ramírez-González et al. 2018). RNA-Seq data originated from Li et al. 2015 which looked at expression profile of spring wheat (cv ‘Manitou’) under room temperature (23°C) and cold temperature (4°C) condition.

For the QTL *QWs.ugw-4A.2, QWs.ugw-4B, QWs.ugw-5A.1, QWs.ugw-5A.2 and QWs.ugw-7A*, large number of high-confidence genes were identified within the surrounding 5Mb region (113, 134, 150, 93 and 72 genes, respectively). For theses QTL, no gene stood out to be the
candidate gene due to the inability to narrow down the gene list using expression pattern and homology analysis.

2.5 Discussions

2.5.1 Image-based phenotyping tools

2.5.1.1 Correlation and heritability

This study utilized image-based phenotyping tools (both captured in the air or on the ground) to evaluate the in-field winter survival of winter wheat. Image-based methods had strong and significant correlation with winter survival value that was estimated visually. Sankaran et al. (2015), also observed strong association ($r = 0.93$) between the winter survival rating estimated visually and by UAV images, even though different vegetation index was used in the study. In addition, our study demonstrated high broad sense heritability for image-based methods that were comparable to the heritability of the visual estimation. At Elora 2017/2018, higher heritability value was observed for the image-based methods. Since the data capture was performed on the same day for the three methods, environmental and genetic variance can be considered insignificant leaving the level of precision of the methods the only source of error. Therefore, by having higher heritability value, it can be interpreted that image-based methods had higher precision than visual estimation. However, this study observed lower heritability value for NDVI sum when combining the environments. This is likely due to the bias that the image-based methods in this study had. In contrast to visual estimation, the result from our image-based methods were influenced more by the size and the growing stage of wheat. For example, a plot with smaller wheat plants with good survival and a plot with large wheat plants with medium survival would generate similar value using the image-based methods. On the other hand, visual estimation can distinguish the two plots by focusing on the “number” of plants that have survived. The innate bias with the image-based methods described in our study therefore made it difficult to interpret winter survival across different environments since a bigger value at another location can just mean that the plants were in later growth stage. This likely resulted in larger genotype by environment variance for our image-based methods which led to the lower heritability values observed in the study. There are, however, ways in which the “number” of the
wheat plants can be estimated from images. Studies had applied computer vision to count the number of sorghum plants in UAV captured images (Ribera et al. 2017). Similar approach can be applied to improve the estimation of winter survival by directly counting the number of plants that have survived.

2.5.1.2 Strengths and weaknesses

Initially, ground-image approach was included in the study to validate the results generated through the UAV images. It was used as the primary image-based method at environment where drone flight was not possible. This method was laborious as the experimenters were required to carry the tripod throughout the entire field and the data collection process took longer than visual estimation. Optimal field conditions were required to capture the images as the tripod was unable to stand on wet soil. The image analysis step was performed unsupervised and automated by CANOPEO. Ground-based method can be sped up by putting a sensor onto a vehicle, however exploring such option was not the focus of this paper. UAV image approach was able to capture data quite efficiently. Scoring 900 plots for winter survival visually took around two and a half hours, but UAV did the job in less than 30 minutes. This advantage will allow for the characterization of large number of materials across different environments more efficiently which is needed for large breeding program. The image processing step, however, was time-consuming and complicated. For example, it took around 6 hours (unsupervised) to generate the orthomosaic map for the field. The experimenters also spent considerable amount of time learning and utilizing all the software mentioned. The integration of semi-automated or fully-automated pipeline will be needed to expediate the process. For example, Haghighattalab et al. 2016 describes a field-map based method that can generate shapefile for the data extraction step automatically.

2.5.1.3 Application of image-based methods for genetic study

Using NDVI sum, we were able to detect nine quantitative trait loci (QTL), some of which coincided with well-known freezing tolerance loci Fr-A1 and Fr-A2. This supported that image-based phenotyping can be applied for genetic study and can identify QTL for winter survival.
In summary, image-based methods mentioned in the study are viable alternatives for estimating winter survival as it correlated well with visual estimation, had high heritability, demonstrated better precision than visual estimation in certain environment and has the potential to be high-throughput. The data generated was also able to be applied in GWAS and identified QTL for winter survival. However, there are many short-comings that will need to be addressed in order to make it applicable for breeding programs. This includes developing different winter survival evaluation method (counting the plants) to standardize the results and enable data comparison across different environments and studies. Also, there is a need for a user-friendly and automated pipeline to make the whole process high-throughput and easy for plant breeders that do not have prior experience with geographic information system to adopt the technology.

2.5.2 Winter survival index

Traditionally, only spring stand is evaluated to estimate winter survival and poor germination can confound the evaluation. We developed the winter survival index (WSI), which is the difference in vegetation cover overwinter normalized to emergence to eliminate this source of error. WSI has low but significant positive correlation with the other phenotyping parameters that were employed ($r = 0.41-0.47$). For Elora 2016/2017, similar loci were detected by using different phenotyping method. Both WSI and NDVI sum were able to detect $QWs.ugw-5A.3 \ (Fr-A1)$ and $QWs.ugw-5A.4 \ (Fr-A2)$, which were shown in previous studies to contribute significantly to freezing tolerance. However, there were QTL that were only detected by either WSI or NDVI sum in Elora 2017 such as $QWs.ugw-4A.2$ and $QWs.ugw-4D$. Like the other image-based methods within this study, WSI was also influenced heavily by growth stages. Genotypes with early spring vigor will produce high WSI and can confound with the evaluation of winter survival. Therefore, the additional QTL that were only detected in WSI likely correspond to genetic regions associated with spring vigor. Moving forward, WSI can be improved by replacing NDVI sum with the number of plants at each data collection time. This can ensure that WSI account for germination and is not confounded by spring vigor.
2.5.3 Genome-wide association study on winter survival

In total, nine QTL for winter survival were identified by using the image-based phenotyping methods described here. When GWAS was conducted using all genotypes in CWWDP, eight QTL were identified between the three environments. *QWs.ugw-5A.3* was the only locus that was detected repeatedly across different environments (Elora 2017 and CÉROM 2018). The gene *Vrn-A1* was mapped within this locus and is likely the gene underlying the QTL. This study, unlike most freezing tolerance studies, included a small number of spring wheat genotypes, because either they had previously been shown to be winter-hardy or they were used as parents in some current winter wheat cultivars. These spring wheat cultivars have been shown to carry the dominant mutation at the *Vrn-A1* promoter region (*Vrn-A1a*) (Whittal et al. 2018). Using near-isogenic lines of Norstar, homozygous for the two different allele at *Vrn-A1* (*vrn-A1* or *Vrn-A1a*), it has been shown that the spring *Vrn-A1a* allele is associated with reduced frost tolerance (Laudencia-Chingcuanco et al. 2011). Evidence have shown that the transition of shoot apex from vegetative to reproductive meristem reduce wheat’s ability to express genes that are responsible for frost tolerance (Laudencia-Chingcuanco et al. 2011). Therefore, it is generally understood that *Vrn-A1* has a pleotropic effect on frost tolerance by modulating developmental stage transition. Co-incidence of the physical location as well as the previous literature on *Vrn-A1*’s involvement in freezing tolerance supported that *QWs.ugw-5A.3* correspond to *Vrn-A1* and that allele variation at *Vrn-A1* is essential for winter survival.

