Brachypodium sylvaticum: developing a new model to study freezing tolerance in temperate perennial grasses

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ABSTRACT

In the current agricultural context, sustainable crops such as perennial grains have the potential to further secure the world's demand in cereals. Under northern climates, perennial grains are as vulnerable as annual grains to early and late frost events that cause important damages and economic losses. Therefore, the understanding of cold-acclimation in perennials grasses, that is the process by which temperate plants prime their defence against freezing stress, is necessary to cultivate them at their full potential under northern latitudes. Here, we tested the newly developed perennial grasses model *B. sylvaticum* in its capacity to facilitate the study of cold-acclimation and freezing tolerance in perennial grasses. Accordingly, our hypothesis is that *B. sylvaticum* can cold-acclimate in response to low temperatures and increase its freezing tolerance level, and that a phenotypic range in the capacity to cold-acclimate will be observed across different accessions. To verify this, we first determined the survival of non-acclimated and cold-acclimated live plants to freezing stress. We observed an increase of 2°C in the freezing tolerance of cold-acclimated plants in nine tested accessions, themselves differentiated by a diversity in cold-acclimation capacity. This was followed by the determination of cold-responsive genes transcript accumulation profiles in *B. sylvaticum*. Consequently, we determined that three cold-responsive genes *BsCOR413*, *BsCOR410* and *BsCBF2.1* were differentially regulated in response to cold in nine accessions of *B. sylvaticum,* which indicates that this plant most likely possesses the molecular mechanisms that allow cold-acclimation and the subsequent increase in its freezing tolerance. In addition, we tested a high-efficiency transformation protocol for *B. sylvaticum* under our laboratory conditions. We report the transformation of a *B. sylvaticum* plant that overexpresses by thirtyfold the acetyltransferase *GCN5*, an epigenetic modifier that was previously linked to cold response in plants. Therefore, *B. sylvaticum*'s capacity to cold-acclimate through the observed underlying molecular mechanisms as well as its ability to be transformed under laboratory conditions highlights its potential in being used as a model to study cold response in perennial grasses. Findings made in *B. sylvaticum* could thus be transferred and applied to economically important perennial grains crop in order to increase their freezing tolerance.

RÉSUMÉ

Dans le contexte agricole actuel, les cultures durables telles que les céréales vivaces ont le potentiel de mieux sécuriser la demande mondiale en céréales. Sous les climats nordiques, les céréales vivaces sont aussi vulnérables que les céréales annuelles aux gelées hâtives et tardives qui créent d'importants dommages et pertes économiques. Ainsi, la compréhension de l'acclimatation au froid chez les herbacées vivaces, soit le procédé par lequel les plantes tempérées activent leurs défenses pour contrer le stress causé par le gel, est nécessaire afin de tirer le plein potentiel de leur culture sous les latitudes nordiques. Nous testons ici la nouvelle plante modèle *Brachypodium sylvaticum* dans sa capacité à faciliter l'étude de l'acclimatation au froid et de la tolérance au gel chez les herbacées vivaces. En conséquence, notre hypothèse est que *B. sylvaticum* peut s'acclimater en réponse à de basses températures et augmenter sa tolérance au gel, et qu'une gamme phénotypique de capacité d'acclimatation devrait être observée à travers plusieurs accessions. Dans le but de tester ceci, nous avons d'abord déterminé la survie de plantes vivantes soumises à des températures sous zéro; nous observons une augmentation de la tolérance au gel d'environ 2°C chez les plantes acclimatées de neuf accessions testées, elles-mêmes étant diversifiées par leur capacité à s'acclimater. Nous avons ensuite caractérisé l'accumulation de transcrits pour des gènes de réponse au froid en réponse à des conditions d'acclimations. Conséquemment, nous avons déterminé que trois gènes de réponse au froid, soit *BsCOR413*, *BsCOR410* et *BsCBF2.1* sont régulés de façon différentielle en réponse au froid chez neuf accessions de *B. sylvaticum*. Ceci nous indique que cette plante possède des mécanismes moléculaires qui lui permettent une acclimatation au froid ainsi que l'augmentation subséquente de sa tolérance au gel. En plus, nous avons testé et adapté sous nos conditions de laboratoire un protocole de transformation à haute-efficacité chez *B. sylvaticum*. Ainsi, nous rapportons la transformation d'une plante *B. sylvaticum* qui sur-exprime par trente fois l'acetyltransférase *GCN5*, soit un modificateur épigénétique dont l'action est liée à la réponse au froid chez les plantes. De ce fait, la capacité qu'a *B. sylvaticum* de s'acclimater au froid en vertu des mécanismes moléculaires sous-jacents qui ont été observés, ainsi que son aptitude à être transformé sous des conditions de laboratoire lui assure d'être utilisé en tant que plante modèle pour étudier la réponse au froid chez les herbacées vivaces. Les découvertes faites chez *B. sylvaticum* pourraient être transférées et appliquées chez des céréales vivaces d'importance économique dans le but d'améliorer leur tolérance au gel.

