Characterization of *Brachypodium distachyon* as a Grass Model for Research on Freezing Tolerance

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Abstract

Long exposure to low non-freezing temperatures accelerates flowering and triggers cold acclimation in winter wheat and barley. Cold tolerance is a complex but important trait that influences yield considerably. *Brachypodium distachyon* is an annual temperate wild grass species and is a powerful model to study grass biology, but the capacity of this plant to tolerate freezing is unknown. An integrated approach involving double ridge formation, final leaf number, and osmoprotectant content was used to confirm the growth habit and freezing tolerance ability of seven diploid *Brachypodium distachyon* accessions. In addition, expression analyses of orthologs of the major vernalization regulator *VRN1* and *COR* genes including an antifreeze protein and a temperature-induced lipocalin (TIL) were conducted. Our results demonstrate that formerly classified spring accessions behaved more like facultative accessions by showing increased osmoprotectants and *COR* transcripts accumulation upon cold exposure. The confirmed winter accessions acquired vernalization saturation after 49 days and accumulated *COR* transcripts to higher levels although no significant difference was observed between the osmoprotectant content of spring and winter accessions. Transgenic lines overexpressing a TIL were also generated to increase the freezing tolerance capacity of the spring accession Bd21. This study validates *Brachypodium distachyon* as a valuable model system to study freezing tolerance in temperate cereals.

Résumé

Les basses températures au-dessus de zéro accélèrent la floraison et déclenchent l'acclimatation au froid chez le blé et l'orge d'hiver. La tolérance au gel est un trait complexe et important qui influence les rendements considérablement. *Brachypodium distachyon* (*Brachypodium*) est une céréale annuelle de climat tempéré et un puissant modèle pour étudier la biologie des monocotylédones, mais les capacités de tolérance au gel de cette plante sont inconnues. Une approche intégrée impliquant la formation de la ride double, le nombre de feuilles final et la concentration d'osmoprotectants a été utilisée afin de confirmer l'habitude de croissance (de printemps ou d'hiver) et les capacités de tolérance au gel de sept cultivars diploïdes de *Brachypodium*. En parallèle, nous avons analysé l'expression des orthologues du principal régulateur de vernalisation *VRN1* et des *COR* gènes incluant une protéine antigel et une lipocaline induite par la température (TIL). Nos résultats démontrent que les cultivars de printemps se comportent plutôt comme des cultivars facultatifs puisque leur contenu en osmoprotectants ainsi que les transcrits *COR* s'accumulent au froid. Les cultivars d'hiver ont atteint la vernalisation maximale après 49 jours au froid et ont accumulé plus de transcrits *COR*. Toutefois, aucune différence n'a été observée dans le contenu en osmoprotectants des cultivars de printemps et d'hiver. Afin d'augmenter la tolérance au gel du cultivar de printemps Bd21, des lignées transgéniques surexprimant une TIL ont été générées. Cette étude valide l'utilisation de *Brachypodium* comme modèle pour l'étude de la tolérance au gel chez les céréales de climat tempéré.

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Contributions of Authors to Manuscripts

The presentation of this thesis is manuscript-based and complies with the rules of the 'Thesis Preparation and Submission Guidelines'. Chapters 3 and 4 represent two separate manuscripts. The co-author of these manuscripts is Dr. Jean-Benoit Charron. The contribution of each author to the manuscripts is described in detail at the beginning of each chapter.

I have contributed to all the work pertaining to this thesis. This includes: reviewing the pertinent literature, setting the experimental design, conducting the experiments in the laboratory, analysing the data, and writing the manuscript. Dr. Jean-Benoit Charron has provided guidance in creating the experimental design of experiments, teaching of laboratory techniques, and editing and reviewing the manuscript. Dr. Jean-Benoit Charron has also provided the funds necessary to conduct this work.

List of Abbreviations

AFP	Antifreeze protein
Bd	*Brachypodium distachyon*
BLAST	Basic local alignment search tool
bp	Base pairs
Bradi	*Brachypodium distachyon*
CA	Cold acclimated
CBF	C-repeat binding factor
CBF/DREB	C-repeat binding factor/dehydration-responsive binding
CEC	Compact embryogenic calli
COR	Cold-regulated
COR/LEA	Cold-regulated/late embryogenesis abundant
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DR	Double ridge
DW	Dry weight
FLN	Final leaf number
Fv/Fm	Chlorophyll fluorescence
FW	Fresh weight
GFP	Green fluorescent protein
IRI	Ice recrystallization inhibition
Kan^R	Kanamycin resistance
LB	Luria-Bertani
LT	Low temperature
mRNA	Messenger RNA
MS	Murashige and Skoog
Myr	Million years
NA	Non-acclimated
ORF	Open reading frame
Osm	Osmolality
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCR	Structurally conserved region
Sm/Sp^R	Streptomycin-spectinomycin resistance
TIL	Temperature-induced lipocalin
VRN	Vernalization
WSS	Water soluble sugars

Licenses

In order to import transgenic plants from the Boyce Thompson Institute, we completed an application for permit to import plants and other things under the Plant Protection Act (Canadian Food Inspection Agency, D-96-13: Import Requirements for Plants with Novel Traits, including Transgenic Plants and their Viable Plant Parts) (Jacqueline Van Acker 514-283-3815 poste#4268, jacqueline.vanacker@inspection.gc.ca, Plant Health Import Permit Office, Canadian Food Inspection Agency, 59 Camelot Drive, Ottawa, Ontario, K1A 0Y9).

Chapter 1 Introduction

1.1 General Introduction

The province of Québec is characterized by three major vegetative zones which are the tundra comprising the Ungava peninsula and the Labrador coast, the taiga comprising the Hudson plateau and valley (south of the tundra and north of the 50° N latitude), and the temperate zone which is located south of the 50° N latitude. Since agriculture is impossible north of the 50° N latitude, all of Québec's agriculture is practiced in the temperate vegetative zone which represents approximately 25% of the province. The temperate vegetative zone is divided according to the two types of climate that prevail, namely the maritime climate and the continental climate. A continental climate prevails in the south-western part of the province where most agricultural activities take place. Warm summers, cool falls, cold winters, and cool to slightly warm springs characterize the continental climate of south-western Québec (Doucet, 1994). The temperate climate of Québec allows a wide variety of crops such as cereals, forages, corn, soybeans, potatoes, blueberries, and Christmas trees to be grown (Government of Quebec, 2009).

The global population is currently growing at an exponential rate and is expected to follow the same trend in the future which will inevitably force agricultural production toward mass production. In fact, we foresee an ever increasing demand for plant derived products which will only be satisfied by increasing crop yields and by dedicating new lands for agricultural production. Since there is a generalized stagnation in crop yield improvement worldwide, we will likely turn our attention toward the development of cultivars that will resist more efficiently abiotic stresses. Also, according to a study conducted in northern Canada and Alaska, there are 39 million hectares of arable land located upward of the 55° N latitude that could be used to cultivate annual crops, forages and pastures even though the climate is not optimal (Mills, 1994). Therefore, for cereals and grass forages to thrive in these northern areas, cold resistant cultivars need to be developed.

One of the most important constraints influencing crop cultivation is the length of the frost-free growing season. In Québec, this period varies from 60 to 160 days and dictates how the agricultural territory is used (Doucet, 1994). Low and most of the time freezing temperatures encountered late in spring, early in fall, and during winter have a negative impact on plant survival and, hence, reduce yield potential considerably. In fact, Québec's agricultural insurance program administered by La Financière agricole du Québec, provides insurances for losses in yield that can be caused by bad weather conditions such as cold and freezing episodes causing crop damages. Most agricultural producers subscribe for a 20% deductible premium insurance meaning that, if the yield obtained by the end of the growing season is lower by 20% or less compared to the average yield of a typical farm specialized in this production, they financially absorb it. However, the fact that farmers decide every year to insure 80% of their crop production is a good indicator of the risk associated with growing cold sensitive crops in Québec (Leclerc, 2010).

Most temperate cereals are cold and freezing tolerant. This is the case of winter annuals like wheat, barley and rye, and all the perennial forages like timothy, brome, alfalfa, etc., some of them being more tolerant than others. However, freezing tolerance can only be achieved if a period of cold acclimation, exposure to low non-freezing temperatures, occurs prior to the first frost. In temperate climates, frost episodes can occur suddenly late in spring and early in fall which results in damage to non-acclimated plants.

Research efforts aimed at improving cold hardiness of temperate cereal crops have been one of the leading strategies to increase cereal production. However, such efforts are being challenged by different factors. Traditional plant-breeding methods for cereals, *i.e.* crossing parents that show a high potential for cold hardiness, have resulted in small and stagnating improvements in the expression of this trait (Limin and Fowler, 1991; Thomashow, 1999). Moreover, attempts to cross different species with good potential for cold hardiness have also failed to enhance the trait in the new species generated. As an example, the

genome of rye (*Secale cereale*), which has one of the greatest cold hardiness response, has been introduced in wheat to create a wheat-rye hybrid known as triticale without significantly enhancing the cold hardiness capacity of the new plant (Limin and Fowler, 1991).

Functional genomics approaches applied to model plants offers the possibility to unravel the phenomenon of cold tolerance in grasses and to develop resistant cultivars that are better yielding. Several laboratories worldwide are studying the effect of cold-regulated genes by producing transgenic lines of *Arabidopsis*. However, the lack of an amenable model plant for cereal species which represent the world's most economically important crops is slowing the development of cold resistant crops. In this study, we validate *Brachypodium distachyon*, a temperate wild grass species, as a valuable model for research in freezing tolerance. We also developed transgenic lines of *Brachypodium* in order to study the function of a cold-regulated gene (a temperature-induced lipocalin) in cereals.

1.2 Hypotheses

1. *Brachypodium distachyon* is a valuable model to understand cold tolerance in temperate grasses.

2. Vernalization response can be used as an indicator of the cold acclimation response of different *Brachypodium distachyon* accessions.

3. Natural phenotypic variation for cold acclimation and the development of freezing tolerance exists among *Brachypodium distachyon* accessions.

4. Transgenic *Brachypodium distachyon* overexpressing a temperature-induced lipocalin will show increased freezing tolerance capacity.

1.3 Objectives

1.3.1 Study 1:

Characterization of *Brachypodium distachyon* as a grass model for research in freezing tolerance

Objective 1: To analyse the effect of vernalization on the phenological development of seven accessions of *Brachypodium distachyon*.

Objective 2: To analyse the accumulation of proline and water soluble sugars in response to cold acclimation in different accessions of *Brachypodium distachyon*.

Objective 3: To identify and characterize the putative vernalization and cold-regulated genes in the newly sequenced genome of *Brachypodium distachyon*.

Objective 4: To analyse the expression of the major vernalization gene in two *Brachypodium distachyon* during vernalization.

Objective 5: To analyse the expression profile of cold-regulated genes in two *Brachypodium distachyon* accessions during cold acclimation.

1.3.2 Study 2:

Characterization of the phenotype of a transgenic line of *Brachypodium distachyon* overexpressing a temperature-induced lipocalin

Objective 1: To identify and characterize the temperature-induced lipocalin (*TIL*) genes in the newly sequenced genome of *Brachypodium distachyon*.

Objective 2: To physically clone the coding sequence of *BradiTIL-2* and to insert it in a binary construct containing the marker gene GFP.

Objective 3: To overexpress *BradiTIL-2* in the diploid inbred cultivar Bd21.

Objective 4: To characterize phenotypically the new *BradiTIL-2* overexpressing lines under low non-freezing and freezing temperatures.

Chapter 2 Literature Review

2.1 Model Systems

Over the past years, the dicotyledonous *Arabidopsis thaliana* has been used extensively as a model system in many areas such as agriculture and medical research. The choice of this model organism for plant science research is unquestionable because its biology suits all the criteria required for a universal model system. Indeed, this inbreeding plant has a rapid life cycle and a small stature making it easy to grow in laboratory settings. Moreover, whole-plant transformation methods with *Agrobacterium tumefaciens* (*Agrobacterium*) have allowed ambitious research in functional genomics and the production of several transgenic lines (Meinke et al., 1998). However, the use of *Arabidopsis* for conducting functional research applicable to the Poaceae grass family, including all of the world's cereal crops, is questionable. In fact, it was found that colinearity between two genes in dicots and monocots can be conserved, but that long sequences of genes were often not. It is also expected that many rearrangements have occurred since dicots and monocots diverged approximately 140 to 150 million years (Myr) ago (Chaw et al., 2004). Therefore, *Arabidopsis* has the potential to be used in grass research for closely linked genes, but a model that is phylogenetically closer to grasses is needed to conduct research with isolated genes particularly those influencing agronomic traits (Keller and Feuillet, 2000).

In an effort to overcome the major drawback encountered with *Arabidopsis*, rice (*Oryza sativa*) was considered as a potential model system for grass species. Even though rice, subfamily Ehrhartoideae, is phylogenetically closer to temperate cereals of the Pooideae subfamily, they diverged approximately 50 Myr ago (Gaut, 2002), its use as a model system for temperate crops is also questionable. Indeed, rice does not exhibit some important agronomic traits typical to temperate crops such as cold and freezing tolerance and vernalization. Also, rice is a large outbreeding plant that has rigorous growth requirements and a long life cycle making it laboratory unfriendly. Finally, only specialized

laboratories in the world master the difficult technique of rice transformation (Draper et al., 2001).

Grasses are the most important source of food worldwide and may reduce our dependency on fossil fuels by acting as basal products for sustainable energy production. In an attempt to find a valuable model system for research in the field of temperate grasses, the scientific community reviewed the attributes of all the members of the tribe Triticeae. Finally, in 1995, *Brachypodium distachyon* (*Brachypodium*) was first pointed out as a potential model system that could reach a high level of performance for Triticeae research where *Arabidopsis* and rice have failed in the past (Bablak et al., 1995). Since then, a lot of research has been conducted in order to characterize the biological and the physiological genomics of this promising model system. Also, tools such as a BLAST server and a genome browser specific to *Brachypodium* have been developed to facilitate research in functional genomics (Oregon State University, 2010). Germplasm collections and inbred lines are available to researchers. The USDA National Plant Germplasm System (NPGS) (www.ars-grin.gov/npgs/) holds 198 accessions which are made freely available for research laboratories interested to work with this plant. The Brachyomics company of the University of Aberstwyth (www.aber.ac.uk/plantpathol/germplasm) also propagates and supplies *Brachypodium* germplasm (ABR lines) to academic and industrial research laboratories. A list of most accessions is available at www.brachypodium.org/stocks. Finally, the development of genetic markers (Vogel et al., 2009), a genetic linkage map (Garvin et al., 2010), bacterial artificial chromosome (BAC) libraries (Huo et al., 2006; Huo et al., 2008), physical maps (Gu et al., 2009), microarrays and databases (www.brachybase.org) are all contributing to ease the use of *Brachypodium* by the scientific community (Vogel et al., 2010).

2.2 Biological Attributes of *Brachypodium*

The natural habitat of *Brachypodium*, also known as purple false brome, is the Mediterranean and Middle East (Opanowicz et al., 2008; Vogel et al., 2010).

This wild annual grass is a member of the Pooideae subfamily like most of the other temperate grasses including wheat (*Triticum*), barley (*Hordeum*) and rye (*Secale*) (Vogel et al., 2010). *Brachypodium* has many biological attributes making it a valuable model system. Indeed, *Brachypodium* is self-fertile which considerably reduces the efforts that have to be put in for breeding unless if one needs to outcross the plant. Self-fertility also allows the production of homozygous lines which is advantageous for maintaining independent genotypes (Vogel and Bragg, 2009). The stature of the plant is small, ranging between 10 cm and 50 cm in height, allowing a plant density as high as 1000 plants per m^2 (Vogel and Bragg, 2009). The generation time of *Brachypodium* also confers an important advantage for using it as a model system. Indeed, under 24 hours of light, the Bd21 (PI 254867) variety developed from seed to floral spike in three weeks and to mature seeds in two months (Garvin, 2007). Finally, both spring and winter diploid accessions are available allowing research in the fields of freezing tolerance and vernalization (Vogel and Bragg, 2009). Transformation of *Brachypodium* with *Agrobacterium* has been developed and routinely reaches an average 50% efficiency rate, with 86% being the largest efficiency rate obtained to date, allowing the production of mutants which is essential for studies in functional genomics (Alves et al., 2009). More than 10000 mutant lines have been created to date by the group of Dr. Philippe Vain (http://www.brachytag.org/) and the United States Department of Agriculture (USDA) (http://brachypodium.pw.usda.gov/TDNA/) (Bevan et al., 2010).

In 2010, the diploid inbred *Brachypodium* accession Bd21 was sequenced using whole-genome shotgun sequencing by the International *Brachypodium* Initiative group (Vogel et al., 2010). Among the most important discoveries that were generated following this sequencing project is first that *Brachypodium* has a genome comprising 272 megabase pairs (Mb) which is considered very compact if compared to other grasses' genomes (Vogel et al., 2010). In terms of comparisons, the whole genome of *Brachypodium* is twice as large as the *Arabidopsis* genome (125 Mb) (Initiative, 2000), smaller than the rice genome (389 Mb) (Rid, 2005), approximately the same size as an average chromosome

arm of barley (Garvin, 2007) and almost three times smaller than the sorghum genome (730 Mb) (Paterson et al., 2009). Second, 25532 protein coding gene loci have been predicted which suggests that the number of genes was maintained across grasses from different subfamilies with rice having 28236 and sorghum 27640. Moreover, it was estimated that 77 to 84% of the gene families were conserved in the *Brachypodium*, rice and sorghum subfamilies. Phylogenetic tree analysis predicted that 62 gene families found in *Brachypodium* were distributed similarly in rice and sorghum which reinforces the fact that *Brachypodium* is a good model to study functional genomics in grasses. Third, retrotransposons and DNA transposons cover respectively 21.4% and 4.77% of the *Brachypodium* genome. Even though many of these retrotransposons are active and may contribute to a genome expansion, the genome of *Brachypodium* has stayed relatively small because recombination between these removes the expanded DNA sequences. One exception to this rule was found to apply to chromosome number 5 where retrotransposon activity contributes to the expansion of the genome in this particular area. Fourth, based on the substitution rate of orthologous gene pairs, it was estimated that *Brachypodium* diverged from wheat, rice and sorghum 32-39, 40-53, and 45-60 Myr ago, respectively (Vogel et al., 2010).

In terms of ploidy, *Brachypodium* accessions possess either 5, 10 or 15 chromosome numbers. It was recently reported that accessions with 1n = 5 such as Bd21 and accessions with 1n = 10 are true diploids whereas accessions with 1n = 15 are tetraploids derived from progenitor genomes similar to the diploid accessions (1n = 5 and 1n = 10) (Bevan et al., 2010). It is almost worthless saying that accessions with 1n = 5 are the ones that are considered as model systems due to their smaller genome (Bevan et al., 2010).

2.3 Abiotic Environmental Stresses

Abiotic environmental stresses such as drought, salinity and low temperatures limit plant productivity and have been the most important causes of crop loss worldwide (Boyer, 1982). To date, many genes have been found to be

induced by abiotic environmental stresses and to influence the ability of plants to survive adverse conditions by enabling them to respond both biochemically and physiologically. The products of these genes have been classified in two groups according to their functions in the stress response. The first group of products directly protects the plant cells and includes, among other types of proteins, antifreeze proteins and detoxification enzymes. The second group of products regulates gene expression and signal transduction and includes transcription factors, protein kinases, and enzymes involved in the phosphoinositide metabolism. However, for the purpose of this research, the attention will be given to the physiological and molecular events induced by cold temperatures even though it was found that there is overlap in the mechanisms enabled by the different abiotic environmental stresses (Seki et al., 2003).