To more precisely identify the genetic factors within winter wheat that affect winter survival, we conducted GWAS after removing the genotypes carrying the *Vrn-A1a* spring allele within the CWWDP. The *QWs.ugw-5A.4* QTL was then detected across different environments and, based on its physical position, appeared to most likely correspond to the *Fr-A2* locus. Seventeen genes from the *C-repeat binding factor (CBFs)* family were mapped within this linkage block (Appels et al. 2018). Out of the 17 genes, the effect of *CBF-A12, A14* and *A15* on cold tolerance and winter survival have been well-characterized by previous work. Soltész et al. (2013) have shown that the heterologous expression of *CBF-A14* and *A15* from wheat in barley can improve frost tolerance via up-regulation of *Cold Regulated (COR)* genes. Two haplotypes (*Fr-A2-T/ Fr-A2-S*) were observed between *CBF-A12* and *CBF-A15*. The haplotype was well
conserved across wheat cultivars from different growing regions and multiple studies have demonstrated that Fr-A2-T is associated with better frost tolerance (Zhu et al. 2014; Würschum et al. 2017; Babben et al. 2018). Würschum et al. (2017) also demonstrated that this haplotype, along with copy number variation of CBF-A14, can explain 40% of the genetic variance in winter survival for European winter wheat. It is therefore likely that QWs.ugw-5A.4 corresponded to Fr-A2 and is responsible for the difference in winter survival.

2.5.4 Candidate genes

This study made use of the recent release of the annotated wheat genome and the available RNA-Seq data to identify the putative candidate gene for each QTL. TraesCS4A01G107100 was identified as a candidate gene for QWs.ugw-4A.1 based on its differential expression pattern in cold treatments and literature on its respective ortholog (Li et al. 2015). OsRIC2-5, an ortholog of TraesCS4A01G107100, has subcellular localization within the cellular membrane and its overexpression of conferred better drought tolerance in rice (Li et al. 2014). This suggests that OsRIC2-5 is involved in signal transduction for stress response. Freezing-induced desiccation is one of the stress factors that is attributed to winterkill. It seems probable, then, that similar to OsRIC2-5, TraesCS4A01G107100 can confer tolerance to winter desiccation through similar mechanism as its role in drought tolerance.

Next, TraesCS4B01G020300 was identified to be the candidate gene for QWs.ugw-4B. TraesCS4B01G020300 was one of the few genes within the list of 134 candidate genes that shown differential expression in response to cold. Its respective ortholog in Arabidopsis was determined to be AtGRP7, which belongs to a group of glycine-rich RNA-binding proteins. Overexpression of AtGRP7 improved the freezing tolerance in Arabidopsis (Kim et al. 2008). Evidence suggests that glycine-rich RNA-binding proteins (GRP) are functionally conserved across different plant species. Kim et al. (2010) demonstrated that glycine-rich RNA-binding proteins (OsGRP6) in rice can rescue the phenotype of cold-sensitive Arabidopsis grp7 mutant by conferring freezing tolerance. Coincidentally, GRP has been shown to function in the development of freezing tolerance in wheat as well. In Christov et al. 2007, TaGRP2 has shown increase expression level after cold acclimation. By using BLAST (Basic Local Alignment
Search Tool) on the TaGRP2 sequence (accession number AB272227 in the DDBJ database) against the wheat reference genome, we confirmed that it matched with TraesCS4B01G020300. Recent work on TaGRP2 shown that it regulates vernalization by preventing the accumulation of VRN1 (Xiao et al. 2014). We therefore hypothesize that TaGRP2 affect winter survival through the modulation of flowering time.

For the testing environment at Elora 2018, GWAS did not detect Vrn-A1 and Fr-A2, however QWs.ugw-5A.2 was found to be associated with winter survival in analyses with and without the genotypes with Vrn-A1a spring allele. The method could not narrow down the list of candidate genes for QWs.ugw-5A.2. However, QWs.ugw-5A.2 co-localized with a QTL (QDFLNv.usw-5A) that was identified by Fowler et al (2016) to be associated with time required for vegetative to reproductive transition (VRT). Out of the 93 high confidence genes identified, one gene (TraesCS5A01G286800) had the protein function annotation of MADS-Box transcriptional factor (IPR002100) (Data S1). MADS-box transcriptional factors are known to have key roles in plant growth and development; for example VRN-1 gene is the main determinant of growth habit in wheat (Yan et al. 2003). We therefore hypothesize that like Vrn-A1, TraesCS5A01G286800 has pleiotropic effect on frost tolerance and ultimately winter survival by modulating the timing of VRT. However, the expression pattern of TraesCS5A01G286800 and results from characterization of orthologs in rice and Arabidopsis suggested otherwise. RNA-seq data from different developmental stages of the wheat (Ramírez-González et al. 2018) showed specific expression of TraesCS5A01G286800 in the reproductive organ (spike and grain) and induced expression was observed during the reproductive stage at the spike. The expression pattern suggested that this gene is involved in reproductive organ development. Previous literature has identified orthologs of TraesCS5A01G286800 in rice (OsMADS8) and Arabidopsis (SEP3) (Appels et al. 2018). Silencing of OsMADS8 along with the functionally redundant OsMADS7 using RNA interference in rice led to severe alteration to floral organs (Cui et al. 2010). Similarly, in Arabidopsis, the triple mutant of SEP3 with the functionally redundant SEP1 and SEP2 caused the conversion of petals and stamens into sepals (Pelaz et al. 2000). While these studies provide the evidence that TraesCS5A01G286800 likely plays a role in floral determinacy, it is uncertain whether TraesCS5A01G286800 can also
modulate of the timing of VRT. Further functional characterization will need to be conducted on TraesCS5A01G286800 to see if it has related functions that can affect winter survival.

The candidate genes mentioned above have been identified by applying the newly available tools in wheat genetics. Although further gene characterization and expression study will need to be performed to confirm their involvement in winter survival, our study illustrated the power of applying these new tools to identify the candidate gene underlying the identified QTL.

2.5.5 Significance of in-field winter survival testing

There was variation in winter survival among the genotypes and the genotype by environment (G by E) interaction effect on winter survival was significant ($P<0.0001$). Different genetic loci were detected for the two testing-years at Elora. For Elora 2016/2017 and CÉROM 2017/2018, Vrn-A1 and Fr-A2 on chromosome 5A were detected to be associated with winter survival, while another genetic locus was detected for Elora 2017/2018. These results showed that different genetic mechanisms can be responsible for winter survival at different testing environments. Based on the genes identified, freezing tolerance seems to be key for winter survival in Elora 2016/2017 and CÉROM 2017/2018 while tolerance to another stress factor might play a more important role for winter survival in Elora 2017/2018. Previous works have also observed significant cultivar by trial interaction for winter survival (Thomas et al. 1993) and that tolerance to different winter-related stress (e.g. freezing temperature, ice) is associated with winter survival depending on the testing-location and year (Andrews et al. 1997). Therefore, genetic study based on multi-year/location winter survival data can supplement in-door freezing tolerance study to identify additional genetic factors that are responsible for winter survival for a specific region.

2.5.6 Conclusions

This study illustrated the importance of genetic studies based on in-field winter survival to supplement results that are based on indoor frost tolerance studies to better understand the genetic basis of overall winter survival. Our study, to the best of our knowledge, was the first to demonstrate the feasibility and precision of using image-based phenotyping data for conducting
quantitative genetic studies of in-field winter survival. In total, nine QTL, including \( Vrn-A1 \) and \( Fr-A2 \), were identified to be essential for the in-field winter survival of wheat. Using the recently published annotated wheat genome sequence and the RNA-Seq online expression data resources, three putative candidate genes were identified including \( TaGRP2 \). The effect of these candidate genes on in-field winter survival will need to be confirmed in future studies.
Chapter 3 : Characterization of Haplotype Diversity at Fr-A2 and Copy Number Variation of Vrn-A1 and CBF-A14 Within Canadian Winter Wheat Germplasm

The study reported in this chapter has been written as a manuscript to be submitted to Molecular Breeding.