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LIST OF ABBREVIATIONS

ADOMET: *s-AdenOsylMEThionine DeCarboxylase proenzyme* functional domain

AP2: *Apetalla 2*

BROMO: *Bromodomain* 2

BsCBF2.1: *Brachypodium sylvaticum C-repeat Binding Factor 2.1*

BsCOR410: *Brachypodium sylvaticum COld-Regulated 410*

BsCOR413: *Brachypodium sylvaticum COld-Regulated 413*

BsEF1: *Brachypodium sylvaticum Elongation Factor-α 1*

BsGAPDH: *Brachypodium sylvaticum GlycerAldehyde-3-Phosphate-DeHydrogenase*

BsGCN5: *Brachypodium sylvaticum General Control Non-repressible 5*

BsSAMDC: Brachypodium sylvaticum S-AdenosylMethionine DeCarboxylase proenzyme

BsUBC16: Brachypodium sylvaticum UBiquitin Conjugating enzyme 16

CA7-14: Cold-acclimated for 7 or 14 days

CBFIII-IV: *C-repeat Binding Factors* class *III* or *IV*

CBFs: *C-repeat Binding Factors*

CORs: *COld-Regulated* genes

DHNs: *Dehydrins*

EF1: *Elongation Factor-α 1*

GAPDH: *GlycerAldehyde-3-Phosphate-DeHydrogenase*

*G_TR_*2: *Translational-type guanine nucleotide-binding domain 2*

GCN5: *General Control Non-repressible 5*

GNAT: *Gcn5-related AcetylTransferase* domain

LT50s: Lethal Temperature for 50% of a population

NA: non-acclimated control

SAMDC: S-AdenosylMethionine DeCarboxylase proenzyme

UBCs: UBiquitin Conjugating enzymes

WPFT: Whole-Plant Freeze Test

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PREFACE

The entirety of this thesis is composed of original work organized in six chapters. The contribution of co-authors to the thesis is as follow. All experiments were designed by Gabriel Lambert-Rivest and Jean-Benoit Charron, and performed by Gabriel Lambert-Rivest with the help of Maria Kyriakidou for accessing genomic data used to complete the work presented under Chapter 4, section 4.1. Furthermore, primer pairs targeting the genes *GCN5* and *GCN5*/*AcV5* as presented under Table 2 in Chapter 3, and used to complete work presented under Chapter 4, section 4.4.2 were designed and provided by Alexandre Martel. The pANIC-6A-*BdGCN5* vector (Figure 18) used in the transformation study as presented under Chapter 4, section 4.4 was also designed and provided by Alexandre Martel. All data analysis was performed by Gabriel Lambert-Rivest.

Chapter 1: GENERAL INTRODUCTION

1.1 Introduction

Cereal crops represent an important constituent of the worlds' diet just as they are widely used for forage and biofuels production (Lal, 2005; Mochida *et al.*, 2013). Considering that the world population is projected to increase to 9 billion of humans by 2050, ensuring global food security by developing a sustainably more productive agriculture becomes a priority (Alexandratos *et al.*, 2012; Mochida *et al.*, 2013). In such context, many experts are advocating for the development and future use of perennial grain crops (Glover *et al.*, 2010). Indeed, perennial crops offer many advantages toward sustainable agriculture; their persistent root systems reduce soil erosion as well as water and nutrient leakage. Once established, this type of crop requires fewer input in fertiliser and pesticide, therefore reducing the labor that is necessary for field maintenance. In the end, the use of perennial grain instead of annual grain crops fosters a 'cleaner' agroecosystem (Cox *et al.*, 2006; Glover *et al.*, 2010). For this reason, many researchers are actively working on developing marketable varieties of perennial grains crop. Their effort recently lead to the development of a variety of perennial wheat grass (*Thinopyrum intermedium*) which could be used for the production of a flour that is suitable for human consumption (Marti *et al.*, 2015). In addition, the use of this crop in the field will have a positive, lasting impact on the soil and water quality where it is grown (Culman *et al.*, 2013). Hence, *T. intermedium* embodies the sustainably more productive grain agriculture that could play a key role in securing tomorrow's agriculture (Cox *et al.*, 2006; Culman *et al.*, 2013).