2.4 Freezing Process

Water is the most important constituent of a plant cell and of its surrounding solution contained in the extracellular space (Hsiao, 1973). There is no doubt that water plays an important role in plant freezing injuries. In fact, the cooling rate and the chemical potential of water both influence the stability of the cell at low temperatures. It is now well established that the plasma membrane, which separates the intracellular space from the extracellular space in the plant cell, acts as the primary site for freezing injury in plants (Steponkus, 1984; Thomashow, 1999). As the temperature drops, the solutions found in both the intracellular and extracellular portions supercool allowing the formation of ice nucleators in the extracellular space. Ice formation causes the solute concentration to increase in the extracellular solution until the chemical potential of the unfrozen water and the ice are in equilibrium. Equilibrium must also be reached between the extracellular ice and the intracellular solution of the cell and results either in cell dehydration or intracellular ice formation. Two important properties of the plasma membrane will allow such equilibrium. First, the plasma membrane acts as a barrier to the extracellular ice preventing ice formation intracellularly. The absence of ice nucleators in the intracellular space also aids to prevent ice

formation. Second, the plasma membrane is semi-permeable allowing osmosis between the extracellular and the intracellular spaces. Therefore, the greater concentration of solutes in the extracellular space will cause the solution from the intracellular space to move out which results in dehydration of the cell. However, this mechanism is only possible if the temperature drops slowly. When the temperature drops fast, the cell is predisposed to intracellular ice formation that can be caused by nucleation and/or penetration from extracellular ice. Above -38°C, water in the intracellular space remains in a supercooled state in the absence of heterogeneous ice-nucleating agents whereas, below this temperature, homogeneous ice-nucleation occurs (Steponkus, 1984). At -10°C, the osmolality (Osm) of the unfrozen extracellular solution is 5 Osm indicating that most of the water from the intracellular space has crossed the plasma membrane which results in cell dehydration (Steponkus, 1984). As a result of dehydration, the membrane can be damaged by expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (Steponkus, 1984; Thomashow, 1999).

2.5 Freezing Injury

The extent of freezing injury is dependent on three factors: the freeze-thaw cycle including the cooling rate and the minimum temperature encountered, the hardiness of the tissue (acclimated versus non-acclimated protoplasts), and the composition of the suspending solution (Steponkus, 1984). At high freezing temperatures ranging from -3 to -5°C, expansion-induced lysis appears to be the major cause of freezing injury in non-acclimated protoplasts. This form of injury is caused by the sensitivity of the plasma membrane to osmotic contraction and expansion during freeze-thaw cycles. During the freezing period of the cycle, dehydration of the protoplast occurs which leads to contraction of the plasma membrane and, hence, reduction in its dimension by endocytosis. During the thawing period of the cycle, the protoplast solution volume increases more rapidly than the rate of expansion of the plasma membrane resulting in cellular lysis (Steponkus, 1984).

Lamellar-to-hexagonal-II phase transition occurs primarily in non-acclimated protoplasts. During a freezing period, water content in the vicinity of the plant cell is approximately 20% which forces bilayers of protoplast and chloroplasts to come into contact. As a result, the phospholipids constituting the two bilayers undergo a conformational change from a lamellar to a hexagonal II phase. During thawing, the plasma membrane can never regain its semi-permeable property which eventually results in cell death (Steponkus, 1984). The lipid composition of cold acclimated protoplasts was found to reduce the formation of hexagonal-II type phospholipids (Uemura et al., 1995).

Fracture jump lesions are thought to be caused by interlamellar attachment and fusion between the plasma membrane and other endomembranes when these come into contact as a result of cellular dehydration. This type of damage would affect membrane stability in non-acclimated protoplasts and at very low temperatures if protoplasts are cold acclimated only for a short period of time (Uemura et al., 1995).

2.6 Oxidative Stress

Production of active oxygen species such as singlet oxygen ($^1O_2$), superoxide (O^-_2) and hydrogen peroxide (H_2O_2) are normal by-products of biological redox reactions such as the ones involved in photosynthesis. During normal growth conditions, plants are able to minimize the damage caused by these active oxygen species (Foyer et al., 1994). However, adverse environmental conditions such as the combination of low temperature and light perturbs the photosynthetic activity in chloroplasts because of excessive excitation energy. As a result of an excess of electron transport to oxygen, there is an increase in the photoreduction of oxygen and there is production of reactive oxygen species (ROS) such as superoxides and peroxides. Oxidative stress can severely affect the stability of membranes and enzymes and, in the most severe case, cause cell death (Breton et al., 2000).

2.7 Cold Acclimation

Cold acclimation is the phenomenon by which low non-freezing temperatures trigger the initiation of freezing tolerance in plants. The extent of freezing tolerance varies greatly depending on the level of cold acclimation among the different temperate plant species. As an example, temperatures between -5°C and -10°C usually kill non-acclimated wheat and rye. However, wheat and rye that have been cold acclimated prior to freezing exposure can survive temperatures as low as -20°C and -30°C, respectively (Thomashow, 1990). At one extreme, creeping bentgrass, a perennial forage grass, was found to survive temperatures as low as -39°C after being acclimated to low non-freezing temperatures occurring during the fall season (Limin and Fowler, 1987). It is now well established that plants acclimated to low non-freezing temperatures prior to frost exposure are able to resist lower freezing temperatures by limiting freezing injury at the cellular level (Thomashow, 1999). Indeed, cold acclimation enhances plasma membrane stability which limits the damages caused by expansion-induced lysis and the formation of hexagonal II phase lipids. Among the different compounds that form as a result of cold acclimation, modified sterols and cerebrosides, simple sugars such as sucrose, and hydrophilic polypeptides all contribute to stabilize the membrane (Thomashow, 1998).

In 1970, it was first suggested that cold acclimation signals, particularly short days and low non-freezing temperatures, induce changes in both the biochemical events and biophysical properties of woody plant cells as a result of altered gene expression (Weiser, 1970). In 1985, Guy et al. reported the first evidence of altered gene expression during cold acclimation. Indeed, when compared to non-acclimated leaves, the abundance of specific mRNAs was found to be different in cold acclimated leaves and the expression of two new species of mRNA was correlated with an increase in freezing tolerance (Guy et al., 1985). Following these pioneering results, several genes were assumed to play important roles in freezing tolerance and many groups concentrated their efforts on

identifying cold-responsive genes and their proteins. Since then, genes and proteins that contribute to freezing tolerance in plants have been discovered.

2.8 Cold-Responsive Genes

Freezing tolerance is considered to be a multigenic trait because the transcription of cold-regulated (*COR*) genes that have additive effects is required to confer complete freezing tolerance (Thomashow, 1990). So far, over 50 different *COR* genes have been identified in *Arabidopsis*, barley, wheat, alfalfa, spinach, canola, and other plants (Thomashow, 1999). Studies involving plant transformation with these *COR* genes have allowed their classification into three distinct functional categories.

The first category comprises genes that code for structural proteins which protect the plant cell during low temperature stresses. These proteins all contribute to increase freezing tolerance and are known as antifreeze proteins, ice nucleators and proteins of groups II and III cold-regulated/late embryogenesis abundant proteins (COR/LEA proteins). Antifreeze proteins have an affinity for ice and recrystallization inhibition activity which limits the expansion of ice crystals by inhibiting the binding of supplemental water molecules to the existent ice structure and favours the formation of small ice crystals rather than large ones (De Vries and Price, 1984; DeVries, 1986; Knight et al., 1984). Antifreeze proteins also have thermal hysteresis activity which increases the difference between the freezing and melting temperature of ice (Griffith and Yaish, 2004). These two properties limit physical damage to the membrane. A study reported the presence of two antifreeze proteins in wheat for which the transcripts accumulate during cold acclimation and that possess ice recrystallization inhibition activity (Tremblay et al., 2005). Antifreeze proteins could potentially be used as markers to differentiate between tolerant and sensitive cereal accessions in response to freezing. Ice nucleators are thought to increase the production of intracellular ice at high freezing temperatures which would promote gradual cell dehydration and reduce the damage caused by sudden intracellular freezing (supercooling). Ice nucleators in cold acclimated plants were found to be

mediated by a protein component as well as by phospholipids and carbohydrates (Brush et al., 1994), but the identity of the gene and the protein responsible for inducing ice nucleation have yet to be determined. Finally, some regions of COR/LEA proteins are able to form amphipathic α-helices which increases the stability of membranes under freezing and dehydrative conditions by reducing their tendency to form hexagonal II phase lipids (Close, 1996; Danyluk et al., 1998; Sarhan et al., 1997; Thomashow, 1999).

The second category comprises genes that code for enzymes involved in the biosynthesis of osmoprotectants, the desaturation of lipids and the antioxidative response. The first group of enzymes catalyzes reactions in which natural substrates present in plants are converted into osmoprotectants. Catalysis of these enzymes is mainly caused by a reduced osmotic pressure in the cytoplasm under freeze-induced dehydration. Osmoprotectants protect the plant cell by increasing the osmotic pressure in the cytoplasm and by stabilizing proteins and membranes under salt and cold stresses (Guy, 1990). These compatible solutes are highly soluble and are either derived from amino acids which is the case for proline and glycine betaine or from sugars which is the case for sucrose, raffinose, sorbitol and fructans (Bohnert and Shen, 1999; McNeil et al., 1999). Cold acclimation was shown to result in an increase in the activity of sugar and proline synthesizing enzymes especially in winter cereals (Charest and Ton Phan, 1990; Savitch et al., 2000). Proline biosynthesis from the amino acid glutamate has been proposed to act as a compatible osmolyte, a ROS scavenger and a molecular chaperone stabilizing the structure of proteins (Verbruggen and Hermans, 2008). Proline accumulation can be a way to store carbon and nitrogen, to buffer cytosolic pH and to balance cell redox status, and be part of the stress signal influencing adaptive responses (Verbruggen and Hermans, 2008). Sugars have been proposed to stabilize membranes and proteins in the dehydrative state induced by freezing by replacing water in the maintenance of those structures (Crowe et al., 1992). Sucrose and trehalose can also form a glass phase which can slow down chemical reactions which stabilizes the cells under dehydrative stress (Bruni and Leopold, 1992; Crowe et al., 1998). The second group of enzymes, which comprises

acyltransferases and desaturases, catalyzes reactions in which lipids of the plasma membrane are desaturated, mainly during cold acclimation, to maintain the membrane's fluidity (Shanklin and Cahoon, 1998). More specifically, an increase in the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine and a decrease in the proportion of cerebroside and sterol containing lipids is thought to increase the stability of the membrane (Uemura et al., 1995). The third group comprises enzymes that are involved in the antioxidative response. During environmental stresses, plants increase the synthesis of non-enzymatic (tripeptide glutathione, vitamin C, and vitamin E) and enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase and catalases) that scavenge ROS (Noctor and Foyer, 1998).

The third category comprises the regulatory genes that act as global regulators (also known as the master switch) in that they control the overall mechanism of the low temperature response. When cold is detected by the plasma membrane or by the cytoplasmic and chloroplastic molecular complexes, a signal is sent by a cascade of kinases and phosphatases which activates specific transcription factors leading to the transcription of some of the *COR* genes. As an example, one of these transcription factors, known as C-repeat binding factor/dehydration-responsive binding protein (CBF/DREB), binds to the specific cis-acting regulatory element CCGAC in the promoter sequence of some *COR* genes which activates their transcription conferring freezing tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Stockinger et al., 1997).

2.9 Temperature-Induced Lipocalins

Lipocalins are small ligand-binding proteins found in bacteria, invertebrate and vertebrate animals. In 2002, the first true lipocalins from plants were identified in wheat and *Arabidopsis* and named *T. aestivum* L. temperature-induced lipocalin (*Ta*TIL) and *A. thaliana* temperature-induced lipocalin (*At*TIL) respectively (Frenette Charron et al., 2002). Lipocalins from plants are characterized by three structurally conserved regions (SCRs) and share homology

with the human Apolipoprotein D, the *E. coli* outer membrane lipoprotein Blc precursor and the American grasshopper Lazarillo precursor (Frenette Charron et al., 2002). Cold acclimation was found to induce the accumulation of the *Tatil* mRNA transcripts in both spring and winter wheat with a greater level of accumulation in the winter ecotype (Frenette Charron et al., 2002; Frenette Charron et al., 2005). Furthermore, it was found that monocotyledonous species possess genes encoding two different members of the TIL group (*TIL-1* and *TIL-2*) which are both regulated by abiotic stresses (Frenette Charron et al., 2005). The plasma membrane was found to be the subcellular location of TILs (Frenette Charron et al., 2005). Following a recent study conducted with T-DNA knock-out *Arabidopsis* lines for the *AtTIL* gene, plasma membrane-associated TILs were found to enhance plant protection against the oxidative stress induced by freezing. Indeed, during oxidative stress, TILs would bind and scavenge peroxidated lipids which helps the membrane to recover and regain its integrity (Charron et al., 2008).

2.10 Vernalization

Vernalization is the process by which a period of cold exposure initiates the transition from the vegetative to the reproductive growth phase. This mechanism is essential for the survival of winter cereals because it prevents exposition of the sensitive floral meristems to the fatal conditions of winter. In opposition, spring cereals do not have a vernalization requirement because they never have to face the freezing temperatures of winter. Allelic differences in the *VRN1*, *VRN2* and *VRN3* vernalization genes determine the natural variation in vernalization requirement of temperate cereals (Distelfeld et al., 2009). *VRN1* encodes a MADS-box transcription factor highly similar to *Arabidopsis APETALA1*, *CAULIFLOWER*, and *FRUITFUL* (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Deletions of the *VRN1* gene in diploid wheat (*Triticum monococcum*) inhibited flowering indicating that this gene is essential for flowering in this species (Shitsukawa et al., 2007). The transcript level of *VRN1* is very low in winter genotypes (*vrn1* allele) until they are vernalized, but once the

transcripts reach a critical threshold level, *VRN1* is expressed to high levels which initiates flowering (Danyluk et al., 2003; Loukoianov et al., 2005; Trevaskis et al., 2003; Yan et al., 2003). In spring genotypes, *VRN1* is constitutively expressed (*Vrn1* allele) (Danyluk et al., 2003; Loukoianov et al., 2005; Trevaskis et al., 2003; Yan et al., 2003). Spring growth habit is mainly due to mutations in the promoter and first intron of *VRN1* (Fu et al., 2005; Pidal et al., 2009; Yan et al., 2004a). As an example, it was shown that deletions in the CArG-box (MADS-box-binding site) in the promoter region of *VRN1* results in a spring growth habit (Pidal et al., 2009). During the short and cold days of winter which is synonymous of vernalization, *VRN1* is up-regulated and is coupled with a down-regulation of *VRN2* (Kane et al., 2005). *VRN2* encodes proteins with a putative zinc finger in the first exon and a CCT domain in the second exon that repress flowering (Yan et al., 2004b). The vernalization requirement of winter cereals is associated with the presence of the dominant *Vrn2* allele as compared with spring varieties that carry the recessive *vrn2* allele (Yan et al., 2004b). A 2.8 kb deletion within the first intron of *VRN1* present in several accessions of spring wheat and barley was found to inhibit binding of the *VRN2* gene flowering repressor (Fu et al., 2005). *VRN2* also plays a role in the repression of *VRN3* which is a homolog of *FLOWERING LOCUS T* in *Arabidopsis* and a strong flowering promoter (Yan et al., 2006). The *Vrn3* allele is associated with early flowering lines (greater transcript levels of *VRN3*) and the *vrn3* allele with late flowering lines and this allelic variation influences the vernalization requirement (Yan et al., 2006). Transformation of winter wheat with the dominant *Vrn3* allele resulted in conversion into a spring-habit genotype confirming that *VRN3* is a strong flowering promoter (Yan et al., 2006). Similarly, overexpression of *VRN3* in wheat increased the transcript levels of *VRN1* and resulted in early flowering (Li and Dubcovsky, 2008). Vernalization response is also induced by changes in photoperiod. More specifically, vernalization occurs when temperatures range from 0 to 7-10°C depending on the species under short days (Distelfeld et al., 2009; Fowler et al., 1996b). In summary, once the period of exposure to low non-freezing temperatures is long enough to induce vernalization saturation, both

VRN1 and *VRN3* are expressed to high levels whereas *VRN2* is minimally expressed and flowering is initiated.

2.11 The Connection Between Vernalization and Freezing Tolerance

Cold acclimation and vernalization response are two processes that plants have evolved in order to survive the freezing temperatures of winter. The induction of both cold acclimation and vernalization requires exposure to the same range of low non-freezing temperatures suggesting that these two processes are connected. Spring genotypes of cereals that are able to flower without exposure to cold exhibit lower freezing tolerance than winter genotypes that require cold exposure to achieve both low-temperature tolerance and vernalization saturation (Limin et al., 2007). In order to clarify the relationship between the two processes, time sequence studies have shown that low-temperature tolerance of winter wheat genotypes increases, reaches maximum and then decreases after a long-term exposure to low non-freezing temperatures (Fowler et al., 1996a; Fowler and Limin, 2004; Fowler et al., 1996b). Maximum low-temperature tolerance is achieved slightly before vernalization saturation indicating that the cold acclimation process is completed before the apical meristem switches from the vegetative to the reproductive phase also known as the double ridge (DR) stage (Fowler et al., 1996c; Limin and Fowler, 2006). Moreover, it was shown that winter wheat genotypes in the vegetative phase could re-acclimate following a de-acclimation period and reach high levels of low-temperature tolerance, but that plants in the reproductive phase have a poor ability to re-acclimate (Mahfoozi et al., 2001a; Mahfoozi et al., 2001b). Since low-temperature tolerance is substantially reduced once the plant has full capacity to flower, *VRN1* was pointed out as a potential candidate for repression of low-temperature tolerance (Limin and Fowler, 2006). The *VRN1/FR1* locus was found to harbor one locus for vernalization (*Vrn1*) and one locus for freezing resistance (*Fr1*) and that both were linked (Sutka and Snape, 1989). A second locus designated *FR*-2, located in the vicinity of *VRN1*, was also found to harbor at least 11 *CBF* genes (Miller et al., 2006). Winter genotypes (*vrn1* allele) express the cold-induced *CBF* genes to a

higher level than spring genotypes (Stockinger et al., 2007). Moreover, once vernalized the *CBF* transcripts in winter genotypes decrease and a strong expression of *Vrn1* is observed (Stockinger et al., 2007). This is suggesting that *Vrn1/Fr1* plays a role in the attenuation or repression of the *CBFs* located at *FR-2* (Stockinger et al., 2007). It was later shown that mutants of diploid wheat without any functional *Vrn1* allele (plants that never flower), had increased freezing tolerance and increased transcript levels of several *CBF* and *COR* genes compared with plants carrying at least one functional *VRN1* copy (Dhillon et al., 2010). *VRN1* is required for the initiation of the regulatory cascade that down-regulates the cold acclimation pathway, but *VRN1* alone is not sufficient to down-regulate the *COR* genes suggesting that other genes may be involved in this process (Dhillon et al., 2010). Previous genetic studies have shown that *FR1* cosegregates with *VRN1* indicating that this gene may not be independent, but rather a pleiotropic effect of *VRN1* (Galiba et al., 1995; Hayes et al., 1993; Limin and Fowler, 2006; Stockinger et al., 2007; Sutka et al., 1999). Dhillon et al. (2010) have shown that allelic differences in *VRN1* is sufficient to determine differences in freezing tolerance indicating that the quantitative trait loci that were found are likely a pleiotropic effect of *VRN1* rather than a distinct effect of the *FR1* locus. Taken together, allelic variation in *VRN1* and the expression of this gene are powerful tools that can be used to understand vernalization, cold acclimation and freezing tolerance in plants.

2.12 Research with *Brachypodium distachyon*

Even though *Brachypodium* is considered as the new revolutionizing grass model system since 1995 (Bablak et al., 1995), very few studies have been conducted with this plant so far. We can foresee a boom of studies reporting the genes involved in tolerance to abiotic stresses and the flowering patterns in this plant with its genome now sequenced (Vogel et al., 2010). So far, four suitable reference genes have been found and can be used to normalize the gene expression data of various tissues of control, hormone-treated and environmentally stressed plants (ubiquitin-conjugating enzyme 18 gene; *UBC18*,

polyubiquitin genes; *Ubi4* and *Ubi10* and S-adenosylmethionine decarboxylase gene; *SamDC*) (Hong et al., 2008). It was also shown that *Brachypodium* accessions broadly group into spring and winter annuals by changing the time to flower when submitted to cold and short photoperiod regimes (Schwartz et al., 2010). More specifically, cold and a short day photoperiod significantly accelerated flowering in winter-annual *Brachypodium* accessions (Schwartz et al., 2010). Moreover, it was shown that vernalization can be achieved by submitting either the seeds or the seedlings to a cold treatment (Schwartz et al., 2010). Four putative *VRN* genes very similar to wheat and barley *VRN* genes were found to be expressed in *Brachypodium* under non-vernalization conditions (Schwartz et al., 2010). Another study has classified 57 *Brachypodium* accessions according to their drought response by using chlorophyll fluorescence (Fv/Fm) and leaf water content as phenotypic evaluation parameters (Luo et al., 2011). The tolerant accessions had minimal leaf wilting and greater leaf water content and Fv/Fm (Luo et al., 2011). Moreover, it was shown that the concentration of water soluble sugars (WSS) increases in response to drought stress, but that no difference can be observed between the tolerant and susceptible accessions (Luo et al., 2011). Finally, *Brachypodium* was validated as a valuable model to study Fusarium head blight and other *Fusarium* diseases of wheat (Peraldi et al., 2011).