Contribution:

Yi Chen and Dr. Alireza Navabi designed the study. Yi Chen collected phenotypic data and performed the analyses. Dr. Mina Kaviani collected the DNA samples for the panel. Yi Chen and Dr. Mina Kaviani conducted the genotyping. Dr. Michel S. McElroy tested the diversity panel in Quebec. Yi Chen prepared the first draft of the manuscript. Dr. Istvan Rajcan and Dr. Mina Kaviani reviewed the manuscript and provided input on the writing.
3.1 Abstract

Winter survival is an essential trait for winter wheat (*Triticum aestivum* L.) cultivars that are being grown in high latitude growing region including eastern Canada. In-door freezing tolerance studies have identified candidate genes that influence freezing tolerance. In addition, copy number variation of certain candidate genes were also shown to affect freezing tolerance. However, there are currently no studies that characterize the allele variation of these freezing tolerance gene for Canadian winter wheat. The objective of this study is therefore to characterize the Canadian winter wheat germplasm for allele variation of *CBF*-A12 and *CBF*-A15 and copy number variation of *Vrn*-A1 and *CBF*-A14 for 415 Canadian winter wheat. The effect of these gene variation on winter survival of winter wheat were also evaluated in three location-year combinations. The majority of the winter wheat (77.3%) were shown to carry three copies of *Vrn*-A1 and the Fr-A2-T haplotype (for *CBF*-A12 and *CBF*-A15) which was shown in our study to be the minimum allele combination for stable winter survival in eastern Canada.
3.2 Introduction

Wheat is an important crop globally and it accounts for almost a fifth of the calories consumed by humans (FAOSTAT 2017). However, high latitude growing regions such as Canada are susceptible to harsh winters that can cause severe winter-kill in winter wheat (Statistic Canada 2017). Winter-kill can have significant economic impact on the producer, there winter survival is an essential trait for winter wheat production in high-latitude growing regions.

Winter survival is a complex trait as there are numerous biotic and abiotic winter-related stress factors that can decrease the overall survival of winter wheat (Sãulescu and Braun 2001; OMAFRA Field Crop Team 2017). However, most studies have suggested that freezing tolerance is an integral part of winter survival. For example, in growing regions such as Western Canada and Lithuania, freezing tolerance has a strong negative correlation with winter survival, with correlation coefficients being -95% and -97%, respectively (Fowler et al. 1981b; Gorash et al. 2017). A significant correlation between freezing tolerance and winter survival was also observed in 4 winter wheat cultivars tested in Eastern Ontario for studying the effect of planting date and winter survival (Andrews et al. 1997). However, in some years, ice tolerance was shown to have stronger association to winter survival than freezing tolerance did in Eastern Ontario. These studies demonstrated that freezing tolerance is likely the major contributor to winter survival for most high-latitude growing regions; whereas additional winter-related stress tolerance, such as ice tolerance, might further improve overall winter survival in specific growing regions.

Frost tolerance has been studied extensively. It is well known that Frost Resistance-2 locus on chromosome 5A (Fr-A2) is among the key components of freezing tolerance and winter survival in wheat (Vágújfalvi et al. 2005; Würschum et al. 2017). Fr-A2 contains a cluster of at least eleven C-repeat binding factors (CBFs) (Miller et al. 2006; Appels et al. 2018), which are transcriptional factors that can activate the expression of Cold Regulated (COR) genes and induce better freezing tolerance in wheat (Dhillon et al. 2010, Bága et al. 2007; Motomura et al. 2013; Zhu et al. 2014; Würschum et al. 2017). Two conserved haplotypes (Fr-A2-T/ Fr-A2-S) were observed between CBF-A12 and CBF-A15 and the Fr-A2-T haplotype had been shown to
confer better freezing tolerance in an in-door study (Zhu et al. 2014). Copy number variation (CNV) of CBFs at the Fr-A2 also influences winter survival. Würschum et al. (2017) demonstrated that CNV of CBF-A14 explained 24.3% of the genotypic variance while Fr-A2 haplotype only explained 0.7%, therefore, highlighting the importance of CNV of CBF-A14 on winter survival for European winter wheat. The importance of CNV of CBF-A14 was further supported by a study done in European durum wheat. CBF-A14 CNV has been shown to be correlated with winter survival and to explain 91.6 % of genotypic variance within genotypes that carry the Fr-A2-T haplotype (Sieber et al. 2016). In short, the Fr-A2-T haplotype and higher copy number of CBF-A14 at Fr-A2 are associated with better freezing tolerance and winter survival.

In addition, Vernalization-A1 (Vrn-A1) on chromosome 5A has been shown to affect freezing tolerance in winter wheat. Vrn-A1 encodes MADS box protein and affect timing of transition from vegetative to reproductive growth and has pleotropic effect on freezing tolerance (Laudencia-Chingcuancoc et al. 2011). Genotypes with higher copy number of Vrn-A1 (3 copies) have better freezing tolerance and better winter survival in the field (Zhu et al. 2014; Würschum et al. 2017). However, higher copy number of Vrn-A1 was also associated with a delay in flowering time in a growth room study (Díaz et al. 2012). This can cause concern for breeders, because wheat producers in high latitude regions typically favor earlier flowering winter wheat for benefits such as disease avoidance and earlier harvest (Fowler 2012; OMAFRA Field Crop Team 2017). Therefore, it is important to verify the effect of copy number of Vrn-A1 on flowering time in the field.

The main objective of this study is to characterize the Canadian winter wheat germplasm for variation in Fr-A2 haplotype and in the copy number of Vrn-A1 and CBF-A14. Secondly, we wanted to evaluate the effect of these candidate genes on winter survival of winter wheat in Eastern Canada to identify the optimal allele combination for winter survival. Lastly, this study evaluated whether copy number variation of Vrn-A1 could affect flowering time within field conditions in Eastern Canada.
3.3 Materials and Methods

3.3.1 Plant material and DNA extraction

The Canadian Winter Wheat Diversity Panel (CWWDP; n =450; Sidhu et al. unpublished) includes representative winter wheat cultivars that have been grown in Canada since the 1880s, current Canadian commercial winter wheat cultivars, advanced breeding materials from a number of breeding programs, key contributing genotypes in the ancestry of the Canadian winter wheat (Robert Graf, Personal communication), and a number of spring wheat cultivars that were specifically bred to be frost tolerant (Whittal et al. 2018).

The DNA was extracted at the four-leaf stage using the Qiagen DNeasy Plant DNA Mini Kit (Qiagen Gmbh, Maryland, USA). Since this study was interested in the winter survival of winter wheat specifically, the panel was genotyped with a Kompetitive Allele Specific PCR (KASP) assay wMAS000033 following procedure described in Grogan et al. (2016) to detect the cultivars carrying the Vrn-A1a dominant mutation that confers spring growth type. Winter survival data from cultivars that carry the Vrn-A1a allele (n=35) was not included in the statistical analysis resulting in the panel size of 415 genotypes.

3.3.2 Field experiments and phenotypic data collection

CWWDP was grown at the Elora Research Station, Ontario, Canada (43°38' 27.0456" N, 80°24' 18.6948" W) in the 2016/2017 (planted on Oct 14th, 2016) and 2017/2018 (planted on Oct 21th, 2017) growing seasons. The panel was grown in Centre de Recherche sur les Grains, Quebec, Canada (CÉROM) (45° 34' 57.12" N, 73° 14' 10.62"W) in the 2017/2018 growing season (planted on September 27, 2017). Each year and location combination was considered an environment. The experimental design in all three environments was a two-replication α-lattice design (Patterson and Williams 1976; Piepho et al. 2006). For 2016/2017 Elora and 2017/2018 CÉROM, each experimental unit was a 1 m long plot with 6 rows with 17.8 cm row-spacing. In 2017/2018 Elora, each experimental unit was a 2 m plot with 6 rows, with 17.8 cm row spacing.

For visual estimation of winter survival, plots were inspected visually and scored based on how much plant stand has remained (0= no plant stand visible in the plot area, 100= full plant
stands remained after the winter). Estimation of winter survival is performed once the snow has melted and the plants were given some time to recover. Winter survival evaluation was performed on May 08th 2017 for Elora 2016/2017, May 14th, 2018 for Elora 2017/2018 and May 9th, 2018 for CÉROM 2017/2018. Heading day was determined for each cultivar as the day in which 75% of the plants within the experimental unit has fully visible spike above the stem.