Under temperate climate, freezing stress due to early fall or late spring frost events is a major cause of damages and economic losses to crop production (Xin and Browse, 2000). Perennial grasses crops are as vulnerable to freezing events as their annual counterpart, especially in the case of newly, first-year established perennial plants (Lorenzetti *et al.*, 1971; Humphreys and Eagles, 1988; Tcacenco *et al.*, 1989, Clifton-Brown and Lewandowski, 2000; Ebdon *et al.*, 2002; Hulke *et al.*, 2008). Therefore, an understanding of cold-acclimation and freezing tolerance in perennial grasses is crucial to enable their proper cultivation under northern climates. Although some studies have investigated freezing tolerance in the perennial grass *Lolium perenne* (perennial ryegrass) (Lorenzetti *et al.*, 1971; Humphreys and Eagles, 1988; Tcacenco *et al.*, 1989, Ebdon *et al.*, 2002; Hulke *et al.*, 2008), few have focussed on characterizing the underlying molecular mechanisms

that prepares perennial grasses to undergo freezing stress (Tamura and Yamada, 2007; Zhang *et al.*, 2009, Tamura and Yonemaru, 2010; Yu *et al.*, 2015). In fact, perennial grasses molecular research would benefit from a diversification of the genomic tools and resources available to researchers (Steinwand *et al.*, 2013). For instance, Mochida *et al.* (2013) advocates that a better understanding of cereals crop genetics is crucial to improve their productivity and is enabled by the use of genetic model plants that are easily studied under laboratory conditions. Hence, feeling the need for additional perennial grasses model plants, the scientific community started to develop genomic tools and resources for *Brachypodium sylvatcium* (Fox *et al.*, 2013; Steinwand *et al.*, 2013), a cold-seasoned Eurasian perennial grass that is closely related to *Brachypodium distachyon*, an already established powerful model of temperate cereal crops (Vogel, 2009).

In the present project we propose to test the proficiency of *B. sylvaticum* as a model for the study of the response to cold in perennial grasses. The following two sections respectively state our hypotheses regarding the species response to cold and outline our work objectives.

1.2 Hypotheses

General Hypothesis

Exposition to low temperatures (4°C) in *B. sylvaticum* will activate molecular mechanisms of cold-acclimation, thus resulting in an observable increase of the plant's freezing tolerance when exposed to freezing temperatures.

Sub-hypothesis

A phenotypic range in the molecular and physiologic capacities to cold-acclimate will be observed across accessions of *B. sylvaticum* from geographically diverse background.

1.3 Objectives

A. The main objective of this study is to characterize *B. sylvaticum*'s cold-acclimation and freezing tolerance capacities. This objective is sub-divided as follow:

    i. to determine the survival of whole, live *B. sylvaticum* plants exposed to sub-zero temperatures

ii. to characterize the molecular responses of *B. sylvaticum* to low, non-freezing temperatures by determining the transcripts accumulation profiles of the cold-responsive genetic markers *COR413*, *COR410* and *CBF2.1*.

B. To test and adapt a transformation protocol for *B. sylvaticum* as described in Steinwand *et al.* (2013) under our laboratory conditions, and to initiate the production of transgenic plants.

Chapter 2: LITERATURE REVIEW

2.1 The Phylogeny of the *Brachypodium* Genus

Early comparative mapping of cereal genomes by Restricted Fragment Length Polymorphism (RFLP) revealed important similarities between three major groups of the crop grasses: *Triticea* or *Pooideae* (e.g. wheat, barley, rye), *Orizea* later renamed *Ehrhartoideae* (e.g. rice) and *Panicoidea* (e.g. maize, sugar canes, foxtail millet, sorghum). Those similarities are referred to as 'the crop circle', which forms a platform of commonly shared genetic traits coded by multiples chromosomal orthologous regions (Moore *et al*, 1995; Devos *et al*, 2005). In fact, the evolution of the species from 'the crop circle' can be narrowed down to five ancestral genomes that had diverged in 30 rice-independent linkages mostly due to gene duplication and neo-functionalization (Bolot *et al*., 2009). In other words, all of the modern cereal crops along with some contemporary wild grasses like *Brachypodium* spp. would have evolved from six major divergence events that involved five common ancestors. Notably, the first split that occurred an estimated 50 million years ago had isolated rice from all other crops.