Connecting Statement

This chapter describes both the vernalization and cold acclimation physiological and molecular responses of several *Brachypodium distachyon* accessions. We believe it is important to understand the response of *Brachypodium* to low non-freezing temperatures in terms of reproductive and freezing tolerance capacities. Consequently, we studied flowering efficiency, osmoprotectant accumulation and vernalization and cold-regulated gene expression under cold temperatures. Armed with these findings, the research community will profit from the widespread exploitation that we anticipate of this powerful model system to develop knowledge regarding complex traits such as freezing tolerance. This chapter provides the launch pad to the creation of freezing resistant cereal crops.

I have contributed to all the work pertaining to the following chapter which includes basic production of seeds of the seven *Brachypodium* accessions, microscopy of double ridge formation, final leaf number count, proline and sugar assays, and identification, alignments, and expression analysis of *Bradi1g08340.2* and *Bradi5g27350.1* (orthologs of *VRN1* and *IRI*, respectively).

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Chapter 3

Characterization of *Brachypodium distachyon* as a Grass Model for Research in Freezing Tolerance

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3.1 Abstract

Long exposure to low non-freezing temperatures accelerates flowering and triggers cold acclimation mechanisms in winter wheat and barley. Cold tolerance is a complex but important trait that influences yield considerably. *Brachypodium distachyon* (*Brachypodium*) is an annual temperate wild grass species and is the new model system for studying grass biology, but little is known about the capacities of *Brachypodium* to cold acclimate and develop freezing tolerance. An integrated approach involving double ridge formation, final leaf number, and osmoprotectant content was used to confirm the growth habit and freezing tolerance ability of seven diploid *Brachypodium* accessions. In addition, expression analyses of orthologs of the major vernalization regulator *VRN1* and the *COR* gene *IRI* which encodes an ice recrystallization inhibition protein were conducted. Our results demonstrate that formerly classified spring accessions behaved more like facultatives by showing increased osmoprotectants and *BradiIRI* transcripts accumulation upon cold exposure. The confirmed winter accessions acquired vernalization saturation after 49 days and accumulated *BradiIRI* transcripts to higher levels although no significant difference was observed between the osmoprotectant content of spring and winter accessions. This study validates *Brachypodium* as a valuable model system to study freezing tolerance in temperate cereals.

3.2 Introduction

One of the most important constraints restricting crop cultivation in temperate climates is the length of the frost-free growing season. Freezing temperatures encountered late in spring, early in fall, and during winter severely alter plant survival and hence, reduce yield potential considerably. Freezing tolerance in plants is induced by exposure to low non-freezing temperatures, a process known as cold acclimation (Thomashow, 1999). As a consequence, winter cereals have the ability to resist freezing temperatures encountered during winter whereas spring genotypes have a poor ability to develop freezing tolerance (Limin et al., 2007). Numerous studies have characterized in depth the cold acclimation process in cereals and *Arabidopsis* and have established that this process is associated with numerous physiological and genetic alterations (Badawi et al., 2007; Crosatti et al., 1996; Danyluk et al., 1994; Galiba et al., 2009; Grossi et al., 1998; Houde et al., 1992; Jaglo-Ottosen et al., 1998; Limin et al., 1997; NDong et al., 2002; Stockinger et al., 1997; Thomashow, 1999; Van Buskirk and Thomashow, 2006; Vogel et al., 2005). Among the many strategies that plants have evolved to cope with freezing temperatures is one that involves the expression of antifreeze proteins (AFPs). AFPs are known to cause thermal hysteresis and recrystallization inhibition. Thermal hysteresis is the difference between the melting and the freezing temperature of ice in an organism and allows plants to supercool in the presence of ice. Thermal hysteresis is known to be low in plants and recrystallization inhibition is probably the main quality of AFPs in these organisms. Ice recrystallization inhibition (IRI) proteins favour the formation of small ice crystals rather than large ones which can cause irreversible damage to the fragile membrane and structures of the apoplast (Kuiper et al., 2001). Recently, a member of the IRI protein family was found to be present in winter wheat (TaIRI) and to significantly inhibit the growth of ice crystals, confirming its function as an IRI protein (Tremblay et al., 2005). Other adaptive features are necessary for the plant to protect critical cell structures and vital physiological processes during freezing and they include increased levels of carbohydrates, soluble proteins, proline, and organic acids, the appearance of new

enzyme isoforms, and modifications in the lipid membrane composition (Hughes and Dunn, 1996; McNeil et al., 1999; Thomashow, 1999). All these alterations are regulated by a complex multigenic system controlled by the expression level of cold-regulated (*COR*) genes (Thomashow, 2001).

Subzero temperatures are fatal to the sensitive floral meristems of cereals. Winter cereals thus have a vernalization requirement that delays the transition from the vegetative to the reproductive phase. The exposure to a period of low non-freezing temperatures up-regulates or down-regulates the expression of vernalization genes, which in turn promotes flowering only in the milder conditions of spring. Several studies have pointed that freezing tolerance can be associated with vernalization in cereals by showing that the transition from the vegetative to the reproductive phase is associated with the down-regulation of *COR* genes and up-regulation of the vernalization gene *VRN-1* (Dhillon et al., 2010; Fowler et al., 1996a; Fowler et al., 1996b; Mahfoozi et al., 2001a; Mahfoozi et al., 2001b). As a result, full expression of cold hardiness genes only occurs in the vegetative phase, and plants in the reproductive phase have a limited ability to cold acclimate. In addition, plants that are still in the vegetative phase have the ability to re-acclimate following periods of exposure to warm temperatures, whereas plants in the reproductive phase only have a poor ability to re-acclimate (Mahfoozi et al., 2001a).

The lack of an amenable model system for temperate cereals has restricted the discovery of genes and mechanisms involved in cold tolerance and vernalization. *Brachypodium distachyon* (*Brachypodium*) was first pointed out in 1995 as a potential model system that could reach a high level of performance for Triticeae research (Bablak et al., 1995). *Brachypodium* is an annual temperate wild grass species phylogenetically closer to temperate cereals than rice and *Arabidopsis* and originates from Mediterranean and Middle East countries where subzero temperatures are frequently observed (Opanowicz et al., 2008; Vogel et al., 2010). *Brachypodium* has many biological attributes including self-fertility, small stature, short generation time, and efficient transformation (Alves et al.,

2009; Garvin, 2007; Vogel and Bragg, 2009). Moreover, *Brachypodium* has a small and sequenced genome (272 Mbp) and spring and winter diploid ecotypes are available (Vogel et al., 2010). There is strong chromosomal synteny between *Brachypodium* and other cereals and about 77% of the genes retrieve significant match in the Triticeae EST database (Huo et al., 2009; Vogel et al., 2010). *Brachypodium* diverged just prior the Pooideae genera and the divergence time with wheat is estimated between 32 and 39 Myr ago (Draper et al., 2001; Vogel et al., 2010). This evidence indicates that, at the phylogenetic level, *Brachypodium* is a good model to study functional genomics of temperate cereals. This model has already proved its value in a number of vernalization and stress tolerance studies (Luo et al., 2011; Peraldi et al., 2011; Schwartz et al., 2010). These studies clearly demonstrate that *Brachypodium* holds promise for a powerful model system to improve abiotic stress tolerance in temperate cereals.

To date, little information is known on the capacities of *Brachypodium* to cold acclimate and develop freezing tolerance. The objective of this research was to determine if *Brachypodium* is a valuable model for research in freezing tolerance for the Triticeae. To achieve this goal, an integrated approach involving double ridge formation (DR), final leaf number (FLN) and osmoprotectant content was used to confirm the growth habit and freezing tolerance ability of seven diploid *Brachypodium distachyon* accessions. In addition, expression analyses of orthologs of the major vernalization regulator *VRN1* and an antifreeze protein were conducted. This methodology allowed us to clearly demonstrate that *Brachypodium* has the capacity to cold acclimate and develop freezing tolerance. Our study is thus the first to suggest that *Brachypodium* is a valuable tool to understand the molecular and genetic basis of freezing tolerance in cereal crops.

3.3 Materials and Methods

3.3.1 Plant Material and Growth Conditions

In this study, we used seven diploid accessions of *Brachypodium distachyon* that are originating from temperate regions surrounding the Mediterranean Sea

where sub-zero temperatures are encountered (Opanowicz et al., 2008). These accessions were preliminarily classified as spring- or winter-type (Schwartz et al., 2010). Seeds of *Brachypodium* spring accessions Bd2-3, Bd3-1, Bd21, and Bd30-1, and winter accessions Bd1-1, Bd18-1, and Bd29-1 were soaked for two hours in sterile distilled water at room temperature after which the lemma was removed. The seeds were then sterilized in 70% ethanol, rinsed with sterile distilled water and sterilized again in 1.3% sodium hypochlorite solution according to Alves et al. (2009) and Vain et al. (2008). The seeds were placed between two sterile filter papers imbibed with sterile distilled water and placed in a Petri dish at 4°C in the dark for one week. This stratification treatment is essential for the activation and synchronisation of germination in *Brachypodium*. Seeds were planted in pots containing Agro Mix® (Plant Products Co. Ltd) and grown for 10 days at 20°C with a 16 hour photoperiod and a photon flux density of 260 µmol m^{-2} sec^{-1}. At the end of this period, control plants were maintained under the same conditions of light and temperature. Cold acclimation was performed by subjecting 10-day-old germinated seedlings to a temperature of 4°C ±1°C with a 8 hour photoperiod and a photon flux density of 150 µmol m^{-2} sec^{-1} for different periods of time as specified for each experiment. For each harvesting time point, the plants were placed back at 20°C under a 16 hour photoperiod.

3.3.2 Phenological Development and Vernalization Saturation Point Determination

Two methods were used to determine the stage of phenological development: double ridge (DR) formation and final leaf number (FLN) measurements. Dissecting the crown and analysing the shoot apex development identified the DR stage (Kirby and Appleyard, 1987). Ten plants for each accession were dissected at each of the ten harvesting time points and the mean number of days required to achieve DR formation was recorded to determine the influence of the LT treatment on the rate of phenological development. Leaves on the main shoot were counted and the plants were grown until the flag leaf emerged and FLN could be determined (Wang et al., 1995). Vernalization

saturation was considered complete once the length of the LT treatment no longer reduced the FLN. A minimum of ten plants was used at each of the ten harvesting time points representing one biological replicate and three biological replicates were used.

3.3.3 Proline and Soluble Sugar Assays

Free proline content was analysed by colorimetric assay according to an established protocol (Ábrahám et al., 2010). Free proline was extracted by grinding 100 mg of whole fresh plants with 500 µl of 3% (w/v) sulfosalicylic acid. Three independent samples of 100 mg (approximately three plants) per accession per LT treatment were used. The extract was centrifuged at 13000 rpm for 5 min at room temperature. 0.1 ml of the extract was mixed with 0.5 ml of a solution of acidic ninhydrin [40% acidic ninhydrin (0.625 g ninhydrin, 15 ml glacial acetic acid and 10 ml 6M orthophosphoric acid), 40% glacial acetic acid and 20% of 3% sulfosalicylic acid]. The samples were incubated for 60 min at 96°C and the reactions were terminated on ice for 5 min. The samples were extracted by adding 1 ml of toluene and vortexing for 20 sec. The absorbance at 520 nm was measured using toluene as a reference. The standard curve was made using L-proline (Alfa Aesar) in a range of 0 to 57.5 µg ml^{-1}. Free proline content (µmol per gram of fresh weight) was calculated according to equation (1) (Bates et al., 1973).

(1) [(µg proline/mL x mL toluene) / 115.5 µg/µmole] / [g sample) / 5]

Total water soluble sugar (WSS) content was analysed according Luo et al. (2011) with modifications to allow the data to be collected using a microplate reader (Galicia et al., 2009). Total WSS was extracted from 5 mg of plant dry tissues with 1 ml of distilled water by vortexing. Twenty plants were harvested, immediately dried for one hour, grinded and mixed together, and three independent samples of 5 mg per accession per LT treatment were used. The extract was placed at 70°C for 45 minutes, vortexed every 15 minutes and centrifuged at 13000 rpm for 20 min at room temperature. The supernatant was diluted 1:9 and 50 µl of this sample was mixed with 100 µl of anthrone solution in

a 96 well microplate (100 mg anthrone in 100 ml 95% (w/w) H_2SO_4). The microplates were shaken at 150 rpm for 10 minutes and incubated at 100°C for 4 minutes. After cooling, the absorbance at 630 nm was measured. The standard curve was made using sucrose in a range of 0 to 200 µg ml^{-1}. The WSS content (mg per gram of dry plant material) was calculated according to equation (2). A dilution factor of 10 was used throughout the experiment.

(2) [(mg of sucrose / mg of starting dry plant material) * dilution factor].

3.3.4 Expression Analysis by Quantitative Real-Time PCR

3.3.4.1 RNA Isolation and cDNA Synthesis

Accessions Bd21 (spring) and Bd29-1 (winter) were used in this assay and 10-day-old seedlings were harvested after 0 (non-acclimated control), 7, 14, 21, 28, 35 and 42 days at 4°C. Each sample consisted of three seedlings (approximately 100 mg of fresh aerial plant tissues) and three independent samples per accession per harvesting time point were used. Total RNA was isolated using the RNeasy plant mini kit (Qiagen) and the samples were treated with DNase I (Qiagen). Purified RNA was reverse transcribed in a 20-µl reaction using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene Products Division, La Jolla, CA) according to the manufacturer's recommendations. Parallel reactions were run for each RNA sample in the absence of AffinityScript RT (no RT control) to assess any genomic DNA contamination. The cDNA products were diluted in water to 400 ng $µl^{-1}$ and stored at -20°C.

3.3.4.2 Design of Gene-Specific Primers

Primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/) and BLASTN search were conducted in order to assess primer specificity. Primers were designed for amplification of 100 to 200 bp fragments in the 3' portion of the coding sequence of the gene of interest. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Primers used in this study are listed in Table 1.

3.3.4.3 PCR Amplification

Quantitative real-time PCR assays were conducted in triplicates in a Mx3000 real-time thermal cycler (Agilent Technologies, Cedar Creek, TX) using 18S ribosomal RNA as the housekeeping gene with Brilliant III Ultra-Fast SYBR® Green QPCR master mix (Stratagene Products Division, La Jolla, CA). Amplification was performed in a 15 µl reaction containing 1x SYBR Green master mix, 350 nM each primer, 30 nM reference dye ROX and 2 µl cDNA template (one-ninth dilution). The PCR thermal-cycling parameters were 95°C for 2 min followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The experiment was repeated three times.

3.3.4.4 Data Analysis

Data was analyzed with the Mx-Pro QPCR software (Stratagene Products Division, La Jolla, CA). The relative expression ratios of the target gene versus the 18S ribosomal RNA reference gene were calculated for each LT treatment using the following algorithm (3) (Zhao and Fernald, 2005).

(3) R0=1/(1+E)^CT

3.3.5 Experimental Design and Statistical Analysis

All the experiments were carried out using randomized complete block design. Two general treatments were used throughout this study: 1) non-acclimation which is defined by 20°C and 16 hour photoperiod and 2) cold acclimation which is defined by 4°C and 8 hour photoperiod for 7 to 63 days. For DR and FLN, ten plants per accession per treatment per assay were used. For proline and qRT-PCR, three independent samples of three seedlings (100 mg of fresh plant tissues) were used per accession per treatment. For WSS, three independent samples of 5 mg of dry plant tissues were used per accession per treatment. All data are presented as means ± standard deviation of the mean. Comparisons between cold acclimated plants and control plants were performed using one-way analysis of variance with PROC GLM (SAS Institute, Version 9.2,

Cary, NC, USA) and least significance difference for comparison between means with a 5% level of significance (SAS Institute Inc., 2009).

3.4 Results

3.4.1 Vernalization Response in *Brachypodium distachyon*

Results presented in Figure 1A show the vegetative/reproductive transition in *Brachypodium* accession Bd21 starting with the shoot apex (vegetative phase), the DR stage (reproductive phase) and the immature spikelet. Four of the accessions, Bd2-3, Bd3-1, Bd21 and Bd30-1, entered the reproductive phase when grown at 20°C for their complete life cycle indicating that those accessions have, as expected, a spring growth habit (Figure 1B). The number of days required to reach DR varied significantly among those accessions clearly indicating natural genetic variation. Interestingly, a 28 days cold treatment under short day conditions accelerated the switch from the vegetative to the reproductive phase in three of the spring accessions. Indeed, by subtracting 28 days and the number of days to DR from the number of days to DR in the non-vernalized seedlings, Bd2-3, Bd3-1, and Bd21 formed DR 23, 6, and 8 days earlier when vernalized for 28 days when respectively compared to plants kept in normal conditions. In comparison, prolonged exposure to cold (42 days) accelerated flowering only in the accession Bd2-3 by 20 days. The three other accessions entered the reproductive phase after a minimum of 14 days at 4°C for Bd1-1 (159 days to DR, data not shown) and 28 days for Bd18-1 (49 days to DR) and Bd29-1 (93 days to DR) indicating that those accessions require a vernalization treatment in order to switch to the reproductive phase thus corroborating their classification as winter accessions. Vernalization for 42 days accelerated flowering by 4, 13 and 47 days in Bd1-1, Bd18-1 and Bd29-1, respectively, as compared with the 28 days treatment.

Exposure to LT also had a significant effect on the final leaf number (FLN) in all the accessions. The FLN of the four accessions that entered the reproductive phase without vernalization decreased with a LT treatment when compared to the

non-vernalized controls (Figure 2). The FLN dropped from 11-13 leaves (no LT treatment) to 7-8 leaves after 35 days of LT treatment. Exposure to LT for periods longer than 35 days did not further reduce the FLN. These results are consistent with the results obtained for DR formation since vernalization in those accessions is not essential for flowering, but greatly accelerates the process. The three other accessions showed elevated FLN (15 to 21 leaves) at the minimal LT treatment necessary for DR formation (14 and 28 days; Figure 1E). Furthermore, no significant change were observed for exposure at 4°C longer than 49 days (8-12 leaves) in these winter type accessions. This result indicates that vernalization saturation in these accessions is probably reached after spending 49 days at 4°C.

To further understand the relationship between the vernalization and cold acclimation responses of *Brachypodium distachyon*, we have decided to monitor the mRNA expression pattern of the closest *VRN1* ortholog in this plant. The gene (*Bradi1g08340.2*) was selected for its 91% sequence identity (e-value of 0) to *Triticum aestivum vegetative to reproductive transition-1* (*TaVRT-1*) (GenBank AY280870.1) (Figure 3A). *Bradi1g08340.2* codes a MADS-box transcription factor and it was previously suggested that it could act as *VRN1* in *Brachypodium* (Schwartz et al., 2010). Bradi1g08340.2 shares high homology with TaVRT-1 (84% identity and 89% similarity) (GenBank AAP33790.1) and VRN1 of *Triticum monococcum* (89% identity and 92% similarity) (GenBank ACI24357.2) (Figure 3B). Most plant MADS domain proteins contain three conserved regions, the MADS, I, and K domains (M-I-K region) and the C-terminal domain can diverge between orthologs (Theissen et al., 1996). Those three conserved regions are found in the Bradi1g08340.2 protein with the C-terminal region showing reduced sequence identity with wheat orthologs (Figure 3B). Bradi1g08340.2 is made of 243 amino acids while TaVRT-1 and VRN1 (*T. monococcum*) are made of 244 and 243 amino acids, respectively. Sequence comparison of Bradi1g08340.2 and APETALA1 (AP1) of *Arabidopsis thaliana* (GenBank AEE34887.1) (data not shown) revealed that both proteins are highly similar suggesting that Bradi1g08340.2 is probably an important flowering regulator in *Brachypodium* (Ferrándiz et al., 2000). Sequence analysis further revealed that

Bradi1g08340.2 possesses a bipartite nuclear targeting domain that is conserved in all the MADS box transcription factors (Danyluk et al., 2003). A conserved phosphorylation site which may potentially affect several cellular signalling processes involved in the vernalization response is present in the C-terminal region of Bradi1g08340.2 (Blom et al., 1999).