3.3.3 Genotyping

3.3.3.1 Candidate genes (Fr-A2: CBF-A12&A15)

Fr-A2 haplotype of the diversity panel was characterized using the primers described by Zhu et al. (2014). All PCRs were carried out in 25 µl reactions containing 12.5 µl 1x PCRBIO Ultra Mix (PCR Biosystems, Foster City, London, United Kingdom), 10 µM for forward and reverse primer for each primer pair, 300 ng of genomic DNA and 5% DMSO. PCR was performed on a 96-well plate following program described in supplementary materials (Appendix VI). PCR product of CBF-A12 was digested using 5 units of ZraI (New England Biolabs, Massachusetts, United States), 1 µg of PCR product and 1x NEBuffer (New England Biolabs, Massachusetts, United States) to final volume of 25 µL. PCR product of CBF-A15 was digested using SalI (Thermo Scientific, Massachusetts, United States) 1 µL of FastDigest Enzyme, 1 µg of PCR product and 1x FastDigest Buffer were mixed to give a final volume of 30 µL. Both digestion reaction was incubated at 37°C for 60 minutes to produce the restriction product. Visualization of restriction product was done using QIAxcel Advanced System (QIAGEN, Hilden, Germany).

For the frost susceptible haplotype (Fr-A2-S), SalI digestion of CBF-A15 produce two fragments of 403/605 bp while ZraI digestion of CBF-A12 produce two fragments of 706/476 bp. For the frost tolerant haplotype (Fr-A2-T), SalI digestion of CBF-A15 produce one band of 1017 bp while ZraI digestion of CBF-A12 produce three fragments of 400/304/476 bp. There was no recombination detected between CBF-A12 and CBF-A15 within CWWDP.

3.3.3.2 Taqman® assays to determine Vrn-A1 and CBF-A14 copy number

A Taqman® Assay was used to determine copy number variation of Vrn-A1 and CBF-A14. Vrn-A1 copy number assay was performed following the protocol described by Diaz et al (2012) with modification of using [FAM-QSY] for the target (VRN-A1) and [VIC-QSY] for the control
CONSTANS2 (CO2). CBF-A14 copy number assay follow the protocol described by Zhu et al (2014) with modification of using [FAM-MGM] for the target (CBF-A14) and [VIC-QSY] for the control CONSTANS2 (CO2). For both Taqman® Assay, 1x TaqPath ProAmp Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5µM of each primer pair for both target and control genes, 0.1 µM of both probes and 10 ng of DNA were mixed to give a final volume of 10 µl duplex reaction. Reactions were run on QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Thermo-Fisher Scientific Inc., Waltham, MA, USA) (Appendix VII). The reactions were done in triplicate. For Vrn-A1, the copy number was determined with the comparative CT (ΔΔCt) method where the cultivar Norstar serves as the calibrator using Copy Caller v2.1® (Applied Biosystems, Foster City, CA, USA). Norstar has 3 copies of Vrn-A1 (Zhu et al. 2014). Copy number variation of CBF-A14 is reported as a relative quantity of CBF-A14 of each cultivar to the calibrator, Norstar (\(\Delta C_T^{\text{variety}} / \Delta C_T^{\text{Norstar}}\)).

3.3.4 Statistical analysis

The following statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was conducted on phenotypic trait data (winter survival and heading date) of each environment separately using the PROC GLIMMIX procedure with variety being the fixed effect while block and incomplete-block(block) as random effects. ANOVA was performed on the combined data of all environments for winter survival, with variety as the fixed effect and environment, block (environment), incomplete-block \(\times\) block(environment) and variety by environment as random effects. For both ANOVA, residuals were computed and plotted against predicted values in PROC SGPLOT to validate the assumptions made by the statistical model. The PROC UNIVARATE procedure was then used to perform a test of normality on the residuals based on Shapiro-Wilk test (Shapiro and Wilk 1965) at a Type I Error rate of \(\alpha=0.01\). Least squares means (LSMEANS) of each trait was generated for each genotype using the LSMEANS statement in PROC GLIMMIX.

Single gene effect of the Fr-A2 haplotype, Vrn-A1 copy number variation, and CBF-A14 copy number variation on winter survival was tested individually for each environment using the PROC GLM procedure, with the winter survival (LSMEANS) as the dependent variable and the
gene as the independent variable. LSMEANS were generated for each genotype at each environment using the LSMEANS statement. T-test was used to compare means of the genotypes within each environment. Fisher’s exact test was used within the PROC FREQ procedure to evaluate the significance of the association between Fr-A2 haplotype and CBF-A14 copy number.

Fr-A2 haplotype and Vrn-A1 copy number were then fit into a linear model using PROC GLM procedure, where combined winter survival across environment (LSMEANS) was the dependent variable and Fr-A2 haplotype, Vrn-A1 copy number and Fr-A2 haplotype × Vrn-A1 copy number were the independent variable. LSMEANS were generated for each Fr-A2 haplotype × Vrn-A1 copy number combination using the LSMEANS statement. Tukey test was used to compare the means of the combinations.

Variance component analysis was conducted on winter survival using the REML algorithm of the PROC VARCOMP with environment, block(environment), incomplete-block × block(environment), Fr-A2 haplotype, Vrn-A1 copy number, Fr-A2 haplotype × Vrn-A1 copy number, genotype (Fr-A2 haplotype), genotype (Vrn-A1 copy number), genotype (Fr-A2 haplotype × Vrn-A1 copy number), Fr-A2 haplotype × environment, Vrn-A1 copy number × environment, Fr-A2 haplotype × Vrn-A1 copy number × environment, genotype (Fr-A2 haplotype) × environment, genotype (Vrn-A1 copy number) × environment, genotype (Fr-A2 haplotype × Vrn-A1 copy number) × environment as random effect. Variance components were generated to calculate broad sense heritability ($H^2$), heritability of the Vrn-A1 copy number and Fr-A2 haplotype combined, and proportion of genetic variance explained by the combination of Vrn-A1 copy number and Fr-A2 haplotype as:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + (\sigma_{G\times E}^2 + y) + (\sigma_{Error}^2 \div (ry))}$$

$$h^2_{Combination} = \frac{\sigma_{Fr-A2 Haplotype}^2 + \sigma_{Vrn-A1 Copy Number}^2 + \sigma_{Fr-A2 Haplotype \times Vrn-A1 Copy Number}^2}{\sigma_G^2 + (\sigma_{G\times E}^2 + y) + (\sigma_{Error}^2 \div (ry))}$$

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where $\sigma^2_G$ is the genotypic variance, $\sigma^2_{GX}$ is the genotype $\times$ environment variance, $\sigma^2_{Error}$ is the residual variance, $r$ is the number of replication and $y$ is the number of environments. Genetic variance accounted for by the combination of $Vrn-1$ copy number and $Fr-A2$ haplotype was estimated as the ratio of $\sigma^2_{Combination}$ to $\sigma^2_G$.

3.4 Results

3.4.1 Candidate gene polymorphism

The CWWDP panel was genotyped by specific markers for allele variation on $CBF-A12$ and $CBF-A15$. There was no recombination observed between $CBF-A12$ and $CBF-A15$ meaning that genotypes with the “T” allele at $CBF-A12$ carried the “T” allele at $CBF-A15$ and genotypes with “S” allele at $CBF-A12$ carried the “S” allele at $CBF-A15$. Within this panel, 86.7% of the winter wheat cultivars carry the $Fr-A2$-$T$ haplotype while only 13.3% of the cultivars carry the $Fr-A2$-$S$ haplotype (Table 3.1).