Together, work by Moore *et al.* (1995), Devos (2005) and Bolot *et al.* (2009) supplies crucial information to the progress of cereal crop research. Indeed, a deeper understanding of 'the crop circle' assists cereal crop scientists in their research effort, especially when those imply notion of grasses phylogeny. For instance, the phylogenetic position of *Brachypodium distachyon*, a member of the *Pooideae* was crucial in the process of its selection and further development as a cereal genetic model (Vogel and Bragg, 2009; The International Brachypodium Initiative, 2010). In the end, this resourceful model organism would facilitate the study of many biological mechanisms in grasses and the subsequent transfer of relevant discoveries into economically important cereals (Brutnell *et al.*, 2015).

Brachypodium is part of the *Poacea* family, subfamily *Pooidea*, tribe *Brachypodieae*, which contains only *Brachypodium* spp. It is hypothesized that the tribe *Diharreanea* act as the sister group to *Brachypodieae* while the latter is the actual sister group for the tribes of the *Poeae* (e.g. *Festuca* spp., oat), *Triticea* (e.g. wheat, barley, rye) and *Bromea* orders. This phylogeny was mainly developed on molecular evidence shown by Yin Shi *et al.* (1993), Hsiao *et al.* (1995) and Catalán *et al.* (1995) and later conveyed in greater details in Catalán *et al.* (1997) (Figure 1, panel A). However, comparative mapping by RFLP conducted on oat, wheat and their relatives by van