Accessions Bd21 and Bd29-1 were chosen to conduct expression analysis because of their contrasting differences in phenological development (Figures 1 & 2). In non-vernalized Bd21 plants, *Bradi08340.2* shows low transcript levels after 0 and 7 days of cold treatment. The transcripts accumulate gradually after 14 and 21 days of cold acclimation and reach maximum accumulation after 28 days of LT exposure (Figure 4A). Vernalization for more than 28 days does not significantly increase *Bradi08340.2* transcripts in Bd21. In Bd29-1, low *Bradi08340.2* transcript levels were monitored in the non-vernalized controls and accumulate gradually with the LT treatment until at least 42 days (Figure 4B). Moreover, the transcript level of *Bradi08340.2* after 42 days of vernalization is 9 times higher than the maximum expression level observed in Bd21 after 28 and 42 days. As shown in the FLN experiment, transition to the reproductive phase and vernalization saturation is reached after 49 days of vernalization in Bd29-1 so we can expect that the characteristic plateau of *VRN1* transcript accumulation is probably reached after the same vernalization treatment.

3.4.2 Cold Acclimation Response and *BradiIRI* Expression

3.4.2.1 Soluble Carbohydrates

In an effort to determine if *Brachypodium* is freezing tolerant, we wanted to determine the effect of cold acclimation on the content of water soluble sugars. Non-acclimated (NA) *Brachypodium* tissues contained minimum values ranging from 43 µg mg^{-1} DW of WSS (Bd1-1) to 57 µg mg^{-1} DW (Bd30-1) (Figure 5A). As a comparison, this range of WSS concentration is similar to the one obtained by Luo et al. (2011) in their non-drought controls indicating that the plants were not stressed before the LT treatment. As soon as the seedlings were acclimated for

7 days, there was a significant increase in the WSS concentration in all accessions (Figure 5A). The maximum WSS concentration was observed after 14 days of LT treatment for all accessions with the exception of Bd2-3 and Bd29-1 who reached maximum WSS concentration at 28 days (Figure 5A). The rate of increase in WSS between NA and 14, 21 and 28 days acclimated seedlings ranged between 139% and 256%. In comparison, the WSS concentration observed after 14, 21 and 28 days of LT treatment ranged between 75 µg mg^{-1} DW (Bd2-3) and 116 µg mg^{-1} DW (Bd18-1) which is very similar to the values Luo et al. (2011) obtained after submitting several *Brachypodium* accessions to drought stress indicating that cold and drought stresses induce the same defense mechanisms in *Brachypodium*. Although Bd2-3 reached a maximum WSS concentration at the 28 days treatment, it is worth noting that this accession contained overall the lowest WSS content among all the accessions tested. WSS concentration decreased slightly at the 21 and 28 days treatment in most accessions. A significant difference in WSS concentration at the 14 days treatment was observed among all accessions with winter accession having a slightly higher concentration suggesting that they may be more freezing tolerant. After one day of de-acclimation, seedlings still had elevated WSS concentrations compared to the NA controls, but a clear reduction of the WSS concentration was observed for all accessions except in Bd2-3 (Figure 5A).

3.4.2.2 Proline Content

We wanted to determine the effect of cold acclimation on the proline content of *Brachypodium* accessions. Accordingly, we submitted seedlings of the seven accessions to 0, 7, 14, 21 and 28 days at 4°C and took samples at each of those time points. We also transferred the 28 days cold acclimated plants back to 20°C and took another set of samples after one day of de-acclimation. We decided not to collect samples beyond 28 days of cold acclimation because preliminary assays showed constant levels of proline and soluble sugars up to 42 days of treatment (data not shown). This time point also correlates with the fact that all the accessions are able to flower after 28 days of treatment. Under NA conditions,

all the accessions showed basal but almost negligible proline content (Figure 5B). However, in seedlings that were cold acclimated for seven days, there was a significant increase in proline content compared to the NA plants (Figure 5B). Bd29-1 showed one of the most robust proline content responses with a maximum reached after 14 days (Figure 5B). The de-acclimated seedlings had a proline content similar to the seedlings acclimated for 7 days in all accessions indicating that the proline content decreases rapidly once the plants are returned to normal growing conditions.

3.4.2.3 COR Gene Transcripts Accumulation

A *Brachypodium* gene encoding an IRI protein was identified by homology search using the *Triticum aestivum* IRI sequences (*TaIRI-1* and *TaIRI-2, GenBank* acc. no. AY968588 and AAX81543.1, respectively) (Tremblay et al., 2005) against the BrachyBase BLAST database and the *Brachypodium* genome browser. A single gene was found as most similar to those sequences: Bradi5g27350.1 (*BradiIRI*). The longest open reading frame is 837 bp and encodes a putative protein of 278 amino acids which is consistent with the size of the protein *TaIRI-1* (280 amino acids) (Tremblay et al., 2005). Search in GenBank revealed high homology with potential IRIs from wheat (>64% identity and >74% similarity), barley (>66% identity and >74% similarity) and perennial ryegrass (*Lolium perenne*) (>61% identity and >73% similarity) (Figure 6A). Sequence analysis revealed the position of a N-terminal cleavage site between amino acids 21 and 22 suggesting the presence of a signal peptide that would target the protein to the extracellular space or to the membrane (Figure 6A). Similarly to TaIRI-1, BradiIRI contains two leucine rich repeat-containing domains located at the N-terminal region (Figure 6B). The IRI domain of BradiIRI shows homology with the two ice-binding sites of the IRI partial sequence of *Lolium perenne* antifreeze protein (LpAFP) (CAB87814.1) (Sidebottom et al., 2000) and with TaIRI-1 (Figure 6C). The XXNXVXG (‘a’ side) and XXNXVX – G (‘b’ side) motifs forming the ice-binding sites are repeated eight times for both BradiIRI and LpAFP compared to five times for TaIRI-1 (Kuiper et al., 2001) (Figure 6C).

The transcripts accumulation level of *BradiIRI* was determined by qRT-PCR in the spring accession Bd21 and the winter accession Bd29-1 accessions. In wheat, *IRI* is expressed at very low levels in NA plants and accumulates to high levels during cold acclimation (Tremblay et al., 2005). As expected, accumulation of *BradiIRI* is also detected during cold acclimation (Figure 7). After a 7 day acclimation period, a strong accumulation of *BradiIRI* in both accessions was observed. In general, the relative transcript abundance in Bd29-1 was significantly higher than in Bd21 by 10 fold for all the acclimation periods. In Bd21, maximum transcript accumulation was reached at 7 days of acclimation after which a steady decrease in accumulation was observed until 42 days of acclimation. In Bd29-1, maximum accumulation was reached at 14 days of cold acclimation. A significant decrease in transcript level was also noted in Bd29-1 after 21 and 28 days of cold exposure, but this reduction was less pronounced than in Bd21 consistent with the winter habit of this accession. Those results thus suggest that late-flowering genotypes (Bd1-1, Bd18-1 and Bd29-1) could exhibit greater freezing tolerance than early-flowering genotypes (Bd2-3, Bd3-1, Bd21 and Bd30-1) due to the higher accumulation of *COR* gene transcripts.

3.5 Discussion

The genus *Brachypodium* is found throughout most temperate regions of the world and its ancestral range is located in the Mediterranean and Middle East countries (Opanowicz et al., 2008). A close examination of the geographic distribution of the seven diploid *Brachypodium* accessions used in this study indicates that they are naturally growing in regions where a temperate climate prevails and where sub-zero temperatures are encountered (Bd1-1 and Bd18-1, Turkey; Bd2-3, Bd3-1 and Bd21, Iraq; Bd29-1, Ukraine; and Bd30-1, Spain) (Garvin et al., 2008; Schwartz et al., 2010). Based on this information and previous findings (Schwartz et al., 2010), we formulated the hypothesis that this plant would carry some if not all the mechanisms responsible for cold acclimation and freezing tolerance. Molecular and physiological studies aimed at understanding the mechanisms of cold tolerance in cereals revealed that this

process is closely associated with the vernalization response (Danyluk et al., 2003; Dhillon et al., 2010; Fowler et al., 1996c). Therefore, with the goal of understanding the cold acclimation pattern in *Brachypodium*, we used a classical approach involving observation of DR formation, FLN, and expression analysis of the ortholog of *VRN1* in an effort to portray the vernalization requirements of this plant.

As a first step, we decided to look at DR formation which clearly indicates the transition from vegetative to reproductive stages in grasses (Kirby, 2002). Observation of DR formation is a method that has been used extensively to classify cereal varieties into spring- or winter-type growth habit (Hemming et al., 2008; Limin et al., 2007). Four of the accessions used in this study (Bd2-3, Bd3-1, Bd21 and Bd30-1) advanced to the DR stage without a vernalization treatment, but showed accelerated transition to the reproductive phase when submitted to cold indicating a positive effect of cold on the flowering efficiency of these *Brachypodium* accessions. Our results are in agreement with previous findings that have shown that Bd2-3, Bd3-1, Bd21 and Bd30-1 (DSNSCA-6) flower without vernalization and that vegetative/reproductive transition is slightly accelerated in Bd2-3 and Bd30-1 when vernalized (Schwartz et al., 2010). Similarly to our results, Schwartz et al. (2010) also found that Bd1-1 requires at least two weeks of vernalization in order to flower whereas Bd18-1 requires at least four weeks and that a six weeks treatment accelerated vegetative/reproductive transition in both accessions.

According to Schwartz et al. (2010), the *Brachypodium* genome contains two genes that are very similar to wheat and barley *VRN1* (*Bradi1g08340.2* and *Bradi1g59250.1*). Another bioinformatics study has proposed that *Bradi1g08340.2* is the only ortholog of *VRN1* of cereals (Higgins et al., 2010). In our study, *Bradi1g08340.2* was chosen to conduct the expression analyses because of its highest sequence similarity and identity to *Triticum monococcum VRN1* (Pidal et al., 2009) and *Triticum aestivum VRT-1* (Danyluk et al., 2003). It has been shown that spring wheat accessions express *VRN1* to high and stable

levels no matter if they are vernalized or not (Danyluk et al., 2003; Kane et al., 2005). On the other hand, *VRN1* is generally expressed to low levels in the first few days of vernalization in winter wheat accessions and progressively until it reaches a plateau beyond the 49 days mark (Kane et al., 2005). Surprisingly, the transcript accumulation pattern of *Bradi1g08340.2* in accession Bd21 resembles the expression pattern observed in winter accessions. The accumulation of *Bradi1g08340.2* is induced by the vernalization treatment. This accumulation reaches a maximum when Bd21 plants are vernalized for 28 days after which a plateau seems to be reached even if some variations in transcripts level are observed. This differential accumulation of *Bradi1g08340.2* in accession Bd21 raises a doubt about the spring habit of this accession. This doubt is further substantiated by our DR and FLN results showing that Bd21 can flower without a vernalization treatment, but that flowering is accelerated after 28 days of vernalization. In addition, we also observed that non-vernalized Bd21 plants often produced small and non-fertile seeds compared to vernalized plants that developed two to three spikelets with most of their seeds fertile. Furthermore, Schwartz et al. (2010) have found that *Bradi1g08340.2* is expressed to lower levels in non-vernalized spring accessions Bd2-3 and Bd30-1 compared to non-vernalized plants of the winter accession Bd18-1. Those observations may indicate that accessions Bd2-3, Bd3-1, Bd21, and Bd30-1 are not typical spring accessions.

Some barley accessions such as Dicktoo were found to have a facultative phenotype. Those plants do not require a vernalization treatment in order to flower, but are photoperiod-sensitive and low-temperature tolerant (Karsai et al., 2005). Upon vernalization under short days, *Brachypodium* accessions Bd2-3, Bd3-1, Bd21, and Bd30-1 advanced to DR more rapidly and reduced their FLN up to 28 days which may be a consequence of the significant accumulation of *VRN1* transcripts at this period. Moreover, the transcripts of the *COR* gene *BradiIRI* were initially elevated after one week of vernalization and significantly reduced at the 28 days mark. Similarly, the facultative barley cultivar Dicktoo is able to accumulate higher levels of COR proteins and acquire higher freezing tolerance

when acclimated under short days (Fowler et al., 2001). This behaviour is thought to be caused by the repression of expression of *VRN1* in the initial weeks of vernalization under short days (Danyluk et al., 2003). Our analysis suggests that the accessions Bd2-3, Bd3-1, Bd21, and Bd30-1 may be in fact better classified as facultative accessions. Further work such as observing DR, FLN, *VRN1* and *COR* gene expression of the plants of these accessions when submitted to a long day vernalization treatment would reinforce this classification. Indeed, studies have shown that long day vernalization of Dicktoo causes constitutive expression of *VRN1* thus giving rise to a faster transition from the vegetative to the reproductive phase and stable FLN as well as lower levels of COR proteins (Danyluk et al., 2003; Fowler et al., 2001; Limin et al., 2007).

The genome of the barley facultative cultivar Dicktoo is known to carry recessive *vrn1* alleles and a physical deletion associated with *VRN2* (Karsai et al., 2005). A genotype analysis would confirm allelic variation in *VRN1* and *VRN2* in *Brachypodium*, but we suspect the accessions Bd2-3, Bd3-1, Bd21, and Bd30-1 to have the allelic constitution *vrn1vrn1*/*vrn2vrn2*. With this typical facultative genotype, the *vrn1* allele is not repressed by *vrn2*, but it may be repressed by other genes like photoperiodically activated transcription factors (Kosová et al., 2008). Currently, there is contradiction in the literature as of whether the gene *VRN2* is absent or present in the genome of *Brachypodium*. One study claims that *VRN2* is absent in Bd21 (Higgins et al., 2010) whereas another study has found an ortholog of *VRN2* in Bd21 (*Bradi3g10010.1*) (Schwartz et al., 2010). Preliminary qRT-PCR analysis performed in the framework of this study did not reveal a typical *VRN2* transcript accumulation pattern for *Bradi3g10010.1* in vernalized *Brachypodium* seedlings (data not shown) thus indirectly confirming the data presented by Higgins et al. (2010). These findings again suggest that the spring accessions may be in fact better classified as facultative accessions.

The winter accessions used in this study typically behaved as winter annuals by reducing their FLN with vernalization and by reaching saturation after 49 days at 4°C (Danyluk et al., 2003; Limin et al., 2007). Moreover, the fact that

Bradi1g08340.2 expression in Bd29-1 after 28 days of vernalization is 10 fold higher than in Bd21 suggests that vernalization is essential for the flowering of *Brachypodium* winter accessions and that saturation is most likely reached after 42 days at 4°C. Furthermore, the massive changes in relative transcript abundance observed in Bd29-1 are in agreement with previous reports that have shown a 3000 fold difference in *VRN1* transcript accumulation in a winter accession of *T. monococcum* upon vernalization (Yan et al., 2003). However, even if the sequence and expression analysis results are strongly suggesting that *Bradi1g08340.2* is in fact *VRN1*, further work will have to be conducted to confirm the exact role of *Bradi1g08340.2*.

In wheat and barley, the progressive accumulation of *VRN1* was shown to be correlated with the down-regulation of *COR* genes (Danyluk et al., 2003; Stockinger et al., 2007). Our findings on the behaviour of *Brachypodium* accessions upon vernalization are very useful in that they indicate that floral transition occurs without a vernalization treatment in early flowering accessions, but that their potential facultative growth habit may be correlated with the presence of freezing tolerance mechanisms. In comparison, winter accessions require a minimum of 14 (Bd1-1) and 28 (Bd18-1 and Bd29-1) days at 4°C to switch to the reproductive stage which suggests that the expression of the *COR* genes involved in the biosynthesis of osmoprotectants starts to be down-regulated near the 28 days mark of the treatment. Our results, showing an induced accumulation of WSS and proline in *Brachypodium* plants upon cold acclimation, are in agreement with these findings. Indeed, maximum WSS and proline accumulation occurred between 14 and 28 days at 4°C in all accessions. Interestingly, the accession Bd1-1 accumulated less proline than the two other winter accessions which is probably correlated with its capacity to flower earlier at 14 days as opposed to 28 days at 4°C. Earlier reports have also shown an increase in WSS (Vágújfalvi et al., 1999; Yoshida et al., 1998) and proline (Naidu et al., 1991; Petcu and Terbea, 1995) in cold acclimated wheat. However, the clear difference in WSS and proline concentration generally observed between spring and winter genotypes of wheat is not observed in *Brachypodium*. This

might be a characteristic that sets apart the *Brachypodium* model since Luo et al. (2011) also did not find a distinctive difference in WSS even though clear differences in drought tolerance capacities were observed among *Brachypodium* accessions. Soluble carbohydrates change in quantity and form when plants are stressed. Those compounds are known to increase stress tolerance in plants by increasing the cell osmotic potential (McNeil et al., 1999). Previous studies have shown that there is a positive correlation between freezing tolerance and soluble carbohydrate accumulation during cold acclimation (Hurry et al., 1995; Olien and Clark, 1993; Vágújfalvi et al., 1999; Yoshida et al., 1998). Proline concentration is also known to increase in plants that are subjected to hyperosmotic stress in response to conditions of water deficit, elevated soil salinity and exposure to low temperatures (Hare et al., 1999). Moreover, proline content can be used as a biochemical marker for frost tolerance in winter wheat (Petcu and Terbea, 1995) because it accumulates with cold acclimation (Charest and Ton Phan, 1990; Naidu et al., 1991) and protects the plant cells by increasing the osmotic pressure in the cytoplasm and stabilizing proteins and membranes under cold stress (Guy, 1990). Since spring accessions may be in fact be facultative accessions this may explain that similar accumulation pattern of osmoprotectants was detected in both groups of accessions as facultative cultivars are known to be frost-tolerant when acclimated under short day conditions (Fowler et al., 2001).

Variations in the expression of *COR* genes are correlated to variation in freezing tolerance in cereals (Danyluk et al., 1994; Danyluk et al., 1998; Fowler et al., 1996b; Houde et al., 1992; Limin et al., 1997). The *Brachypodium* genome contains several genes with high relatedness to cereal *COR* genes. The *BradiIRI* transcripts abundance analysis reported here provides evidence that some of the molecular mechanisms of freezing tolerance observed in hardy grasses are present in *Brachypodium*. This strongly suggests that phenotypic variations in freezing tolerance exist between spring and winter accessions. Indeed, the close sequence homology between BradiIRI, TaIRI-1 and LpAFP as well as the transcript accumulation pattern of *BradiIRI* show evidence that *Brachypodium* possess active cold acclimation mechanisms. The transcript accumulation pattern indicates

higher levels of *BradiIRI* transcripts in cold acclimated *Brachypodium* plants compared to controls. *BradiIRI* transcripts level were 10 fold higher in the winter accession Bd29-1 compared to levels observed in the spring/facultative accession Bd21 which clearly suggests higher freezing tolerance capabilities. Furthermore, a progressive decrease in transcript levels was observed after 14 days and 21 days of cold exposure for Bd21 and Bd29-1, respectively. These transcripts accumulation patterns agree well with the floral transition data presented in this manuscript. The reduction in transcript abundance happens more rapidly in the spring accessions which correlates with the fact that those accessions flower more rapidly. Moreover, the decrease in transcripts abundance measured at 21 days of cold acclimation in the winter accession Bd29-1 almost perfectly coincides with the observation that this winter accession acquires its flowering capacities somewhere between day 22 and 28 of cold exposure. This suggests that *COR* gene transcripts become less abundant when the floral transition is about to be reached. Obviously, transcript accumulation data of several *Brachypodium COR* genes will have to be determined to obtain a more precise picture. Moreover, confirmation of active freezing tolerance mechanisms in *Brachypodium* will have to await future studies involving whole plant freezing assays.

This study confirms that natural genetic variation exists among diploid accessions of *Brachypodium* and is the first to demonstrate that this model organism is a valuable tool to study the complex multigenic trait of freezing tolerance.

3.6 Table

Table 1. List of primers used in this study. BdVRN1-137-F and BdVRN1-138-R were designed for amplification of a 220 bp fragment of *Bradi1g08340.2* (*VRN1* ortholog in *Brachypodium*) for qRT-PCR. BdIRI-158-F and BdIRI-159-R were designed for amplification of a 160 bp fragment of *BradiIRI* for qRT-PCR. 18S-117-F and 18S-118-R were designed for amplification of a 131 bp fragment of the housekeeping gene 18S ribosomal RNA in *Brachypodium* for qRT-PCR.