Table 3.1 Frequency of CWWDP cultivars (n=415) within different group of $Fr-A2$ haplotype, $Vrn-1$ copy number and $CBF-A14$ copy number

<table>
<thead>
<tr>
<th>Gene</th>
<th>Copy number/haplotype</th>
<th>Number of genotypes (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Vrn-1$</td>
<td>1</td>
<td>12 (2.9)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>44 (10.6)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>356 (85.8)</td>
</tr>
<tr>
<td></td>
<td>Undetermined</td>
<td>3 (0.7)</td>
</tr>
<tr>
<td>$CBF-A14$</td>
<td>1</td>
<td>21 (5.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87 (21)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>304 (73.2)</td>
</tr>
<tr>
<td></td>
<td>Undetermined</td>
<td>3 (0.7)</td>
</tr>
<tr>
<td>$Fr-A2$</td>
<td>T</td>
<td>360 (86.7)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>55 (13.3)</td>
</tr>
</tbody>
</table>

a Value in the bracket represents percentage of genotypes within the panel carrying the allele

3.4.2 Copy number variation at $Vrn-1$ and $CBF-A14$

At $Vrn-1$, 85.8% of the winter wheat cultivars had three copies of $Vrn-1$, 10.6% has two copies while 2.9% has one copy (Table 3.1). Copy number of $CBF-A14$ is measured as the
relative quantity (RQ) to the calibrator cultivar, Norstar, where higher relative quantity corresponds to higher copy number of CBF-A14. Three separate grouping were observed within this study (Figure S1), group one has the RQ of less than 0.4 to the calibrator, group two has the RQ within 0.4-0.7 and group three has the RQ of higher than 0.7. For CBF-A14 copy number, 73.2% of the cultivars belong to group three, 21% belong to group two and only 5.1% belong to group one (Table 3.1).

![Figure 3.1](image.png)

**Figure 3.1** Frequency distribution of relative quantity of CBF-A14 of the genotypes in the Canadian winter wheat diversity panel (n =415) to the calibrator, Norstar.

### 3.4.3 Effect of the candidate genes on winter survival

Single gene effect of Fr-A2 haplotype, Vrn-A1 copy number and CBF-A14 copy number on winter survival were evaluated for this study. Fr-A2 haplotype was shown to have significant effect on winter survival of winter wheat in the field (P<0.0001) (Table 3.2). The interaction effect of Fr-A2 haplotype and environment was also significant (P =0.0055). Genotype group with the Fr-A2-T haplotype had significantly better mean winter survival than the genotype
group with Fr-A2-S across the three environments (Table 3.3). The mean winter survival difference between the two haplotypes range from 7 to 14% depending on the environment. Vrn-A1 copy number was shown to have significant effect on the winter survival of winter wheat in the field (P<0.0001) (Table 3.2). Across the three environments, genotype group with two copies of Vrn-A1 seemed to have a better winter survival than the group with one copy, however the differences were not statistically significant across all three environments (Table 3.3). The group with three copies of Vrn-A1 has significantly better winter survival than groups with one or two copies of Vrn-A1 across the three environments. CBF-A14 copy number was shown to have a significant effect on winter survival of winter wheat in the field (P<0.0001) (Table 3.2). The interaction effect between CBF-A14 copy number and the environment was also significant (P<0.0001). At CÉROM 2017/2018 and Elora 2017/2018, the group with the highest copy number of CBF-A14 (group 3) had significantly better winter survival than group two and one (Table 3.3). The difference in winter survival between group three and two were not significant at Elora 2016/2017, however, both groups had better winter survival than group one. Therefore, higher copy number of CBF-A14 therefore was associated with a better winter survival.

Table 3.2 General linear model analysis of the single gene effect (Fr-A2 haplotype, Vrn-A1 copy number and CBF-A14 copy number) and environment on the winter survival of Canadian winter wheat diversity panel (n=415)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr-A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fr-A2 haplotype</td>
<td>1</td>
<td>26444.3469</td>
<td>26444.3469</td>
<td>107.99</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Environment</td>
<td>2</td>
<td>121874.6148</td>
<td>60937.3074</td>
<td>248.85</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Fr-A2 haplotype *Environment</td>
<td>2</td>
<td>2551.1557</td>
<td>1275.5778</td>
<td>5.21</td>
<td>0.0055</td>
</tr>
<tr>
<td>Vrn-A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vrn-A1 Copy Number</td>
<td>2</td>
<td>18235.91497</td>
<td>9117.95749</td>
<td>36.53</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Environment</td>
<td>2</td>
<td>45778.95824</td>
<td>22889.47912</td>
<td>91.71</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Vrn-A1 Copy Number*Environment</td>
<td>4</td>
<td>621.28669</td>
<td>155.32167</td>
<td>0.62</td>
<td>0.6466</td>
</tr>
<tr>
<td>CBF-A14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBF-A14 Grouping</td>
<td>2</td>
<td>34860.41280</td>
<td>17430.20640</td>
<td>72.76</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Environment</td>
<td>2</td>
<td>80946.49790</td>
<td>40473.24895</td>
<td>168.95</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CBF-A14 Grouping*Environment</td>
<td>4</td>
<td>6641.02228</td>
<td>1660.25557</td>
<td>6.93</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
Table 3.3 Least squares means for winter survival rate of genotypes with specific \textit{Vrn-A1} and \textit{CBF-A14} copy number and \textit{Fr-A2} haplotype at each environment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Copy number/haplotype</th>
<th>Environments</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Vrn-A1}</td>
<td>1</td>
<td>64.06\textit{a}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>72.20\textit{a}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>77.48\textit{b}</td>
</tr>
<tr>
<td>\textit{CBF-A14}</td>
<td>1</td>
<td>58.79\textit{a}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>77.75\textit{b}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>77.47\textit{b}</td>
</tr>
<tr>
<td>\textit{Fr-A2}</td>
<td>T</td>
<td>77.58\textit{a}</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>70.93\textit{b}</td>
</tr>
</tbody>
</table>

Mean in the same environment followed by different letters are significantly different based on \textit{t}-test at the significance threshold of \( P = 0.01 \)

3.4.4 The allele combination associated with better winter survival

The goal of this part of the study was to identify the combination of haplotype and copy number variation that can ensure stable winter survival in high latitude growing region. \textit{CBF-A14} are also within the \textit{Fr-A2} locus along with \textit{CBF-A12} and \textit{CBF-A15} that makes up the \textit{Fr-A2} haplotype. Cultivars with the \textit{Fr-A2-T} haplotype tends to have higher number of copies of \textit{CBF-A14} (either group two and three) as compared to cultivars with the \textit{Fr-A2-S} haplotype, which had a lower number of copies of \textit{CBF-A14} (either group one or two) (Figure 3.2, Table 3.4). Based on Fisher’s Exact Test, there is a significant association between \textit{CBF-A14} copy number and \textit{Fr-A2} haplotype (\( P = 0.0001 \)).

Table 3.4 Number of genotypes of the Canadian winter wheat diversity panel (\( n = 415 \)) within each genotypic combination grouping of \textit{Fr-A2} haplotype and \textit{CBF-A14} copy number

<table>
<thead>
<tr>
<th>\textit{Fr-A2} Haplotype</th>
<th>\textit{CBF-A14} Grouping</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>T</td>
<td>301</td>
<td>56</td>
</tr>
<tr>
<td>S</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>87</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Three cultivars had undetermined genotypes
Therefore, only the combination of Fr-A2 haplotype and Vrn-A1 copy number was considered to address the goal of the study. Majority of the panel (77.3 %) have the allele combination of Fr-A2-T haplotype with three copies of Vrn-A1 (Table 3.5). The effects of Fr-A2 haplotype and Vrn-A1 copy number on winter survival were significant (<0.0001), but the interaction effect was not (P =0.0679) (Table 3.6). Out of the six possible combinations, the combination of the Fr-A2-T haplotype and three copies of Vrn-A1 had the highest winter survival with the mean survival of 71.57% (Figure 3.3). Additional analysis was conducted to examine whether selecting for higher CBF-A14 copy number within genotypes that carry the Fr-A2-T haplotype and three copies of Vrn-A1 could further improve winter survival. However, the effect of CBF-A14 on winter survival was not significant within the genotypes that carry the Fr-A2-T haplotype and three copies of Vrn-A1 (Data not shown).
Table 3.5 Combined mean winter survival of winter wheat genotypes of the Canadian winter wheat diversity panel (n =415) across the environments within each Fr-A2 haplotype and Vrn-A1 copy number combination group

<table>
<thead>
<tr>
<th>Vrn-A1 CNV</th>
<th>Fr-A2</th>
<th>Winter-Survival ⁹</th>
<th>Number of Cultivars (%) ¹⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>42.77 ⁹a</td>
<td>4 (1.0)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>61.89 ⁹b</td>
<td>8 (1.9)</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>60.01 ⁹b</td>
<td>16 (3.9)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>64.08 ⁹b</td>
<td>28 (6.7)</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>62.50 ⁹b</td>
<td>35 (8.4)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>71.57 ⁹c</td>
<td>321 (77.3)</td>
</tr>
</tbody>
</table>

¹⁰Means followed by the same letter are not significantly different according to Tukey’s test (P=0.01). ¹¹Value in the bracket represents percentage of genotypes within the panel carrying the allele. Three genotypes have undetermined genotypes.