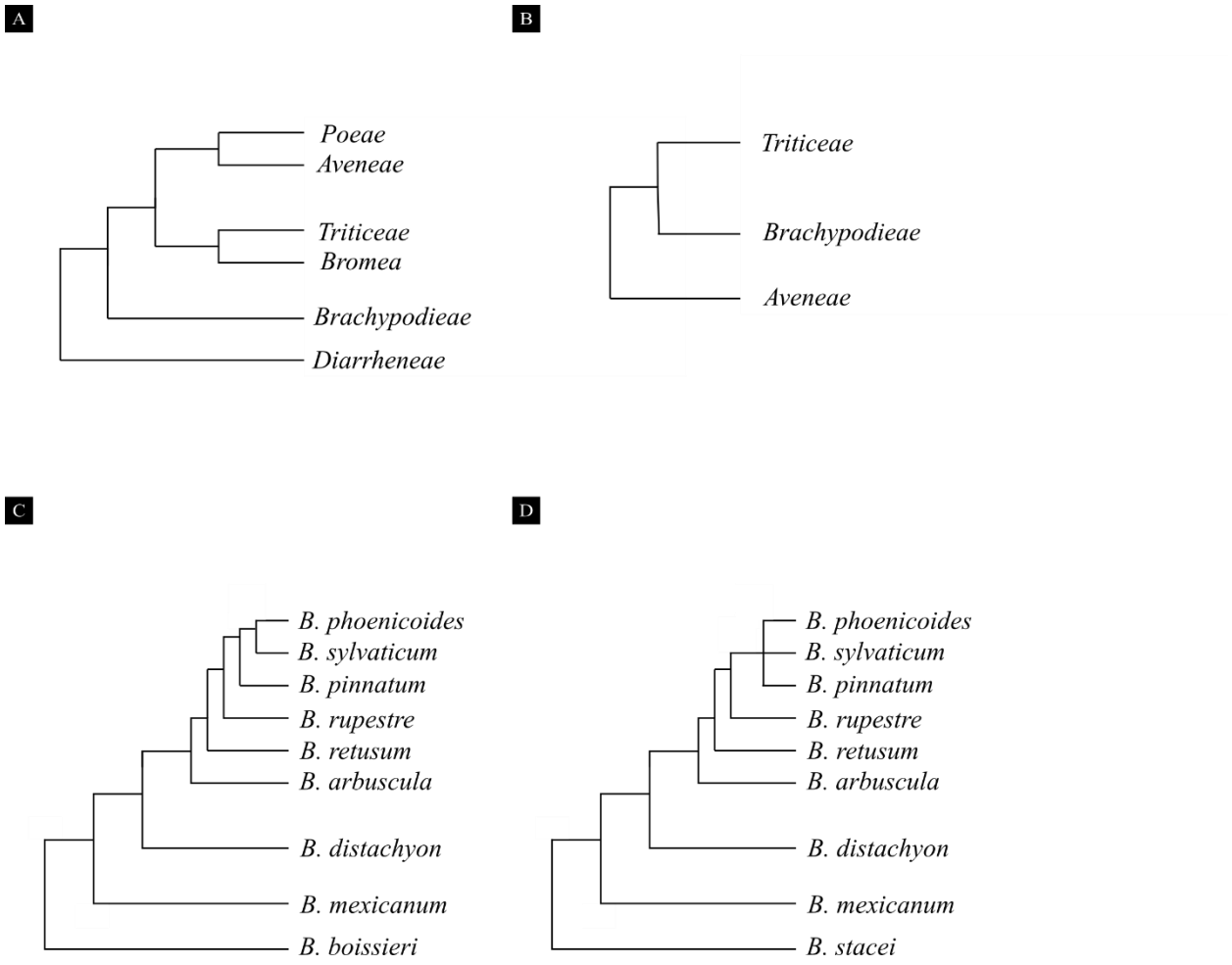

Figure 1. Phylogenetic trees. A: Classification of the subfamily *Pooideae* as presented in Catalán *et al.* (1997). **B:** Classification of the *Pooideae* subfamily as presented in Bolot *et al.* (2009). **C:** Classification of the *Brachypodiaceae* family as inspired from strict consensus trees presented in Catalán *et al.* (2012). **D:** Classification of the *Brachypodiaceae* family as presented in Betekhtin *et al.* (2014).

Deynze *et al.* (1995a-b) suggested a different classification of the core *Pooideae* where *Aveneae* would act as a sister group to the *Brachypodieae*, the *Triticeae* and the *Bromeae* as synthesized in Gaut (2002) and reported in Bolot *et al.* (2009)(Figure 1, panel B). Nevertheless, subsequent molecular studies that compared various type of genetic material amongst pooid grasses gave stronger support to the position of *Brachypodium* as first reported by Catalán *et al*. (1997) (Hilu *et al.*, 1999; Duvall *et al.*, 2007; Davis *et al.*, 2010; Blaner *et al.*, 2014; Hochbach *et al.*, 2015; Saarela *et al.*, 2015). Together those studies show that *Brachypodieae* is one of the first taxon from the core *Pooideae* to have diverged and that it is closely related to the *Poeae* and *Triticeae* (Figure 1, panel A). In other words, the *Brachypodium* genus would act as the sister group to both *Poeae* and *Triticeae* which includes economically important crops such as oat and wheat. To date, twelve species of *Brachypodium* are described, three annuals (the diploids *B. stacei*, *B. distachyon*, and the tetraploid, natural hybrid *B. hybridum*), and nine perennials (the strictly diploid: *B. arbuscula, B. glaucovirens*, *B. retusum* and *B. sylvaticum*, the strictly tetraploid: *B. phoenicoides*, the strictly octoploid: *B. boissieri* and the alternatively diploid or tetraploid: *B. mexicanum* and *B. pinnatum*) (Catalán *et al*., 2012; Betekhins *et al.* 2014). The first molecular phylogenetic studies of the genus by Catalán *et al*. (1995) and Catalán and Olmstead (2000) defined a core annual group and a core perennial group but failed to provide adequate support pertaining to the relative position of the two core groups. In a more recent study, Catalán *et al.* (2012) inferred a classification that places the perennial *B. boissieri* as sister-group to the entire genus from which diverged a group crowned by *B. distachyon* and formed by two core subgroups; an annual one including *B. stacei* and *B. hybridum,* and a perennials one formed by, in order of evolution, *B. arbuscula*, *B. retusum, B. rupestre*, *B. pinnatum*, *B. sylvaticum* and *B. phoenicoides* (Figure 1, panel C). A similar classification was inferred by Betekhtin *et al.* (2014). However, the latter places either *B. stacei* or *B. mexicanum* as the sister-group to *B. distachyon, B. hybridum* and the perennials core group (Figure 1, panel D). Although both Catalán *et al.* (2012) and Betekhtin *et al.* (2014) provide well supported intra-genus classifications, they do not necessarily agree with each other as some nodes received moderate support. Nonetheless, the strong support for the position and composition of the perennials core was validated by Catalán *et al.* (1995), Catalán and Olmstead (2000), Catalán *et al.* (2012) and Betekhtin *et al.* (2014) just as the two latter studies placed in very close relationship *B. distachyon* and *B. sylvaticum*.