Primer Name	**Primer Sequence (5' to 3')**	**Amplicon Size (bp)**	**Primer Tm (°C)**
BdVRN1-137-F	5'-CAGATCCAGAAAGAACCAGCTAA-3'	220	54.5
BdVRN1-138-R	5'-GCGATTACTGATATTTGTTGTTGG-3'		52.6
BdIRI-158-F	5'-AACCGAAAAACTCTAGACGAAGAA-3'	160	54.2
BdIRI-159-R	5'-GACTCCCACTTAGGATTTTGTTGT-3'		55.0
18S-117-F	5'-GAAGTTTGAGGCAATAACAGGTCT-3'	131	55.3
18S-118-R	5'-ATCACGATGAATTTCCCAAGATTAC-3'		53.5

3.7 Figure Legends

Figure 1. Vernalization accelerates the rate of phenological development in all accessions. Shoot apices were dissected and analyzed for the appearance of the double ridge (DR) structure. Sections A to C were viewed under a stereomicroscope with an 1.5X objective and 10X eyepiece and digitally documented with the Zeiss AxioCam MRc digital camera. A) The shoot apex of *Brachypodium* accession Bd21 develops vegetatively and produces leaf primordia. B) Leaf primordia form single ridges (SR) prior to DR formation. C) Floral primordia develop above leaf primordia giving rise to DR that characterize the transition from the vegetative to the reproductive phase in cereals. D) Floral primordia differentiate into floral organs giving rise to the florets. E) DR formation in seven accessions of *Brachypodium*. DR formation was compiled for seedlings that were not vernalized (NA0) and vernalized for 28 and 42 days (CA28 and CA42). Each time point consisted of ten plants and the experiment was terminated 120 days after the vernalization treatments. Data points at 120 days represent plants that did not form DR during the course of the experiment. The experiment was repeated three times with similar results and vertical bars indicate standard deviation of the mean. The difference between the number of days to DR formation of the non-vernalized seedlings (VRN0) and the vernalized seedlings (VRN28 and VRN42 days) is statistically significant at $P<0.0001$.

Figure 2. Vernalization reduced the final leaf number of all accessions. Final leaf number (FLN) was compiled for seedlings that were not vernalized (0 days) and vernalized for 7, 14, 21, 28, 35, 42, 49, 56 and 63 days. Each time point consisted of ten plants and the experiment was terminated 120 days after the vernalization treatments. The experiment was repeated three times with similar results and vertical bars indicate standard deviation of the mean. The difference between the FLN of the non-vernalized spring seedlings (0 days at 4°C) and the vernalized seedlings is statistically significant at $P<0.0001$. The difference between the FLN of the seedlings vernalized for 14 days for Bd1-1 (winter) and

28 days for Bd18-1 (winter) and Bd29-1 (winter) and the seedlings vernalized for 49 days is statistically significant at P<0.0001.

Figure 3. Sequence identity of *Bradi1g08340.2* coding sequence and Bradi1g08340.2 protein with related MADS-box gene and proteins from other species. A) Nucleotide alignment of *Bradi1g08340.2* and *TaVRT-1*. Identical nucleotides and substitutions are shaded in black and gray, respectively. B) Structure and sequence alignments of Bradi1g08340.2 with *Triticum monococcum* VRN1 and *Triticum aestivum* VRT-1. Identical and similar amino acids are shaded in black and gray, respectively. MADS-box domain, DNA-binding domain; I domain, intervening region; K domain, keratin-like domain; C domain, C terminal region; serine stretch, region rich in phosphorylation sites. *, Residues identified as being part of a nuclear targeting signal by PSORT (Nakai and Horton, 1999) (http://psort.hgc.jp). Serine stretch, NetPhos 2.0 (Blom et al., 1999) (http://www.cbs.dtu.dk/services/NetPhos/) predicts phosphorylation sites in this region.

Figure 4. *Bradi1g08340.2* transcripts accumulate continuously and to higher levels in the *Brachypodium* winter accession Bd29-1 in response to vernalization. A) *Bradi08340.2* in vernalized plants of the accession Bd21. B) *Bradi08340.2* in vernalized plants of the accession Bd29-1. *Brachypodium* plants were grown for 10 days at 20°C under a long day (16 h) photoperiod, transferred to 4°C under a short day (8 h) photoperiod, and then sampled at regular intervals (0 days represent non-vernalized controls). qRT-PCR analysis was done using total reverse-transcribed RNA isolated from aerial parts with the forward and reverse primer pair BdVRN1-137-F and BdVRN1-138-R. Data shown represent mean values obtained from three biological replicates and the vertical bars indicate standard deviation of the mean. The experiment was repeated three times with similar results. Means that are shown represent data obtained from one of the three repeated experiments. Means with different letters are statistically significant at P<0.0001.

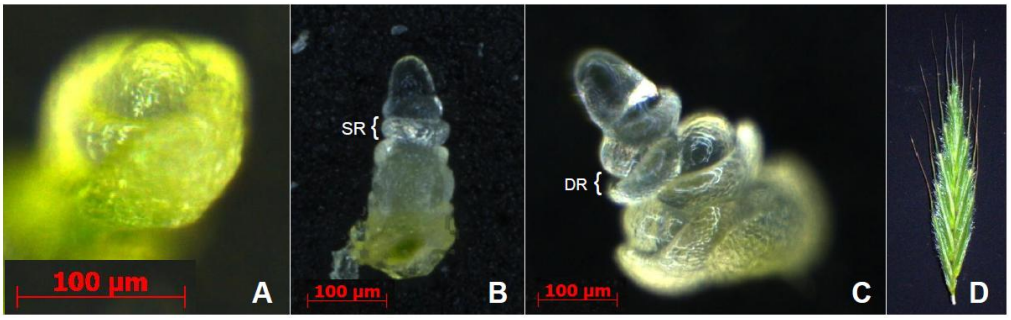
Figure 5. Soluble carbohydrate and proline osmoprotectants accumulate to high levels in all accessions in response to cold acclimation. A) Water soluble sugar content of *Brachypodium distachyon* accessions under control (0 days at 4°C) and cold acclimation periods (7, 14, 21 and 28 days at 4°C). DA1, de-acclimated for one day at 20°C following 28 days of cold acclimation. B) Proline concentration of *Brachypodium distachyon* accessions under control (0 days at 4°C) and cold acclimation periods (7, 14, 21 and 28 days at 4°C). DA1, de-acclimated for one day at 20°C following 28 days of cold acclimation. *Brachypodium* plants were grown for 10 days at 20°C under a long day (16 h) photoperiod, transferred to 4°C under a short day (8 h) photoperiod, and then sampled at regular intervals. Data shown represent mean values obtained from independent biological replicates (n=3) and technical replicates (n=3) and the vertical bars indicate standard deviation of the mean. The experiment was repeated three times with similar results.

Figure 6. The *Brachypodium BradiIRI* gene encodes a bipartite protein. A) Sequence alignment of cereal ice recrystallization inhibition (IRI) proteins with the partial *Lolium perenne* antifreeze protein (LpAFP). Identical and similar amino acids are shaded in black and gray, respectively. The predicted signal peptide targeting the proteins to the apoplast is boxed. B) Alignment of the leucine-rich repeat domains of the *Brachypodium* and wheat proteins. The highly conserved residues are boxed. C) Alignment of the IRI domains of the ryegrass, wheat and *Brachypodium* proteins. The 'a' side and 'b' side consensus repeated sequences follow the representation suggested by Kuiper et al. (2001) for the ice-binding surfaces of LpAFP. The highly conserved residues are boxed.

Figure 7. *BradiIRI* transcripts accumulate to higher levels in the *Brachypodium* winter accession Bd29-1 in response to cold acclimation. A) *BradiIRI* in cold acclimated plants of the accession Bd21. B) *BradiIRI* in cold acclimated plants of the accession Bd29-1. *Brachypodium* plants were grown for 10 days at 20°C under a long day (16 h) photoperiod, transferred to 4°C under a short day (8 h) photoperiod, and then sampled at regular intervals (0 days

represent non-acclimated controls). qRT-PCR analysis was done using total reverse-transcribed RNA isolated from aerial parts. Data shown represent mean values obtained from independent amplification reactions (n=3) and the vertical bars indicate standard deviation of the mean. The experiment was repeated three times with similar results. Means with different letters are statistically significant at $P<0.0001$.

3.8 Figures



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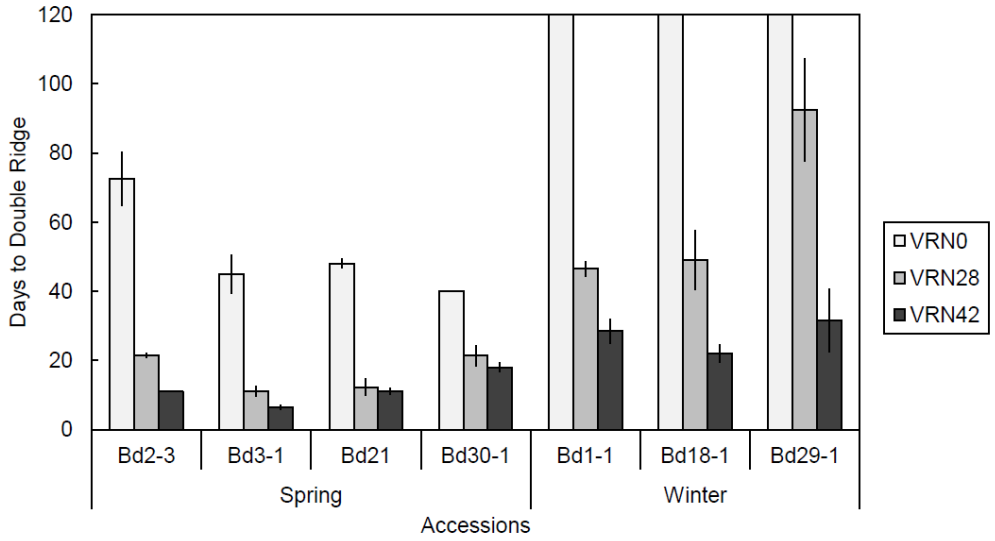

Figure 1. Vernalization accelerates the rate of phenological development in all accessions.

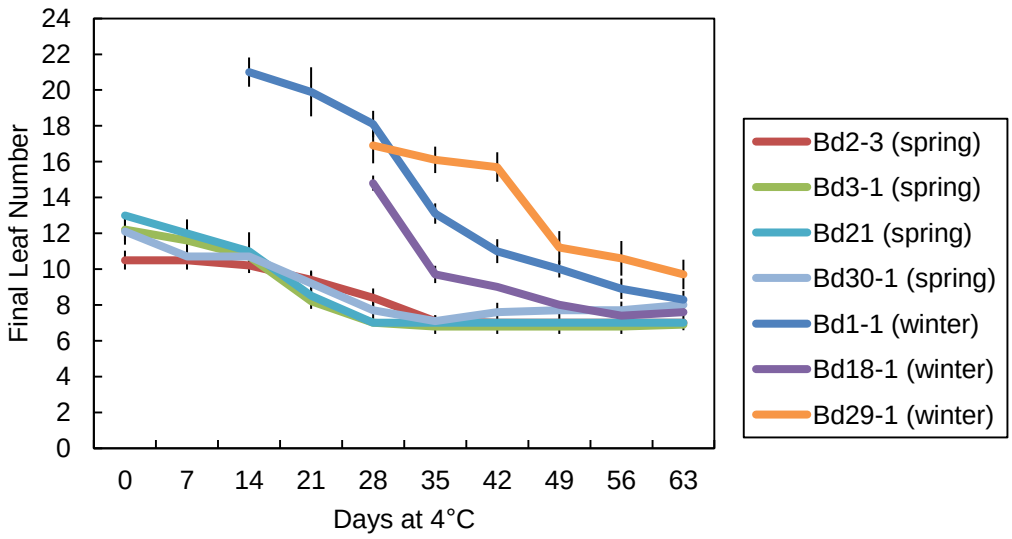

Figure 2. Vernalization reduced the final leaf number of all accessions.

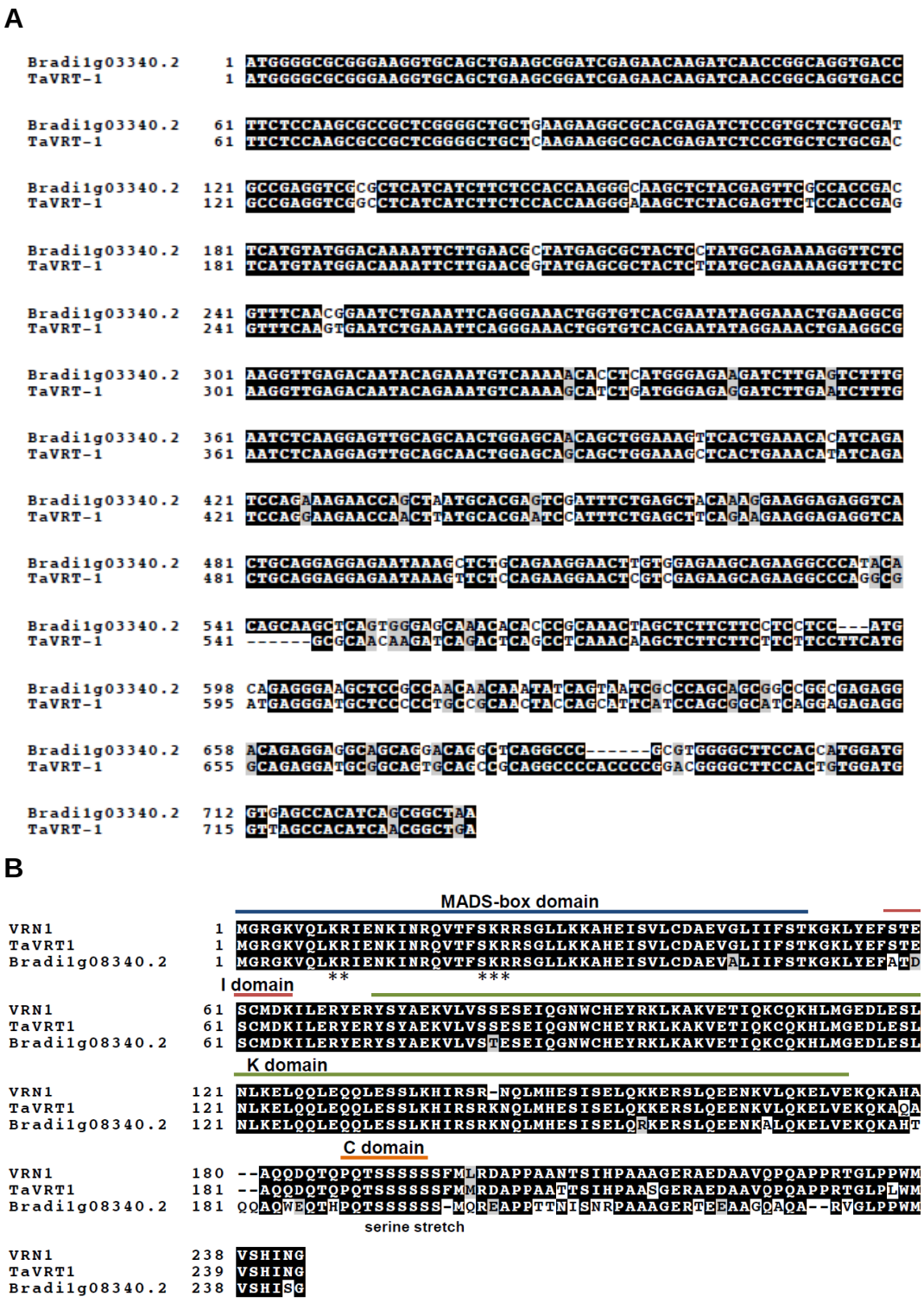

Figure 3. Sequence identity of *Bradi1g08340.2* coding sequence and Bradi1g08340.2 protein with related MADS-box gene and proteins from other species.

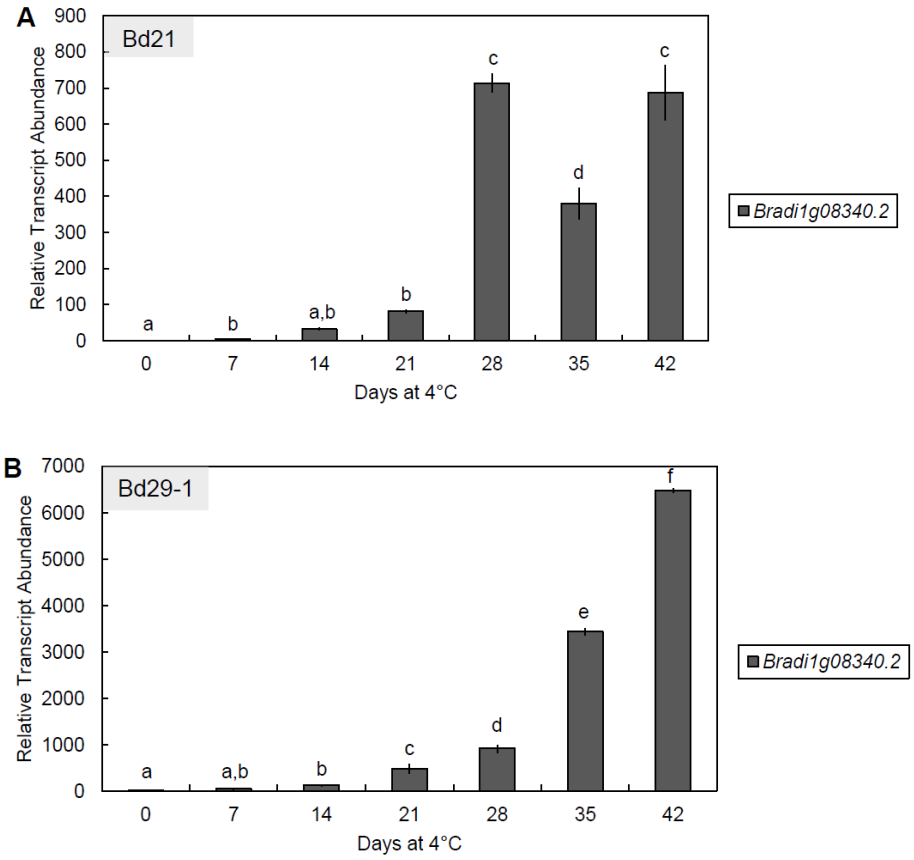

Figure 4. *Bradi1g08340.2* transcripts accumulate continuously and to higher levels in the *Brachypodium* winter accession Bd29-1 in response to vernalization.

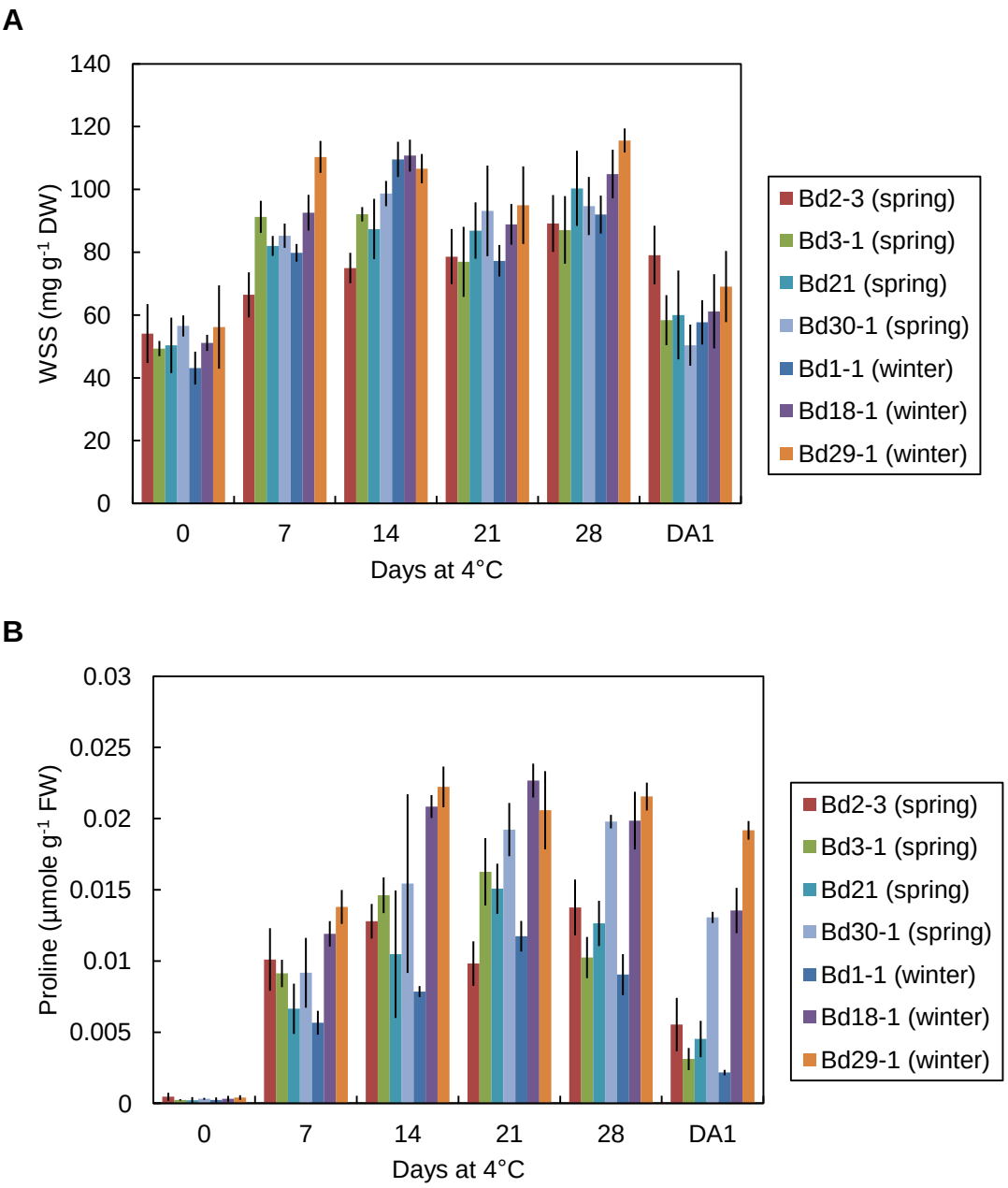

Figure 5. Soluble carbohydrate and proline osmoprotectants accumulate to high levels in all accessions in response to cold acclimation.