Table 3.6 General linear model analysis of the two gene effect (Vrn-A1 copy number and Fr-A2 haplotype) on the winter survival of Canadian winter wheat diversity panel (n =415) combined across the three environments

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vrn-A1 Copy number</td>
<td>2</td>
<td>2561.509307</td>
<td>1280.754653</td>
<td>14.04</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Fr-A2 Haplotype</td>
<td>1</td>
<td>2025.521482</td>
<td>2025.521482</td>
<td>22.21</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Vrn-A1 Copy number* Fr-A2 Haplotype</td>
<td>2</td>
<td>493.975441</td>
<td>246.987721</td>
<td>2.71</td>
<td>0.0679</td>
</tr>
</tbody>
</table>
Figure 3.3 Combined winter survival of winter wheat cultivars across the environments with different combination of Vrn-A1 copy number and Fr-A2 haplotype. Center line represents the median; the box represents the 25th and 75th percentiles; whiskers represent 5th and 95th percentiles. Groups with the same letter are not significantly different according to Tukey’s test (P=0.01).
3.4.5 Broad sense heritability of the candidate gene markers

Variation partitioning of the winter survival for CWWDP indicated that genetic influence was more important than the genotype by environment interaction variance. Broad-sense heritability estimate was high for winter survival (0.77). In addition, the combination of \( Vrn-A1 \) copy number and \( Fr-A2 \) haplotype accounted for a large proportion of genetic variance (67.38\%) (Table 3.7). The phenotypic variance explained by the combination of \( Vrn-A1 \) copy number and \( Fr-A2 \) haplotype was 0.52.

3.4.6 \( Vrn-A1 \) copy number variation does not affect in-field heading time

The objective was to determine if selection for three copies of \( Vrn-A1 \) would lead to a delay in flowering time in the field. Analysis showed that the effect of \( Vrn-A1 \) copy number on heading time was not significant. Although, genotype group with two copies of \( Vrn-A1 \) appeared to have later heading time in comparison to groups with one and three copies, the difference was not significant (Table 3.8).
Table 3.7 Variance partitioning for winter survival of the Canadian winter wheat diversity panel (n = 415)

<table>
<thead>
<tr>
<th>Variance Components</th>
<th>Winter Survival Combined years</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma^2_G$</td>
<td>108.42</td>
</tr>
<tr>
<td>Genotypic variance explained by candidate genes</td>
<td></td>
</tr>
<tr>
<td>$\sigma^2_{Fr-A2}$ Haplotype</td>
<td>73.05</td>
</tr>
<tr>
<td>$\sigma^2_{Vrn-A1}$ Copy Number</td>
<td>35.03</td>
</tr>
<tr>
<td>$\sigma^2_{Fr-A2}$ Haplotype x $Vrn-A1$ Copy Number</td>
<td>0</td>
</tr>
<tr>
<td>Additional genotypic variance</td>
<td></td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Fr-A2 haplotype)}$</td>
<td>1.36</td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Vrn-A1 Copy Number)}$</td>
<td>32.64</td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Fr-A2 Haplotype x Vrn-A1 Copy Number)}$</td>
<td>1.37</td>
</tr>
<tr>
<td>$\sigma^2_{G \times E}$</td>
<td></td>
</tr>
<tr>
<td>$\sigma^2_{Fr-A2}$ Haplotype x Environment</td>
<td>2.38</td>
</tr>
<tr>
<td>$\sigma^2_{Vrn-A1}$ Copy Number x Environment</td>
<td>0</td>
</tr>
<tr>
<td>$\sigma^2_{Fr-A2}$ Haplotype x $Vrn-A1$ Copy Number x Environment</td>
<td>0.74</td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Fr-A2 haplotype) x Environment}$</td>
<td>0</td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Vrn-A1 Copy Number) x Environment}$</td>
<td>40.40</td>
</tr>
<tr>
<td>$\sigma^2_{Genotype(Fr-A2 Haplotype x Vrn-A1 Copy Number) x Environment}$</td>
<td>0.11</td>
</tr>
<tr>
<td>$\sigma^2_{Error}$</td>
<td>103.40</td>
</tr>
<tr>
<td>$H^2$</td>
<td>0.77</td>
</tr>
<tr>
<td>$h^2$ Combination</td>
<td>0.52</td>
</tr>
<tr>
<td>Percent genetic variance explained by the allele combination (%) $^c$</td>
<td>67.38</td>
</tr>
</tbody>
</table>

$\sigma^2_G$ is the genotypic variance, $\sigma^2_{G \times E}$ is the genotype x environment variance and $\sigma^2_{Error}$ is the residual variance. $^a H^2$ is the broad sense heritability. $^b$ Heritability of the combination of Vrn-A1 copy number and Fr-A2 haplotype. $^c$ is calculated as the ratio of genotypic variance explained by candidate genes to $\sigma^2_G$ multiply by 100.
Table 3.8 Mean heading date (Julian date) of winter wheat genotypes from Canadian winter wheat diversity panel (n = 415) within each \textit{Vrn-A1} copy number group across the three environments

<table>
<thead>
<tr>
<th>\textit{Vrn-A1} Copy Number</th>
<th>Location/year</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>162.13 \textit{a}</td>
<td>164.14 \textit{a}</td>
<td>157.64 \textit{a}</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>163.67 \textit{a}</td>
<td>166.19 \textit{a}</td>
<td>158.56 \textit{a}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>162.94 \textit{a}</td>
<td>165.32 \textit{a}</td>
<td>157.57 \textit{a}</td>
<td></td>
</tr>
</tbody>
</table>

*Mean in the same environment followed by different letters are significantly different based on \textit{t}-test at the significance threshold of \(P = 0.01\).*
3.5 Discussion

3.5.1 Allele diversity within the Canadian germplasm

The first goal of this study was to characterize the allele variation of candidate genes for freezing tolerance within the Canadian winter wheat germplasm. Allele variation within the candidate genes was observed, however, the majority (77.3%) of the diversity panel already contained the optimal allele combination for winter survival (three copies of Vrn-A1 and Fr-A2-T haplotype). This is likely because winter survival has been a key trait for growing winter wheat in Canada (Fowler 2012), so the cultivars with such allele combination were favored. It has been reported that upon genotyping a European diversity panel, the frequency of alleles that confer winter-hardiness was higher in a group of cultivars that came from the colder growing region (Würschum et al. 2017). However, within our study, more than 20% of the winter wheat diversity panel did not have the optimal combination. This suggested that Canadian breeding program could still benefit from applying these genetic markers in their breeding program in order to breed for winter wheat with optimal winter survival.