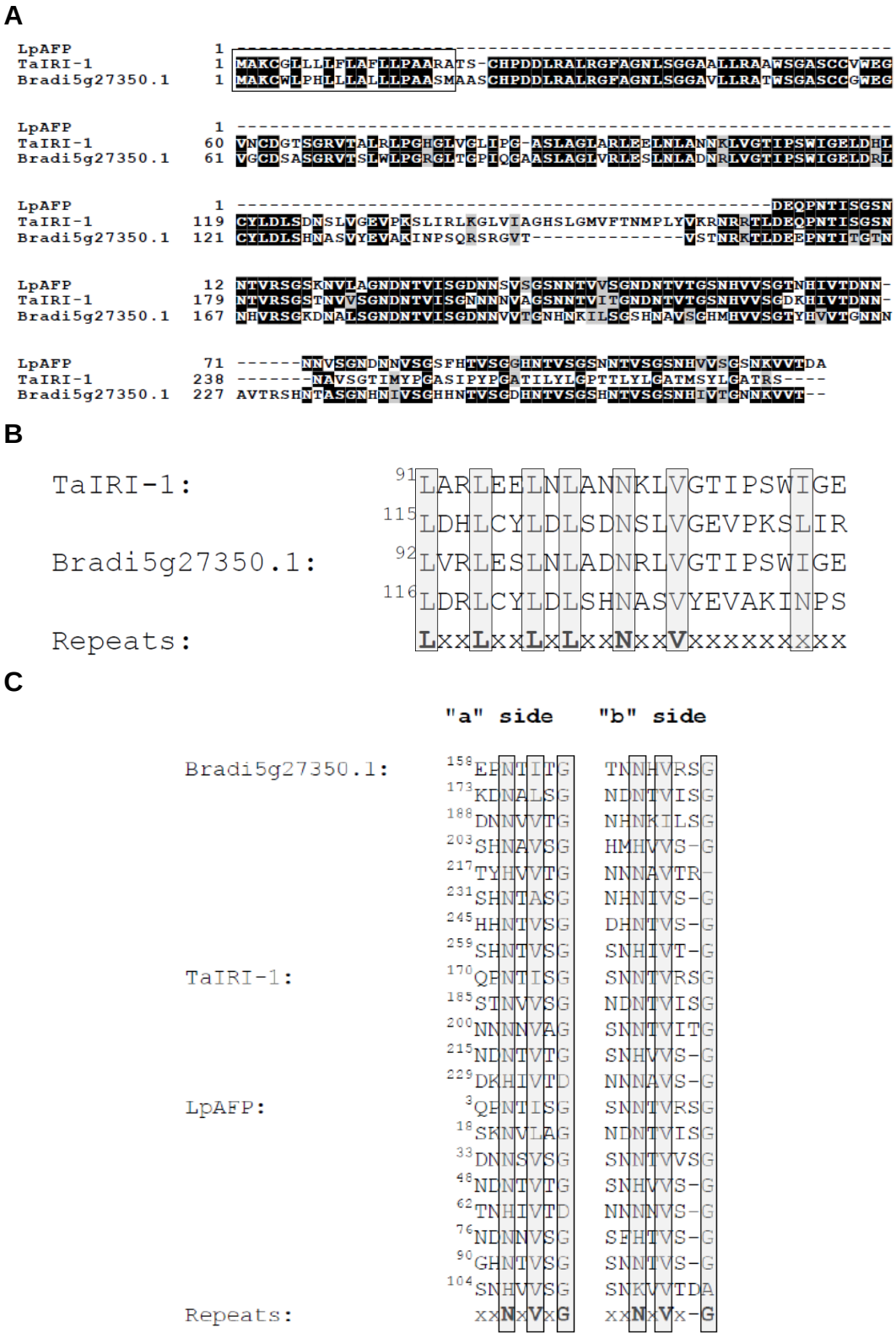

Figure 6. The *Brachypodium BradiIRI* gene encodes a bipartite protein.

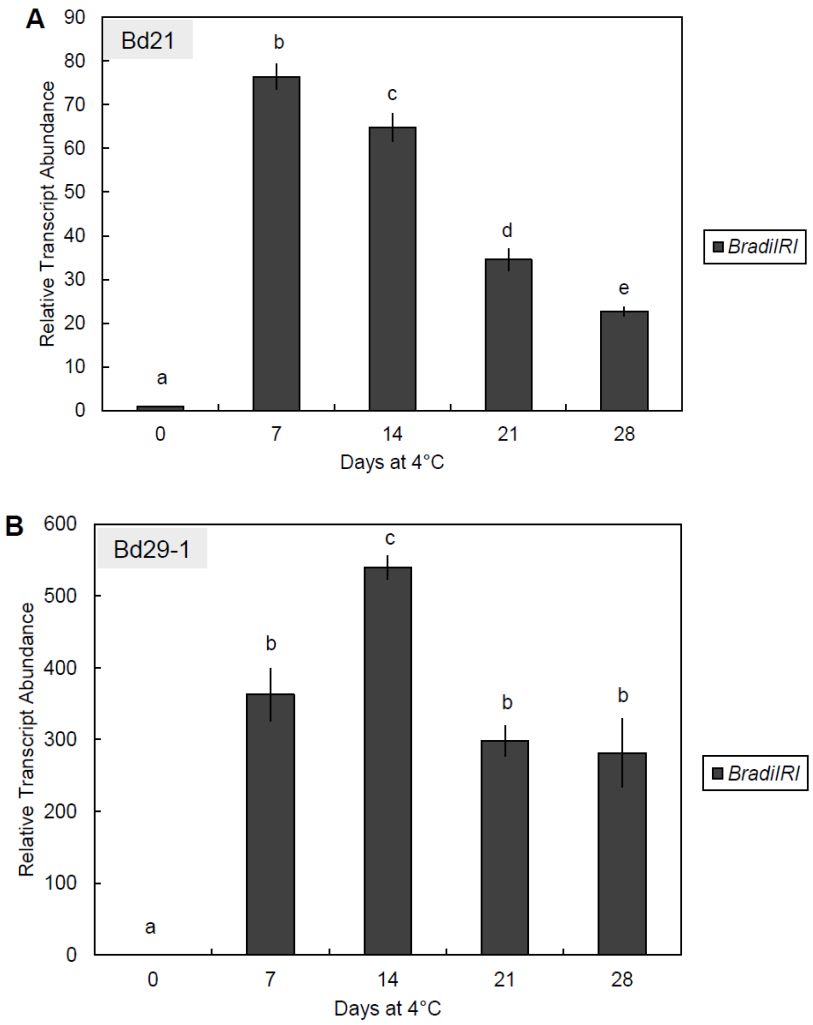

Figure 7. *BradiIRI* transcripts accumulate to higher levels in the *Brachypodium* winter accession Bd29-1 in response to cold acclimation.

Connecting Statement

Having first studied *Brachypodium*'s response to a low non-freezing temperature and validated this plant as a model system for studying freezing tolerance, we turn next to its transformability. The first part of this chapter is devoted to the identification and expression analysis of the lipocalin BradiTIL-2 in *Brachypodium*. This gene was selected based on previous reports showing that members of the lipocalin family enhance freezing tolerance in plants by protecting against oxidative stress. Just as other laboratories worldwide are now producing transgenic lines of *Brachypodium* for various genes of interest, the second part of this chapter describes the production of plants overexpressing *BradiTIL-2* by means of transformation of immature embryos. Overall, this chapter reports the first steps taken towards engineering freezing resistant crops.

I have contributed to all of the work pertaining to the following chapter which includes identification, alignment and expression analysis of *Bradi3g48590.1* (ortholog of the temperature-induced lipocalin-2) in *Brachypodium*, construction of the transformation vector Gateway® pMHb7Fm21GW-UBIL-*BradiTIL-2* containing the lipocalin-2 fused to GFP, and arrangements with the Boyce Thompson Institute for transformation of *Brachypodium* Bd21 accession with the newly created construct.

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Chapter 4

Transformation of *Brachypodium distachyon* Bd21 with the Temperature-Induced Lipocalin-2

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4.1 Abstract

Exposure to low non-freezing temperatures, a process known as cold acclimation, initiates freezing tolerance in winter cereals. During cold acclimation, several genes are up-regulated and code for proteins that protect plant cells against freezing damages. Temperature-induced lipocalins (TILs) are not typical COR proteins as they are believed to protect non-acclimated tissues against sudden drops in temperature. The increased freezing tolerance observed in non-acclimated plants overexpressing TILs is believed to be related to the reactive oxygen species (ROS) scavenging properties of lipocalins. On the other hand, they are expressed upon cold acclimation which is a characteristic that normally defines *COR* genes. The coding sequence of a temperature-induced lipocalin was found in *Brachypodium distachyon* (*Brachypodium*) by homology search using the *Arabidopsis* and *Triticum aestivum* lipocalins. Expression analysis has shown that *BradiTIL-2* is up-regulated during cold acclimation and accumulates to high levels after 28 and at least 42 days in Bd21 and Bd29-1 accessions, respectively. *BradiTIL-2* was cloned in the Gateway® destination vector pMHb7Fm21GW-UBIL and was introduced in compact embryogenic calli of the *Brachypodium* accession Bd21 via *Agrobacterium*-mediated transformation.

4.2 Introduction

Lipocalins are small ligand-binding proteins found in bacteria, invertebrate and vertebrate animals. In 2002, the first true lipocalins from plants were identified in wheat and *Arabidopsis* and named *Triticum aestivum* L. temperature-induced lipocalin (*Ta*TIL) and *Arabidopsis thaliana* temperature-induced lipocalin (*At*TIL), respectively (Frenette Charron et al., 2002). Lipocalins from plants are characterized by the three structurally conserved regions (SCRs) defining the lipocalin family and share homology with three closely related lipocalins: the human Apolipoprotein D, the *E. coli* outer membrane lipoprotein Blc precursor and the American grasshopper Lazarillo precursor (Frenette Charron et al., 2002). Expression studies have shown that cold acclimation induces the accumulation of *TaTIL* transcripts in both spring and winter wheat with a greater level of accumulation in the winter cultivars (Frenette Charron et al., 2002; Frenette Charron et al., 2005). Moreover, the level of TIL accumulation is correlated with oxidative stress and freezing tolerance in plants (Charron et al., 2008). The subcellular location of TILs is the plasma membrane (Frenette Charron et al., 2005). Experiments with a T-DNA knock-out *Arabidopsis* line for the *AtTIL* gene has shown that the plasma membrane-associated TIL enhances plant protection against freeze induced oxidative stress by binding and scavenging peroxidated lipids which helps the plasma membrane to recover and regain its integrity (Charron et al., 2008).

Plant transformation is used in many areas of plant science to overcome the limitations encountered with traditional plant breeding and can be used to develop cold tolerant crops (Sarhan and Danyluk, 1998). This technique consists in silencing, overexpressing or simply introducing genes of particular interest in plants. Indeed, *Arabidopsis* has been widely used to conduct research in cold tolerance particularly for functional genomics (Jaglo-Ottosen et al., 1998). Whole adult *Arabidopsis* plants can be easily transformed when submerged with an *Agrobacterium* inoculum (Bechtold et al., 1993). However, transformation of grasses cannot be done using whole-plant infiltration techniques. Instead,

transformation has shown to be efficient only when conducted on compact embryogenic calli (CEC) derived from immature embryos (Păcurar et al., 2008; Vain et al., 2008; Vogel and Hill, 2008; Vogel et al., 2006a). Recently, a protocol was established for *Agrobacterium*-mediated transformation of *Brachypodium* and has succeeded in generating transgenic lines of Bd21 with an efficiency of 86% (Alves et al., 2009).

Our previous work has shown that *Brachypodium* has the capacity to cold acclimate by enabling the molecular mechanisms responsible for the accumulation of sugars, proline and an antifreeze protein. Data mining in the genome of *Brachypodium* revealed the presence of two lipocalins (*BradiTIL-1* and *BradiTIL-2*) very similar to the wheat and *Arabidopsis* lipocalins. We chose here to characterize *BradiTIL-2* since a considerable amount of work is already available on *TaTIL-1* (Charron et al., 2008; Frenette Charron et al., 2002; Frenette Charron et al., 2005). Expression studies showed that *BradiTIL-2* accumulates during cold acclimation in both *Brachypodium* accessions Bd21 and Bd29-1. Transgenic lines of Bd21 overexpressing *BradiTIL-2* were generated to investigate the function of this gene in *Brachypodium*.

4.3 Materials and Methods

4.3.1 Primer Design and PCR of *BradiTIL-2*

Total RNA was isolated from cold acclimated plant tissues of the *Brachypodium* accession Bd21 using the RNeasy plant mini kit (Qiagen) and the samples were treated with DNase I (Qiagen). Purified RNA was reverse transcribed in a 20-µl reaction using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene Products Division, La Jolla, CA) according to the manufacturer's recommendations. The Primer3 program (http://frodo.wi.mit.edu/primer3) was used to design the primers BdTIL-72-F and BdTIL-73-R for amplification of *BradiTIL-2* (Table 2). The forward primer includes part of the 5' untranslated region of the gene, the start codon (ATG), and a few nucleotides of the coding region. The reverse primer was designed for amplification of the whole coding

sequence of the gene except for the stop codon (TGA) in order to allow continuous transcription of the gene and the green fluorescent protein (GFP). The amplicon (*BradiTIL-2* without a stop codon) was obtained with an initial denaturation at 95°C for 30 seconds and 30 repeated cycles of denaturation (95°C for 30 seconds), annealing (50°C for 30 seconds) and extension (68°C for 1 minute) and with a final extension at 68°C for 1 minute.

4.3.2 Expression Analysis of *BradiTIL-2* by Quantitative Real-Time PCR

4.3.2.1 RNA Isolation and cDNA Synthesis

Brachypodium accessions Bd21 (spring) and Bd29-1 (winter) were used in this assay and 10-day-old seedlings were harvested after 0 (non-acclimated control), 7, 14, 21, 28, 35 and 42 days at 4°C. Each sample consisted of three seedlings (approximately 100 mg of fresh aerial plant tissues) and three independent samples per accession per harvesting time point were used. Total RNA was isolated using the RNeasy plant mini kit (Qiagen) and the samples were treated with DNase I (Qiagen). Purified RNA was reverse transcribed in a 20-µl reaction using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene Products Division, La Jolla, CA) according to the manufacturer's recommendations. Parallel reactions were run for each RNA sample in the absence of AffinityScript RT (no RT control) to assess any genomic DNA contamination. The cDNA products were diluted in water to 400 ng μl^{-1} and stored at -20°C.

4.3.2.2 Design of Gene-Specific Primers

Primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/) and BLASTN search were conducted in order to assess primer specificity. BdTIL-168-F and BdTIL-169-R were designed for amplification of 100 to 200 bp fragments in the 3' portion of the gene of the temperature-induced lipocalin-2. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Primers used in this study are listed in Table 2 (primers BdTIL-168-F and BdTIL-169-R, 18S-117-F and 18S-118-R).

4.3.2.3 PCR Amplification

Quantitative real-time PCR assays were conducted in triplicates in a Mx3000 real-time thermal cycler (Agilent Technologies, Cedar Creek, TX) using 18S ribosomal RNA as the housekeeping gene with Brilliant III Ultra-Fast SYBR® Green QPCR master mix (Stratagene Products Division, La Jolla, CA). Amplification was performed in a 15 µl reaction containing 1x SYBR Green master mix, 350 nM each primer, 30 nM reference dye ROX and 2 µl cDNA template (one-ninth dilution). The PCR thermal-cycling parameters were 95°C for 2 min followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The experiment was repeated three times.

4.3.2.4 Data Analysis

Data was analyzed with the Mx-Pro QPCR software (Stratagene Products Division, La Jolla, CA). The relative expression ratios of the target gene versus the 18S ribosomal RNA reference gene were calculated for each LT treatment using the following algorithm (1) (Zhao and Fernald, 2005).

(1) R0=1/(1+E)^CT

4.3.3 Cloning Strategy and Recombinational Cloning

The *BradiTIL-2* coding sequence was first cloned in pCR®8/GW/TOPO® (Life Technologies, Grand Island, NY). The resulting construct was then digested with BsrGI (New England Biolabs, Pickering, ON) and the *BradiTIL-2* insert was subsequently cloned in the pENTR™ 1A entry vector (Life Technologies, Grand Island, NY). To enhance cloning efficiency, the 5' end phosphate groups of the digested pENTR™ 1A were removed with calf intestinal alkaline phosphatase (New England Biolabs, Pickering, ON). The two fragments (*BradiTIL-2* and pENTR™ 1A) were extracted from an agarose gel using the QIAquick Gel Extraction Kit according to the manufacturer's recommendations (Qiagen, 2008). The two fragments were ligated using ligase (New England Biolabs, Pickering, ON) at 4°C overnight after which the enzyme was inactivated at 65°C for 15

minutes. For transformation, 50 µl of frozen chemically competent *E. coli* cells (Mach1^{TM}-T1^{R}) were thawed on ice followed by addition of 6 µl of the ligation-reaction mixture, incubation on ice for 5 minutes, heating at 42° for 30 seconds, addition of 250 µl of SOC medium and plating on solid Luria-Bertani (LB) medium containing kanamycin (Qiagen, 2001). Ten individual colonies were selected and grown overnight in liquid LB containing kanamycin and plasmid DNA was isolated with the QIAprep Spin Miniprep Kit according to the manufacturer's recommendations (Qiagen, 2006b). The presence and correct orientation of the gene in the entry vector was determined by restriction analysis with enzymes BsrGI, SmaI and PstI (New England Biolabs, Pickering, ON) and positive clones were sent to Genome Québec for sequencing.

The recombinational cloning method was used to clone *BradiTIL-2* in the Gateway® destination vector. In this experiment, the LR recombination reaction was used to clone *BradiTIL-2* from the pENTR^{TM} 1A entry vector into the Gateway® destination vector pMHb7Fm21GW-UBIL. In order to do so, 50 ng of the entry clone (*att*L1-*BradiTIL-2*-*att*L2) and 150 ng of the destination vector (*att*R1-*ccdB*-*att*R2) were incubated together overnight with 1 µl of LR Clonase^{TM} II (Invitrogen Corporation, 2009) enzyme (Int + IHF + Xis) followed by the addition of 0.5 µl of proteinase K and incubation at 37°C for 10 minutes. The product was used to transform chemically competent *E. coli* (Mach1^{TM}-T1^{R}) according to the procedure outlined previously.

The pMBb7Fm21GW-UBIL destination vector (http://gateway.psb.ugent.be/), which transcriptionally fuses a GFP epitope tag onto target proteins in monocots, has a streptomycin-spectinomycin resistance gene (Sm/Sp^{R}) (100 mg L^{-1}) that allows selection of the plasmid in *E. coli* on LB medium containing either spectinomycin or streptomycin. The cells were thus plated on LB containing spectinomycin. As previously described, restriction analysis was done on plasmid DNA followed by PCR screening for *BradiTIL-2* and GFP. Two positive constructs were sent to Genome Québec for sequencing confirming that both constructs contained *BradiTIL-2* fused in frame with GFP.