3.5.2 The allele combination associated with better in-field winter survival

Our study demonstrated that the combination of Vrn-A1 copy number and the Fr-A2-T haplotype had significant contribution to winter survival, explaining 67.38% of the genotypic variance, and cultivars with three copies of Vrn-A1 and Fr-A2-T haplotype have better winter survival. This demonstrated that candidate genes identified from in-door frost tolerance study can translate to better winter survival in the field in Eastern Canada. This is likely because frost tolerance has a strong and significant correlation with in-field winter survival in Eastern Canada (Andrews et al. 1997). Our result concurs with that reported by Zhu et al. 2014 who showed in a bi-parental population that the lines with three copies of Vrn-A1 and the Fr-A2-T haplotype had better frost tolerance in an indoor study. Similarly, Würschum et al. (2017) demonstrated the importance of Vrn-A1 copy number variation and Fr-A2 haplotype for in-field winter survival in Europe. However, the variation at Fr-A2 and CNV of Vrn-A1 only explained 27.3% of the genotypic variation (Würschum et al. 2017). The difference between the latter result and our study may be the result of the difference in genetic composition of the diversity panel being tested. Our study
reported high broad sense heritability (0.52) for the allele combination of Fr-A2 haplotype and Vrn-A1 CNV. This suggests that the gene-specific markers should be incorporated into a breeding program to develop wheat cultivars for high-latitude growing regions. However, it is acknowledged that traits other than frost tolerance such as ice tolerance and snow mold resistance are also important for winter survival. Therefore, the significance of these candidate genes in Eastern Canada may have been overestimated because the environments within our study are historically not associated with these stress factors (Schneider and Seaman 1987; OMAFRA Field Crop Team 2017).

3.5.3 Association between CBF-A14 copy number variation and Fr-A2 haplotype

The association between Fr-A2 haplotype and CBF-A14 copy number was observed in this study where genotypes with Fr-A2-T haplotype were likely to have a higher copy number of CBF-A14. This association was also shown in previous work (Sieber et al. 2016; Würschum et al. 2017). It has been demonstrated in another study that used a diversity panel of durum wheat that the copy number of CBF-A14 explained approximately 90% of the genotypic variance in genotypes with the Fr-A2-T haplotype (Sieber et al. 2016). We did not observe the same contribution of CBF-A14 within the genetic background of Fr-A2-T haplotype and three copies of Vrn-A1. This likely occurred because of the genotypic difference between the diversity panel used in the two studies. The diversity panel used by Sieber et al. (2016) had more copy number variation within CBF-A14 whereas the current study subpopulation only had genotypes with higher copy number of CBF-A14. Therefore, the winter survival improvement that CBF-A14 group 3 had over group 1 cannot be attributed to CBF-A14 alone.

3.5.4 Vrn-A1 copy number and winter survival.

In our study, it has been observed that higher copy number of Vrn-A1 was associated with better winter survival. The expression of Vrn-A1 has been shown to have negative correlation with the expression of frost tolerance genes such as Cold-responsive gene (COR) and CBF genes in barley and wheat (Fowler et al. 1996; Kobayashi et al. 2005). Vrn-A1 likely regulate the expression of these genes through direct interaction as previous work has shown that Vrn-A1
binds directly to the promoter region of CBF2, 4 and 9 in barley (Deng et al. 2015b). This evidence suggested that Vrn-A1 play a direct role in regulating frost tolerance. Furthermore, negative correlation was observed between copy number of Vrn-A1 and its transcript, where the genotype with the highest copy number of Vrn-A1 (3) had the lowest expression level in comparison to genotypes with one or two copies throughout different stages of vernalization (Díaz et al. 2012). The exact mechanism of this negative correlation is unknown. All in all, genotypes that have higher copy number of Vrn-A1 has less Vrn-A1 expression throughout the winter, which ensures a higher expression of frost tolerance gene resulting in better winter survival.

### 3.5.5 Vrn-A1 and heading date in the field

Our study showed that the effect of CNV of Vrn-A1 on heading date was not significant under field conditions. Cultivars with three copies of Vrn-A1 did not have a significantly different heading date compared to this with one or two copies. In contrast, Díaz et al. 2012 observed that higher copy number of Vrn-A1 was associated with later heading in an in-door experiment. The discrepancy was likely due to the difference in the growing conditions between the studies.

Flowering time in wheat are modulated by vernalization and photoperiod (Kumar et al. 2012). Vernalization accelerates the winter wheat flower faster and cultivars with higher copy number of Vrn-A1 required longer vernalization period to reach the same flowering time as those with fewer copies of Vrn-A1. In contrast to in-door experiment which the vernalization period is set (4 weeks in Díaz et al. 2012), the long duration (3-4 months) of Canadian winter ensures that most cultivars reach vernalization saturation (the point where further cold exposure does not affect flowering time). This makes photoperiod sensitivity the main determinant of flowering time in Canada for winter wheat (Whittal et al. 2018) and provides an explanation as to why CNV of Vrn-A1 did not have an influence on flowering time for the in-field study in Canada. The lack of influence is an advantage for the breeders as three copies of Vrn-A1 can be incorporated without affecting flowering time which is an important trait with the optimal time being different depending on the growing region and rotational practice.
3.6 Conclusions

Our study characterized the Canadian winter wheat germplasm and demonstrated that majority of the Canadian winter wheat had three copies of \textit{Vrn-A1} and the \textit{Fr-A2-T} haplotype. In addition, it has been found that the combination of three copies of \textit{Vrn-A1} and the \textit{Fr-A2-T} haplotype is associated with better in-field winter survival in eastern Canada. Lastly, \textit{Vrn-A1} copy number did not have an effect on flowering time in the field. Therefore, selecting for higher copy number of \textit{Vrn-A1} will not have a negative influence on flowering time which has a significant agronomic impact for the Canadian winter wheat growers.
Chapter 4 General Discussion and Future Directions

4.1 General Discussion

Eastern Canada is an important growing region for winter wheat and where approximately 70% of the winter wheat from Canada is produced (Statistic Canada 2017). However, Canada is a high-latitude growing region with colder winters, which can lead to significant winterkill. This makes winter survival one of the core traits for winter wheat cultivars that are being grown in Canada.

The first hypothesis of this study is that Canadian winter wheat germplasm has significant variation in winter survival. This thesis investigated the variation in the winter survival of 450 winter wheat cultivars from across Canada including several spring wheat cultivars that were specially bred to be freezing tolerant. I observed variation in winter survival within the 450 winter wheat cultivars growing in the testing locations. This is likely due to the difference in geographic locations for which the cultivars were bred. For example, winter temperatures in western Canada can often drop below -35°C, whereas in Eastern Canada, the coldest temperature is usually around -25°C (Fowler 2012). Therefore, winter wheat cultivars bred for Western Canada are likely to have better winter survival. This finding suggests that favorable alleles for winter survival are not fixed in the Canadian winter wheat germplasm and that identification of the genomic regions for winter survival may be possible.

The second hypothesis of this study is that ground and UAV images can be used to phenotype winter wheat for winter survival. My results showed that both image-based phenotyping methods had high heritability values and the results had high correlation with winter survival that was estimated visually. In one of the field locations, the image-based methods even showed higher precision in comparison to visual estimation. To my knowledge, this study was the first to compare the precision between different methods of estimating winter survival. The findings agreed with previous literature, which reported that image-based methods were viable alternatives for phenotyping winter survival (Sankaran et al. 2015; Khot et al. 2016). Image-based method can reduce the time for phenotyping and potentially increase the precision of the data collection process (Araus and Cairns 2014).
The third hypothesis was that Canadian winter wheat germplasm contains favorable alleles that influence winter survival. As mentioned earlier, variation in winter survival was observed in the Canadian winter wheat germplasm whereas some cultivars demonstrated great survival across the testing locations. Upon performing GWAS, I identified nine QTLs that were associated with in-field winter survival including well known freezing tolerance loci such as Vrn-A1 and Fr-A2 (Chapter two). By applying image-based phenotyping methods, some novel QTLs were identified. However, it is hard to distinguish whether the QTLs were identified because image-based methods have more precision than visual phenotyping or the results from image-based methods were confounded with spring vigor. Further analysis was done to identify candidate genes using RNA-seq data from a previous cold tolerance study (Li et al. 2015). These candidate genes have homologs in other species that were shown to influence freezing tolerance and winter survival. Therefore, the results of this thesis support the notion that novel QTL identified in this study was associated with winter survival. Furthermore, the finding suggests that the favorable allele at the QTL for winter survival is not fixed in the Canadian winter wheat germplasm and that genetic markers could be used to select for cultivars with great winter survival.

The fourth hypothesis was that the Canadian winter wheat germplasm has allelic variation for CBF-A14 and CBF-A15 and that the variation is associated with winter survival. The fifth hypothesis was that the Canadian winter wheat germplasm has copy number variation for CBF-A14 and Vrn-A1 and that the variation is associated with winter survival. The results of this study suggests that the Canadian winter wheat germplasm has variation for all the candidate genes selected for the study. In terms of CBF-A14 and CBF-A15, it was observed that there was no recombination between the two genes within the panel, therefore, forming two conserved haplotype Fr-A2-S and Fr-A2-T. The haplotype was also conserved in American and European winter wheat germplasm (Zhu et al. 2014; Würschum et al. 2017; Babben et al. 2018). Furthermore, it was shown that Fr-A2-T is associated with higher winter survival, which is in agreement with a previous report (Zhu et al. 2014). This study found copy number variation for Vrn-A1 and CBF-A14 within the Canadian winter wheat germplasm. In agreement with previous studies, higher copy number of either gene was associated with better winter survival in eastern
Optimal allele combination for winter survival in eastern Canada was having three copies of $Vrn-A1$ and the $Fr-A2-T$ haplotype. A previous study also showed that the same combination was optimal for freezing tolerance in an indoor study (Zhu et al. 2014). The copy number of $Vrn-A1$ and the $Fr-A2$ haplotype explained 52% of the phenotypic variance which suggests that they would make for good winter survival genetic markers. Results showed that the majority of the winter wheat (77.3%) within the Canadian germplasm has the optimal gene combination. It has been postulated that this happens because winter survival has been a historically important trait for Canada, therefore, this allele combination was unconsciously selected for (Fowler 2012). In characterizing the allele variation for candidate genes in European winter wheat diversity panel, Würschum et al. 2017 also noticed that cultivars from northern Europe (colder region) had higher frequency of alleles that confer better winter survival. Canadian winter wheat breeding programs can benefit from applying genetic markers linked to these haplotypes to ensure that their material has adequate winter-hardiness in Canada.

4.2 Limitations and Future Directions

This section will outline some of the limitations from this study and suggests future research directions moving forward.

The main goal of this work was to develop a high-throughput phenotyping tool that can be used to screen winter survival and identify genomic regions that are associated with winter survival, which would support the effort of breeding for winter-hardy winter. Winter survival is a complex trait that involves tolerance to many winter-related stresses such as ice tolerance and snow mold tolerance. In the testing sites that are part of this study (Elora and CÉROM), stress factors other than freezing temperature were not observed. Therefore, the ability for these cultivars to withstand other prevalent winter-related stresses was likely not evaluated. In the future, the study can be improved by having test-sites that are associated with specific winter stress factors. For example, a test site in Essex for frost heaving and a test site around Ottawa for ice tolerance and snow mold tolerance (OMAFRA Field Crop Team 2017). Genotype by environment analysis can be performed to identify stable genotypes that have good winter
survival across Ontario. In addition, the stability and the effect of the selected genetic markers on winter survival across Ontario can be evaluated. Lastly, with GWAS, location-specific QTLs for winter survival can be identified to develop genetic markers that can better select winter wheat for specific growing region.

This study described the high-throughput phenotyping methods that can be used to evaluate winter survival. These methods can support the proposed future directions by enabling the evaluation of multiple sites in an efficient manner. Several shortcomings of the image-based methods that were used in the study were identified. The main problem was that winter survival results from image-based methods were confounded by growth stage and spring vigor. This made comparing the results of image-based methods across environment and year difficult. Standardizing the result by counting the number of plants that have survived within the images captured can potentially resolve this problem. Therefore, the future direction is to evaluate whether machine learning can be used to count wheat plants or not and if such method could be applied in the evaluation of winter survival.

This study identified candidate genes for winter survival (Chapter two). The study assumed that genes for winter survival are cold-responsive meaning that they experience a change in expression in response to cold. Developmental stages were shown to affect cold tolerance in previous work (Mahfoozi et al. 2001b). This study’s method of candidate gene identification will likely miss these potential targets. In addition, the method may also miss candidate genes that are regulated through post-transcriptional mechanisms. In short, the method described in the study favored the genes that are regulated by cold and could miss other potential candidate genes. Future directions will be to verify the functions of the three candidate genes identified in the study (Chapter two) and confirm their influence on winter survival in Eastern Canada.
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Yin L (2018) CMplot: Circle Manhattan Plot


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Appendix I Climate Data from Oct 1st, 2016 to June 15th, 2017 at the Elora Research Station (43°38' 27.0456" N, 80°24' 18.6948" W). Light blue line represents the daily minimum air temperature while dark blue line represents the daily minimum soil temperature. Soil temperature was captured at 5cm below ground level. The orange bars represent the average snow depth of each day. Dotted black line is the reference line for 0°C.
Appendix II Climate Data from Oct 1st, 2017 to June 15th, 2018 at the Elora Research Station (43°38’ 27.0456” N, 80°24’ 18.6948” W). Light blue line represents the daily minimum air temperature while dark blue line represents the daily minimum soil temperature. Soil temperature was captured at 5cm below ground level. The orange bars represent the average snow depth of each day. Dotted black line is the reference for line 0°C.
Appendix III Climate Data from Oct 1st, 2017 to June 15th, 2018 at the Montreal International Airport weather station (45°28’ 14.000” N, 73°44’ 27.000” W). This weather station is selected to represent climate at the Centre de recherche sur les grains (CÉROM). Light blue line represents the daily minimum air temperature. The orange bars represent the average snow depth of each day. Dotted black line is the reference line for 0°C.

Appendix IV Quantile-quantile plot for Genome-wide association study conducted on the in-field winter survival data for the Canadian wheat diversity panel (n=450) for the trait of NDVI Sum at Elora 2016/2017. GLM represents general linear model; CMLM represents compressed mixed linear model; K represents kinship matrix; Q represents the population structure; PC represents the principle components; MSD represents mean-squared-difference.
Appendix V Putative candidate genes identified for quantitative trait loci for winter survival

<table>
<thead>
<tr>
<th>QTL</th>
<th>Candidate gene</th>
<th>Orthologous gene in other species</th>
<th>Protein identity a</th>
<th>Orthologous gene description</th>
<th>Respective publication DOI</th>
</tr>
</thead>
<tbody>
<tr>
<td>QWs.ugw-4A.1</td>
<td>TraesCS4A01G107100</td>
<td>OsRCI2-5 (Os03g0286900) in Oryza sativa</td>
<td>84.4%</td>
<td>Over-expression of OsRCI2-5 conferred better drought tolerance than wild type in rice. Cell membrane specific expression.</td>
<td>10.4238/2014.May.23.13</td>
</tr>
<tr>
<td>QWs.ugw-4B</td>
<td>TraesCS4B01G020300</td>
<td>AtGRP7 (At2g21660) in Arabidopsis thaliana</td>
<td>81.5%</td>
<td>Over-expression of GRP7 in Arabidopsis improved freezing tolerance, potentially via the regulation of stomatal aperture.</td>
<td>10.1111/j.1365-313X.2008.03518.x</td>
</tr>
</tbody>
</table>

aOrthologous gene with the highest protein identity in either Oryza sativa or Arabidopsis thaliana was identified using Ensembl Plants (Kersey et al. 2018)

Appendix VI PCR program for Fr-A2 haplotype

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>35 Cycles</td>
<td>95°C 57°C 72°C</td>
<td>15 seconds 15 seconds 20 seconds</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
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</table>

Appendix VII PCR program for performing Taqman® Assay for VRN-A1 and CBF-A14 copy number

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>10 Mins</td>
</tr>
<tr>
<td>40 Cycles</td>
<td>95 60</td>
<td>10 sec 1 Min</td>
</tr>
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</table>