4.3.4 *Agrobacterium* Transformation

Competent cells of *Agrobacterium* were transformed with pMHb7Fm21GW-UBIL-*BradiTIL-2* by electroporation. Two ng of plasmid DNA were added to 40 µl of electrocompetent cells and transferred in a prechilled cuvette and electroporation was done in a Multiporator/Eppendorf Eporator® with a voltage of 1440 V and a time constant (τ) of 5 ms. 400 µl of LB medium was added immediately after electroporation and the culture incubated for one hour at room temperature. The cells were plated on LB containing spectinomycin and carbenicillin (50 mg L^{-1}) for selection of the Ti plasmid (pTiBo542) in the AGL1 super-virulent strain of *Agrobacterium* (Hellens et al., 2000). A control empty vector that carries only the gene for GFP flanked by the ubiquitin promoter (pXHb7FNFI-UBIL) (Figure 8) was also used for plant transformation in order to optimize the transformation protocol and to determine if the vector alone confers certain advantages or disadvantages to the plant.

4.3.5 Production of Immature Embryos

Immature green seeds of Bd21 were harvested when pollination had just occurred. The lemma of the seed was removed and the half naked seed was then soaked in ethanol for 15 seconds, rinsed in sterile water for 15 seconds, and sterilized in 1.3% hypochlorite solution for 15 seconds. The embryo was dissected off by making an incision and opening the seed coat in a plate of sterile water. The embryo was then placed on a basic callus induction medium (MSB3+$CuSO_4$) according to Alves et al. (2009) for culture for 3 weeks at 25°C in the dark. To allow maximum cell generation, the side of the embryo touching the endosperm was placed on the medium while the scutellum was facing up.

4.3.6 Production of Compact Embryogenic Calli

Three fragmentations are necessary for the production of CEC. At week 3, CEC were fragmented in one to three pieces and transferred on new basic MSB3+$CuSO_4$ solid medium for another two weeks at 25°C in the dark. At week

5, CEC were fragmented again in 4 to 6 pieces and transferred on new basic MSB3+$CuSO_4$ solid medium for another week at 25°C in the dark. At week 6, CEC were fragmented one last time in four to six pieces and transferred on new basic MSB3+$CuSO_4$ solid medium prior to transformation with *Agrobacterium*.

4.3.7 *Agrobacterium*-Mediated Transformation of CEC

In order to prepare the *Agrobacterium* inoculum, 1 ml of LB liquid medium containing 100 mg L^{-1} of spectinomycin was inoculated with 5 µl of the stock of transformed bacteria stored at -80°C. The culture was grown overnight in an incubator-shaker at 28°C and 200 rpm. 200 µl of the culture was then plated on LB containing 100 mg L^{-1} of spectinomycin and 30 mg L^{-1} of acetosyringone which increases transformation efficiency (Sheikholeslam and Weeks, 1987). The plates were cultured for two days at 28°C. Half of the plates were scraped and used to inoculate 10 ml of liquid MSB medium containing 45 mg L^{-1} of acetosyringone according to Alves et al. (2009). The culture was incubated for 45 minutes in a 28°C incubator-shaker at 200 rpm until the OD_{600} was equal to 1. 13 ml of this suspension was used to flood the CEC plates in a laminar flow hood. The plates were left in the hood for five minutes to allow infection after which the suspension was completely removed from the plates. CEC were put on a dry sterile filter paper and left uncovered in the hood for a seven minutes dessication treatment. CEC were then transferred on MSB3 containing 60 mg L^{-1} of acetosyringone and cocultured with the bacteria for two days at 25°C in the dark. Cocultured CEC were transferred onto MSB3+Cu0.6 containing 40 mg L^{-1} of hygromycin and 225 mg L^{-1} of timentin for three weeks at 25°C in the dark. Hygromycin is used to select for transformed CEC and both hygromycin and timentin help to limit the growth of *Agrobacterium*.

4.3.8 Selection of Transformed Calli

CEC were screened three weeks after transformation for the presence of small bright GFP sectors using a stereomicroscope with a UV light source. The fluorescent sectors were dissected and grown for another three weeks at 25°C in

the dark as independent transgenic lines on MSB3 solid medium containing $CuSO_4$ (0.6 mg L^{-1}), hygromycin (30 mg L^{-1}) and timentin (225 mg L^{-1}).

4.3.9 Regeneration of Transgenic Plants

After six weeks of culture, calli were screened again for GFP positive segments and the positive calli were transferred on MSR26 solid regeneration medium according to Alves et al. (2009) containing hygromycin (20 mg L^{-1}) and timentin (225 mg L^{-1}) for two weeks at 25°C under a 16 hour photoperiod. After two weeks, calli began to form shoots. These shoots (rooted or not) were transferred in tubes containing MSR63 solid germination medium according to Alves et al. (2009) supplemented with charcoal (7 g L^{-1}) and timentin (112 mg L^{-1}) and were cultured for two to three weeks at 25°C under a 16 hour photoperiod. After 2 to 3 weeks, the plantlets were screened a last time for green fluorescence and only positive plantlets were transferred to soil for seed production.

4.3.10 Assessment of Transformation Efficiency

Several methods were used to assess transformation efficiency. First, only fluorescent calli sectors seen under the stereomicroscope were used to regenerate plantlets. Also, the plantlets were examined under UV to detect the presence of GFP in the nodes and roots. Second, once the plantlets were regenerated, some leaves were use to extract DNA by using the DNeasy Plant Mini Kit following the manufacturer's recommendations (Qiagen, 2006a). The Primer3 software was used to design GFP-79-F and GFP-80-R primers for a 380 bp fragment of the GFP encoding gene (Table 2). The amplicon was obtained with an initial denaturation at 95°C for 5 minutes and 30 repeated cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds) and extension (68°C for 45 seconds) and with a final extension at 68°C for 5 minutes.

4.4 Results

4.4.1 Identification and Molecular Characterization of *BradiTIL-2*

Several monocotyledonous species have been shown to contain genes encoding two TIL lipocalins, *TIL-1* and *TIL-2* (Frenette Charron et al., 2005). Using the *Triticum aestivum* temperature-induced lipocalin sequences (*TaTIL-1* and *TaTIL-2*, GenBank acc. no. AY077702 and DQ222977) as query, two genes were found to be highly similar to the temperature-induced lipocalin sequences: *Bradi3g36970.1* (*BradiTIL-1*) and *Bradi3g48590.1* (*BradiTIL-2*). At the protein level, BradiTIL-1 and BradiTIL-2 share 67% identity and 79% similarity together. The length of the gene is 856 bp and the open reading frame of the mRNA is 582 bp. Similarly to TaTIL-2 which comprises 190 amino acids, BradiTIL-2 is made of 181 amino acids. Search in GenBank database revealed high homology (>83% identity and >89% similarity) with TIL-2 lipocalins from wheat, barley, sorghum, maize and rice. Secondary sequence analysis revealed that the N-terminal portion of the *Brachypodium* TIL lipocalins possesses the three lipocalin SCRs related to features of the β-barrel which are also found in wheat and *Arabidopsis* lipocalins (Frenette Charron et al., 2002) (Figure 9).

4.4.2 *BradiTIL-2* Expression and the Cold Acclimation Response

Compared to TIL-1, very little information is available about the cellular function of TIL-2 in monocots. In an effort to gain more knowledge on lipocalins, the expression level of the putative *BradiTIL-2* was determined by qRT PCR with the spring/facultative accession Bd21 and the winter accession Bd29-1. Expression analysis indicated that the accumulation of *BradiTIL-2* transcripts in cold exposed plants is genotype-dependent with significantly more relative transcript abundance in Bd29-1 than in Bd21 except at the 28 days mark where BradiTIL-2 accumulated to higher levels in Bd21 (Figure 10). Spring/facultative habit *Brachypodium* seedlings cold acclimated for up to 21 days did not show differential expression of *BradiTIL-2* when compared to non-acclimated plants. After 28 days of cold treatment, *BradiTIL-2* transcript level tripled compared to

control plants and the expression level remained higher than the non-acclimated control after 35 and 42 days of cold treatment. In contrast, winter habit *Brachypodium* cold acclimated seedlings showed a three-fold increase in *BradiTIL-2* expression after only 7 days of cold treatment. This increase in *BradiTIL-2* transcript levels remained constant after 14 and 21 days of cold treatment after which increased up to seven fold after 42 days of cold treatment. Those results are consistent with earlier expression studies in which *TaTIL-2* accumulated with cold acclimation in both spring and winter wheat with greater transcript accumulation levels observed in winter wheat cultivars (Frenette Charron et al., 2005). These observations indicate the possible association between *BradiTIL-2* expression and the cold tolerance response in *Brachypodium* and may suggest that accession Bd29-1 is more tolerant to freezing temperatures than Bd21.

4.4.3 Generation of pMHb7Fm21GW-UBIL-*BradiTIL-2*

In order to overexpress *BradiTIL-2* in *Brachypodium*, *BradiTIL-2* was cloned into the Gateway® destination vector pMHb7Fm21GW-UBIL which fuses *BradiTIL-2* to *GFP* (Figure 11A). Table 3 summarizes the important features present on the newly generated construct as well as their physical location. Prior to transformation in compact embryogenic calli, this construct was verified by sequencing the *BradiTIL-2-GFP* open reading frame nucleotide sequence. Sequencing revealed that *GFP* was indeed in frame with *BradiTIL-2* (Figure 11B).

4.4.4 Genetic Transformation

Close to two hundred CEC were generated in this experiment and most of them had a healthy creamy appearance, pearly surface and were hard to the touch. CEC that had a translucent appearance and that were soft to the touch were not used for transformation as their transformation efficiency is generally low (Alves et al., 2009). Many CEC were incubated with *Agrobacterium* transformed with either the control or the construct vector and showed a healthy appearance after spending three weeks on MSB3+Cu0.6 culture medium containing hygromycin.

Other CEC showing browning after inoculation with *Agrobacterium* were discarded. When CEC were screened for GFP fluorescence under an inappropriate stereomicroscope, it was extremely difficult to differentiate between transformed and untransformed callus. Therefore, we relied solely on the hygromycin resistance rather than on the GFP fluorescence at this stage. The healthy looking sectors that were in direct contact with the medium containing hygromycin were used to regenerate plantlets. However, when the DNA of those plants was tested by PCR with the *GFP* primers, no amplicon was obtained (Figure 12). After obtaining this result, we decided to stop the transformation of immature embryos in the laboratory until a high-performance stereomicroscope would be delivered. The transformation was outsourced to the Plant Transformation Laboratory of the Boyce Thompson Institute to complete this project.

4.5 Discussion

4.5.1 Cloning the Temperature-Induced Lipocalin-2

The present study aimed at enhancing freezing tolerance in *Brachypodium* by overexpressing the temperature-induced lipocalin-2. The results clearly demonstrated the recent common origin of wheat and *Brachypodium* and the complexity of the technique of monocot transformation. The temperature-induced lipocalins-2 of *Brachypodium* and wheat share 89% identity and 93% similarity. The divergence time between *Brachypodium* and wheat, defined using orthologous gene pairs, was evaluated as 32 to 39 Myr ago and wheat was found to be the closest ancestor of *Brachypodium* among rice and sorghum (Vogel et al., 2010). Many studies have shown that there is more colinearity between the genomes of *Brachypodium* and wheat than wheat and rice (Bossolini et al., 2007; Draper et al., 2001; Huo et al., 2008; Huo et al., 2009; Vogel et al., 2006b). Species of the subfamily Pooideae which includes *Brachypodium*, wheat and barley, have evolved freezing tolerance after radiation from rice 40 to 54 Myr ago (Sandve et al., 2008; Vogel et al., 2010). Analysis of the evolution of gene families pertaining to stress response such as the MADS box gene family can be used to trace back their initial creation Myr ago. As an example, the main

expansion of the IRI-gene family (comprising the well-studied antifreeze proteins) is expected to have occurred ~36 Myr ago (Sandve et al., 2008). Since *Brachypodium* diverged from wheat fairly recently compared to other grasses, the most ancient adaptive features for cold tolerance may well be present in *Brachypodium* whereas most recent mechanisms may be absent.

BradiTIL proteins share similarity with the wheat lipocalins and three evolutionarily related lipocalins, ApoD, Blc, and Lazarillo (data not shown) (Frenette Charron et al., 2002). The wheat lipocalins are known to accumulate with a cold acclimation treatment (15 times for *TaTIL-1* and close to 3 times for *TaTIL-2* after 56 days at 4°C) and the increase in expression was shown to be greater in winter wheat (Frenette Charron et al., 2005). The fact that *BradiTIL-2* accumulated continuously and significantly until 42 days of cold acclimation in the *Brachypodium* winter accession Bd29-1 indicates that this gene does not behave as a regular *COR* gene which would start to be down-regulated before floral transition. Lipocalins are implicated in many important and different functions (Akerstrom et al., 2000; Bishop et al., 1995; Frenette Charron et al., 2002) and hence *BradiTIL-2* may have other functions than environmental stress response in *Brachypodium*. There is also a positive correlation between the level of expression of lipocalins and the capacity to tolerate freezing temperatures in *Arabidopsis* (Charron et al., 2008). Similar results were observed in *Brachypodium* in this study. *BradiTIL-2* was expressed upon low-temperature exposure in both the spring and winter accessions and to greater levels in the winter accession. BradiTILs share high similarity at the amino acid level to wheat and *Arabidopsis* TILs with the three SCRs and they accumulate similarly with cold exposure. We can thus expect that *Brachypodium* lipocalins have the same protective features against oxidative stress caused by freezing temperatures as it was demonstrated in *Arabidopsis* (Charron et al., 2008). ScanProsite predicts a putative glycosylation site between amino acids 55-58 for BradiTIL-1 and 63-68 for BradiTIL-2 which is consistent with the fact that all TIL homologs show a conserved N-glycosylation site (Frenette Charron et al., 2005; Sigrist et al., 2010).

4.5.2 *Agrobacterium*-Mediated Transformation of *Brachypodium distachyon*

Cereal crops account for two-thirds of the world's food supply (Borlaug, 2002). With an ever increasing world population, it is needless to say that development of high yielding cereals is indispensable to fulfill food demand. Conventional breeding techniques have only contributed to small enhancement in stress tolerance and yield. Biolistics (microprojectile bombardment) and *Agrobacterium*-mediated transformation are two techniques that have been successfully used to transform monocots and that offer a faster and more targeted production of tolerant and yielding cereals (Jones, 2005). *Arabidopsis* transformation is conducted through floral dip into an *Agrobacterium* solution and this technique produces thousands of transformants within the time required to complete one natural life cycle of the plant (Clough and Bent, 1998). On the opposite, monocot transformation is known to be a challenging, time-consuming and labour-intensive technique with efficiencies ranging between 0.3 and 28.6% in rice, maize, wheat and barley (Cheng et al., 2004; Shrawat and Lörz, 2006). In this study, we were following a protocol elaborated for *Agrobacterium*-mediated transformation of *Brachypodium* that suggests an efficiency of 86% (number of independent transgenic plants/number of target callus in the top three experiments) within a time process from wild-type seeds to transgenic T_1 seeds of 8 months (Alves et al., 2009). In this study, enthusiastic and long-term efforts have been put into the transformation of *Brachypodium* with the available Gateway® destination vectors without successful generation of transgenic plants.

Many reasons can explain why transformation was unsuccessful in this study. Transformation of cereals is a challenging technique that only a few laboratories worldwide master. The stereomicroscope that was used to screen CEC could not be operated in complete darkness which made it difficult to precisely differentiate between non-transformed and transformed calli. Also, no positive controls for GFP fluorescence were available at the time of the transformation. Therefore, the identification of true positives was extremely difficult and could have led to the selection of false positives. Later on, the

laboratory received a stereomicroscope that was installed in a dark room. Moreover, seeds from transgenic lines of Bd21 were ordered from the laboratory of Dr. Philippe Vain (Department of Crop Genetics, John Innes Center, Norwich Research Park, Colney, Norwich, UK) and the plants were used as positive controls for GFP fluorescence. The concentration of hygromycin may not have been high enough to allow a strict selection between transformed and non-transformed calli. Indeed, seeds of our regenerated plants did not show any delay in growth compared with the transgenic lines obtained from Dr. Vain when grown on a hygromycin selective media. We also bought a new strain of *Agrobacterium* AGL1 from the reputable stock center ATCC (American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110, USA, distributed to Canada by Cedarlane) in order to eliminate the bacteria as a potential source of error.

Our decision to stop the initial *Brachypodium* transformation efforts in our laboratory has been motivated by the fact that the Boyce Thompson Institute for Plant Research (Joyce Van Eck, Tower Road, Ithaca, New York, 14853-18.1, USA) has launched a service for *Brachypodium* transformation. This organization transforms *Brachypodium* for an extremely competitive price considering the time and materials that are required for the whole transformation protocol in the laboratory and a rapid turnover. Indeed, the service guarantees production of ten independent transformant lines in less than four months which is barely attainable outside such a facility. The Institute uses a similar procedure that was outlined in section 4.2 (Materials and Methods) for plant transformation. Therefore, we sent the two constructs (the Gateway® destination vector pMHb7Fm21GW-UBIL-*BradiTIL-2* and control vector pXHb7FNFI-UBIL) to the Institute. The transformation process was started on November 11th 2011. As of March 28 2012, thirteen plants potentially overexpressing *BradiTIL-2*, eight plants transformed with the control vector, and five non-transformed regenerated plants (controls) have been received. Further work will involve screening the transformants by conducting a PCR with the *GFP* primers to confirm transformation efficiency. Once a sufficient amount of seeds will be collected from those plants, the mutants will be tested for freezing tolerance using whole plant freezing assays. We expect

the mutants to have a greater freezing tolerance than the wild-type Bd21 since they are overexpressing *BradiTIL-2*.

Over the course of this study, we benefited from the numerous advantages offered by the new model system *Brachypodium distachyon*. Indeed, navigation in the genome of *Brachypodium* for isolating the temperature-induced lipocalin-2 has been achieved with the least efforts considering we used query sequences from wheat and *Arabidopsis.* Moreover, the high quality of the genome sequence allowed us to amplify and analyse the expression of this gene. In addition, this study demonstrates the good response of *Brachypodium* to tissue culture for production of compact embryogenic calli and raises hope that successful plant transformation will undoubtedly be routine in the near future of this laboratory especially with the new equipment acquired during the course of this work.

4.6 Tables

Table 2. List of primers used in this study. BdTIL-72-F and BdTIL-73-R were designed for amplification of the temperature-induced lipocalin-2 without the stop codon in *Brachypodium* for cloning in pMHb7Fm21GW-UBIL. GFP-79-F and GFP-80-R were designed for amplification of a 380 bp fragment of the GFP encoding gene in the Gateway vectors pMHb7Fm21GW-UBIL and pXHb7FNFI-UBIL. BdTIL-168-F and BdTIL-169-R were designed for amplification of a 164 bp fragment of the temperature-induced lipocalin-2 in *Brachypodium* for qRT-PCR. 18S-117-F and 18S-118-R were designed for amplification of a 131 bp fragment of the housekeeping gene 18S ribosomal RNA in *Brachypodium* for qRT-PCR.

Primer Name	**Primer Sequence (5' to 3')**	**Amplicon Size (bp)**	**Primer Tm (°C)**
BdTIL-72-F	5'-CGAGTCAGACACAAATTACAAATGG-3'	600	54.5
BdTIL-73-R	5'-TTTACCAAAGAGCGACTTGAACCAC-3'		57.6
GFP-79-F	5'-TTCACCTTGATGCCGTTCTTCTGCTT-3'	380	60.8
GFP-80-R	5'-ACCTACGGCAAGCTGACCCTGAAGTT-3'		63.7
BdTIL-168-F	5'-AGCTCAAGGTCAAGTTCTACGTG-3'	164	57.7
BdTIL-169-R	5'-CTGCACAGAATCCAGAGGTTTT-3'		54.6
18S-117-F	5'-GAAGTTTGAGGCAATAACAGGTCT-3'	131	55.3
18S-118-R	5'-ATCACGATGAATTTCCCAAGATTAC-3'		53.5

Table 3. Features of the pMHb7Fm21GW-UBIL-*BradTIL-2* binary vector.

Feature	**Description**	**Position in the vector (base pairs)**
RB	Signal	77..276
Sm/SpR	Streptomycin/Spectinomycin antibiotic resistance (complement)	5180..6429
LB	Signal	6435..6767
Tnos	Nopaline synthase polyadenylation sequence Genus: *Agrobacterium* Species: *Agrobacterium tumefaciens*	6799..7066
Hyg	Hygromycin resistance gene (complement) Genus: *Escherichia* Species: *Escherichia coli*	7072..8262
p35S	Promoter (complement) Genus: *Caulimovirus* Species: Cauliflower mosaic virus	8268..9295
T35S	Terminator Genus: *Caulimovirus* Species: Cauliflower mosaic virus	9339..9564
attB3	Recombinational cloning site	9646..9666
GFP	Green fluorescent protein (complement) Genus: *Aequorea* Species: *Aequorea victoria*.	9668..10384
Junction	Junction between BdTIL and GFP (complement)	10385..10423
BdTIL	*Brachypodium distachyon* temperature-induced lipocalin (complement)	10424..11002
UBIL	Ubiquitin promoter (complement) Genus: *Zea* Species: *Zea mays*	11003..12988
attB4	Recombinational cloning site (complement)	12990..13

4.7 Figure Legends

Figure 8. The Gateway® control vector pXHb7FNFI-UBIL that carries the green fluorescent protein (*GFP*) gene flanked by the ubiquitin promoter.

Figure 9. Alignment and structure of the *Brachypodium* temperature-induced lipocalins with related lipocalins. Protein alignment of *Brachypodium* TIL-1 and TIL-2 with *Triticum aestivum* TIL-1 and TIL-2 and *Arabidopsis* TIL. Identical residues are in black and similar residues are in gray. The three structurally conserved regions (SCRs) that provide a signature for the lipocalins are indicated above. Red arrows, blue rectangles and green rectangle represent β-strands, α-helix, and 3_{10} α-helix, respectively.

Figure 10. *BradiTIL-2* accumulates continuously and to higher levels in the *Brachypodium* winter accession Bd29-1 in response to cold acclimation. Transcripts level of *BradiTIL-2* in both spring Bd21 and winter Bd29-1 *Brachypodium* under cold acclimation. *Brachypodium* plants were grown for 10 days at 20°C under a long day (16 h) photoperiod, transferred to 4°C under a short day (8 h) photoperiod, and then sampled at regular intervals (0 days represent non-acclimated controls). qRT-PCR were done using total reverse-transcribed RNA isolated from aerial parts. Data shown represent mean values obtained from independent amplification reactions (n=3) and the vertical bars indicate standard deviation of the mean. The experiment was repeated three times with similar results. * represent statistical difference at $P<0.0001$ in the transcript mean between the two accessions at a certain acclimation period. Means with different letters are statistically significant at $P<0.0001$. Dark letters represent significance for Bd21 and gray letters represent significance for Bd29-1.

Figure 11. Map of the pMHb7Fm21GW-UBIL-*BradiTIL-2* plasmid and the fusion of *BradiTIL-2* with *GFP*. A) Map of the binary vector pMHb7Fm21GW-UBIL-*BradiTIL-2* containing a temperature-induced lipocalin (*TIL*) of *Brachypodium* illustrated using the BioEdit software. The numbers inside the vector represent the base pairs. B) *BradiTIL-2* was cloned in frame with *GFP* in

the Gateway® destination vector pMHb7Fm21GW-UBIL. The *BradiTIL-2* ORF with the ATG start codon and no stop codon is in plain text. Highlighted in yellow is the junction between *BradiTIL-2* and *GFP*. Highlighted in green is the N-terminal portion of *GFP*.

Figure 12. PCR analysis confirming that the regenerated plants growing on hygromycin are false positives. A) Gel picture for PCR analysis of the regenerated plants with the GFP primers. M indicates the DNA ladder (100 bp). Lanes 1 and 2 indicate wild-type *Brachypodium* accession Bd21. Lanes 3 to 5 indicate three distinct regenerated plants. Lanes 6 and 7 indicate positive controls which consist of the vectors pMHb7Fm21GW-UBIL and pXHb7FNFI-UBIL, respectively. Lane 8 indicates a negative control with water. Lanes 9 to 13 indicate the quality of the DNA of two wild-type plants and of the three regenerated plants, respectively.

4.8 Figures

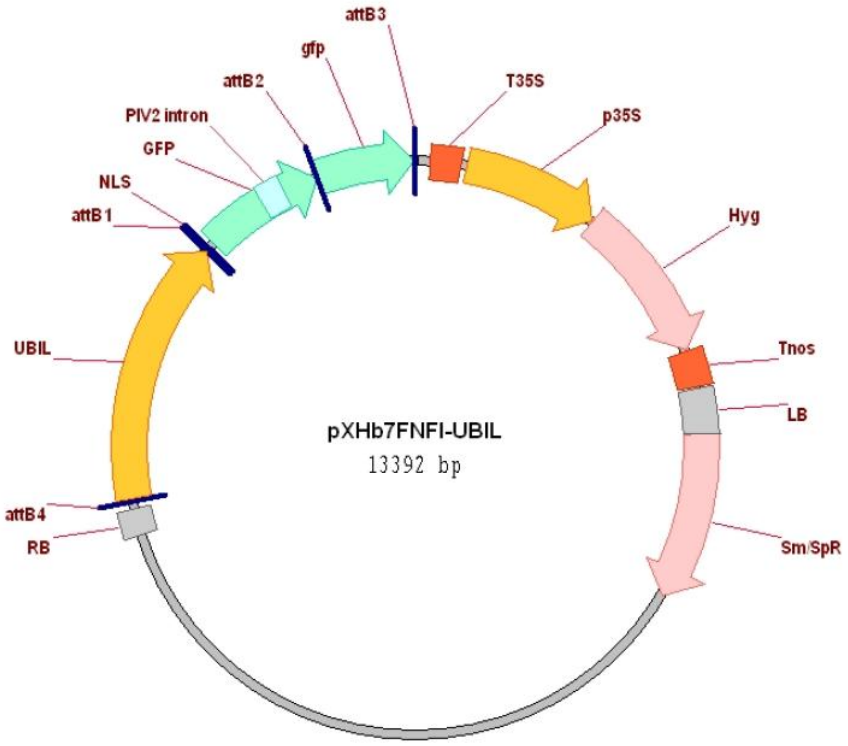


Figure 8. The Gateway® control vector pXHb7FNFI-UBIL that carries the green fluorescent protein (*GFP*) gene flanked by the ubiquitin promoter.

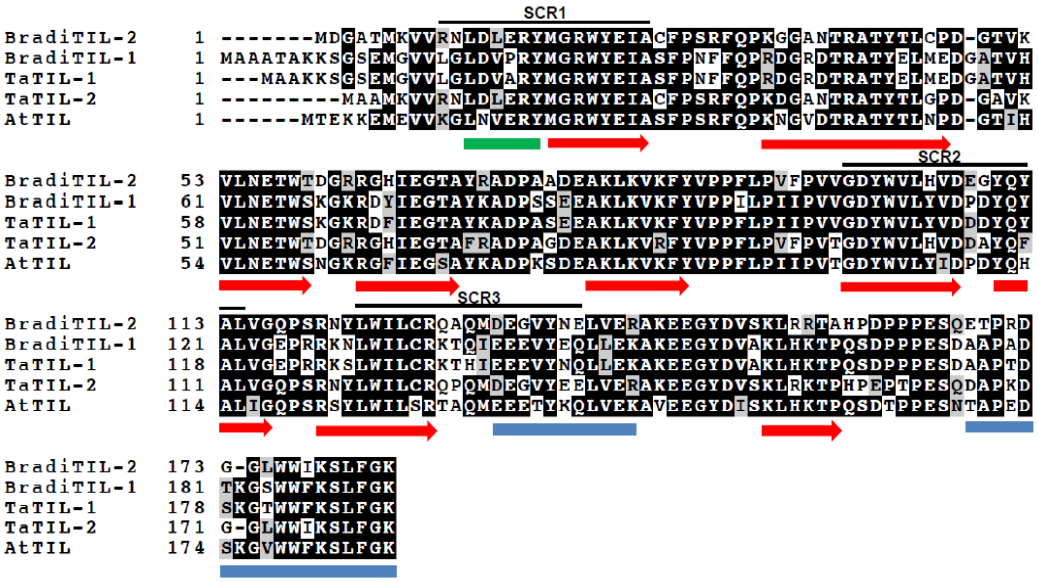


Figure 9. Alignment and structure of the *Brachypodium* temperature-induced lipocalins with related lipocalins.

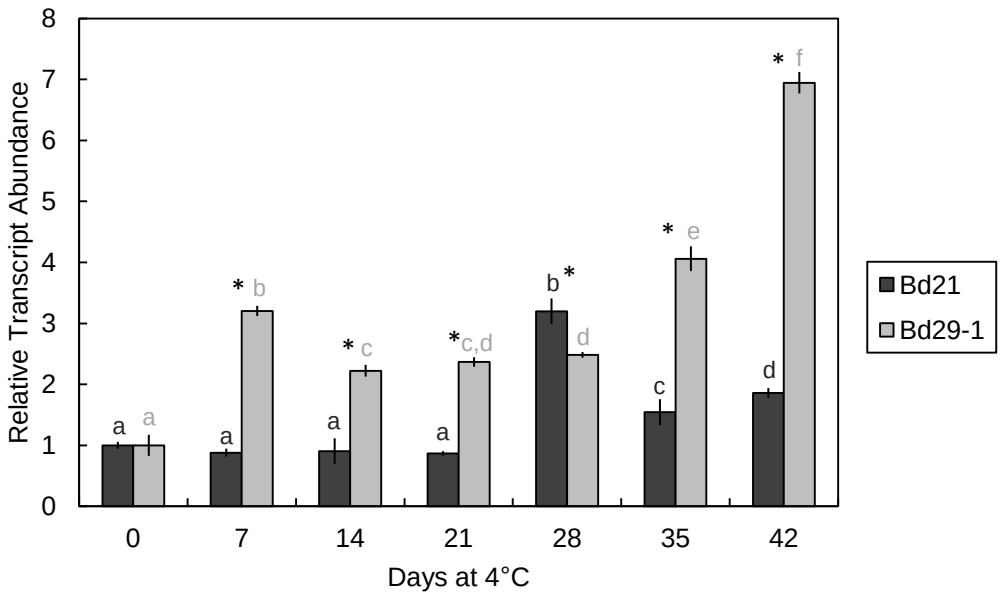
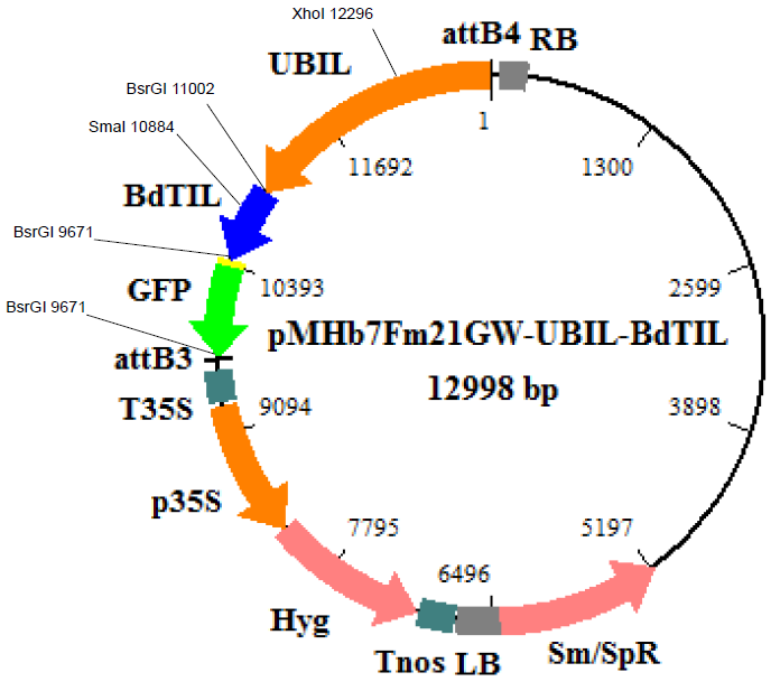


Figure 10. *BradiTIL-2* accumulates continuously and to higher levels in the *Brachypodium* winter accession Bd29-1 in response to cold acclimation.

A



B

ATG GCA GCT GCG ACG GCG AAG AAG AGC GGG AGC GAG ATG_GGG GTG GTT CTT GGT CTG
GAC GTG CCG CGG TAC ATG GGC CGC TGG TAC GAG ATC GCG TCG TTC CCA AAC TTC TTC
CAG CCC CGG GAC GGG CGG GAC ACG CGC GCC ACG TAC GAG CTC ATG GAG GAC GGC GCC
ACG GTG CAC GTG CTC AAC GAG ACC TGG AGC AAA GGG AAG CGC GAC TAC ATC GAG GGC
ACC GCC TAC AAG GCC AAC CCG TCC AGC GAG GAG GCC AAG CCC AAG GTC AAG TTC TAC
GTG CCC CCC ATC CTC CCC ATC ATC CCC GTC GTC GGC GAC TAC TGG GTC CTC TAC GTC
GAC CCC GAC TAC TAG TAC GCC CTC GTC GGC GAG CCC CGC CGC AAA AAC CTC TGG ATT
CTG TGC AGG AAG ACG CAG ATC GAG GAG GAG GTG TAC GAG CAG CTG CTG GAG AAG GCC
AAG GAG GAA GGC TAC GAC GTG GCC AAG CTG CAC AAG ACG CCG CAG AGC GAC CCG CCG
CCG GAG AGC GAC GCC GCG CCC GCC GAC ACC AAA GGG TCC TGG TGG TTC AAG TCG CTC
TTT GGT AAA AAG GGC GAA TTC GAC CCA GCT TTC TTG TAC AAA GTG GGA **ATG** GTG AGC
AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG GTC GAG CTG GAC GGC GAC
GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT GCC ACC TAC GGC
AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC TGG CCC ACC
CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC GAC CAC ATG
AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CC

Figure 11. Map of the pMHb7Fm21GW-UBIL-*BradiTIL-2* plasmid and the fusion of *BradiTIL-2* with *GFP*.

A

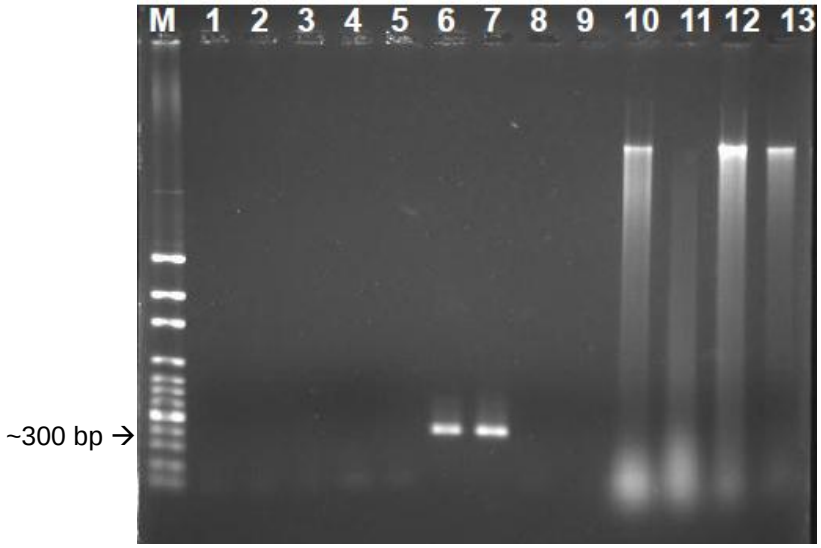


Figure 12. PCR analysis confirming that the regenerated plants growing on hygromycin are false positives.

Chapter 5 General Discussion, Conclusions, Futures Studies and Contribution to Knowledge

5.1 General Discussion and Conclusions

Brachypodium distachyon is a temperate wild grass species that has been proposed by the research community as the new model system for grasses because of its desirable qualities such as small stature, self-fertility and rapid generation time. Remarkable efforts have been put into sequencing the complete genome of this plant and developing an efficient transformation protocol. Results are obvious: the 272 Mb genome of *Brachypodium* is collinear to a large extent with the wheat genome, diploid ecotypes are available, and the plant is readily transformable through *Agrobacterium*-mediated transformation and thousands of T-DNA insertion lines are available from different sources. There is no doubt that *Brachypodium* possesses all the biological attributes of a model system, but whether it is a valuable model to understand how temperate cereals protect themselves against harsh winter conditions still had to be determined.

The first part of this work has shown that the spring and winter accessions of *Brachypodium* behave similarly as spring and winter cultivars of economically important cereals such as wheat and barley. *Brachypodium* winter accessions essentially require a period of vernalization in order to switch to the reproductive phase whereas spring/facultative accessions switch to the reproductive phase naturally without exposure to cold. Unlike wheat and barley spring accessions, *Brachypodium* spring accessions switch to the reproductive phase more rapidly and reduce their final leaf number when exposed to low non-freezing temperatures. Furthermore, our work revealed that these physiological observations could be directly related to the expression of the major vernalization regulator *VRN1*. In addition, osmoprotectant accumulation analyses in plants of spring and winter accessions under cold acclimation conditions revealed that both types of accessions could induce physiological responses in order to cope with their changing environment. However, the osmoprotectants tested were found to accumulate in all *Brachypodium* accessions with no significant differences

observed between spring and winter accessions. On the other hand, expression analysis of a *COR* gene coding for an antifreeze protein, a protein specialized in protection against cellular damages caused by freezing, allowed us to differentiate between spring and winter accessions. Indeed, spring accessions accumulated less of this *COR* gene transcripts when compared to winter accessions thus clearly suggesting that the latter accessions had a higher freezing tolerance potential.

The second part of this study first described the isolation and cloning of the temperature-induced lipocalin-2 of *Brachypodium*. Expression analysis for the temperature-induced lipocalin-2 demonstrated both the sensitivity of the method to distinguish between spring and winter accessions and the higher relative transcript abundance in the winter accession. The second part of this study introduces the numerous steps required for *Brachypodium* transformation. The most critical steps that enhance transformation and screening efficiency are analysed and discussed. The knowledge developed and the instruments received during the course of this study will undoubtedly set off the production of numerous transgenic lines in the laboratory.

In conclusion, the results of this study indicate that vernalization and cold acclimation seem to be two linked processes in *Brachypodium* with *COR* genes attaining maximum expression one to three weeks before vernalization saturation occurs. We also showed that it is possible to study the function of *COR* genes in *Brachypodium* by overexpressing the temperature-induced lipocalin-2. Overall, this study successfully validates the hypothesis that *Brachypodium* is a valuable model to study cold tolerance in temperate cereals.

5.2 Future Studies

By validating *Brachypodium* as a model to study cold tolerance, the opportunities to unravel the complex genetic network of freezing tolerance in grasses are infinite. This current study paves the way to some of the following future studies that could be accomplished in the short to medium term.

1. Evaluation of freezing tolerance of *Brachypodium* with whole plant freezing assays. Such a study would accurately classify the *Brachypodium* accessions as sensitive or tolerant to freezing.

2. Phenotypic and molecular analyses of the transgenic lines overexpressing the temperature-induced lipocalin-2 in order to determine if this gene enhances freezing tolerance by limiting the damage caused by oxidative stress in *Brachypodium*. Likewise, generation of a transgenic line silencing the temperature-induced lipocalin-2 would reinforce our understanding of the function of this gene.

3. Generation of transgenic lines silencing the *Brachypodium VRN1* ortholog to analyse the effect on cold-regulated genes and to better understand the connection between reproductive development and freezing tolerance.

4. Creation of promoter-reporter transgenic lines for the promoter of *BradiIRI* in order to examine the localization of *BradiIRI* expression in planta. Moreover, in vitro expression of the recombinant protein would reveal if BradiIRI possesses ice recrystallization inhibition activity.

5. This study has proved that changes in gene expression and metabolite biosynthesis are triggered by cold exposure in *Brachypodium*. Overall comprehension of the effects of cold on the biology of *Brachypodium* could be achieved using transcriptome profiling (RNA-seq), proteomics and metabolomics approaches.

5.3 Contribution to Knowledge

The summary of the major accomplishments of my thesis are described below.

1. Validation of *Brachypodium distachyon* as a valuable model to study cold tolerance in cereals.

2. Characterization of the vernalization and cold acclimation patterns in *Brachypodium distachyon*.

3. Identification and expression analysis of a major vernalization regulator and two cold-regulated genes in *Brachypodium distachyon*.

4. Construction of a binary vector for overexpression of the temperature-induced lipocalin-2 fused with GFP.

5. Generation of transgenic lines of *Brachypodium distachyon* overexpressing a temperature-induced lipocalin.

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