The fact that *B. sylvaticum* and *B. distachyon* would have evolved from a common ancestor is important to the current project. More specifically, it implies that most of the discoveries made in *B. distachyon* may be reasonably extrapolated to, and compared in *B. sylvaticum* or vice-versa. For example, Bossolini *et al.* (2007) and Qi *et al.* (2010) respectively discovered that both, *B. sylvaticum* and *B. distachyon* share high level of synteny with wheat, but lower similarity with rice. Those studies confirm that *B. sylvaticum* and *B. distachyon* are both members of 'the crop circle', thus highlighting the strategic position and accessibility of the *Brachypodium* plants when used as genetic models.

2.2 *Brachypodium* Species as Model Organisms

Understanding cereal genetics and developing tools to breed highly productive varieties is facilitated by the use of diploid model organisms. Indeed, these allow an easier study of specific genetic traits as compared to the often polyploid cereal crops (Mochida and Shinozaki, 2013). Rice, which has been proposed as a cereal crop model, offers extensive resources such as a sequenced genome, multiple bacterial artificial cloning (BACs) libraries, mutants, and a wide variety of fully characterized accessions (Goff, 1999; IRGS, 2005). However, being a specialized semi-aquatic grass from the sub-familly *Orizea*, rice ecology and phylogeny differ from that of other economically important cereal grain crops such as wheat, oat and barley. As discussed in the previous section, *Brachypodium* grasses are closely related to oat, wheat and barley. Therefore, *Brachypodium* grasses were suggested as alternatively more powerful models for cereal grain crops (Draper *et al.*, 2001). For this reason, the *Brachypodium* genus received an ever increasing attention from cereal crop researchers in the last decades. Indeed, species such as the annuals *B. stacei* and *B. hybridum* have been proposed as good models for phylogenetic or abiotic stresses studies (Catalán *et al.*, 2012; Catalán *et al.*, 2014). The genome of *B. stacei* was recently sequenced (234 Mb arranged in 5 chromosomes, 29,898 loci coding for 36,357 proteins) and is available through the Joint Genome Institute (JGI) platform Phytozome (Phytozome 12.1.3). Other species such as the perennial *B. sylvaticum* or, in particular, the annual *B. distachyon* had been more extensively studied to be developed into important grasses models (Vogel and Bragg, 2009; Fox *et al.*, 2013; Steinwand *et al.*, 2013).

2.2.1 *Brachypodium distachyon*

Brachypodium distachyon possesses many of the desirable characteristics of a genetic model. It is a small, self-fertile plant with a short generation time, which allow for high throughput studies under laboratory conditions (Brkljacic *et al.* 2011). It also has a small genome size (Yin Shi *et al.*, 1993), conveniently one of the smallest for known monocot (Hasterok *et al.*, 2006). To date, *B. distachyon* genome size is approximated at 272 Mb with 5, duplicated chromosomes containing 34,310 loci some of which are coding for 52,972 proteins (V3.1, Phytozome 12.1.3). Furthermore, *B. distachyon* is found in a wide variety of wild accessions for which high phenotypical as well as genotypic diversity is observed (Draper *et al.*, 2001; Vogel *et al.*, 2009; Gordon *et al.*, 2014). In combination to its strategic position within 'the crop circle', aforementioned traits suggested that *B. distachyon* is a resourceful monocot model organism (Draper *et al.*, 2001).

Accordingly, researchers thoroughly investigated *B. distachyon*'s biology. For instance, Khan and Stace (1999) discovered that *B. distachyon* is highly self-compatible and that it is hybridisable with *B. sylvaticum* and both diploid, and tetraploid *B. pinnatum.* Kellogg *et al.* (2013) dissected the early development of inflorescence whereas Sabelli and Larkins (2009), Opanowicz *et al.* (2008) and O'Connor *et al.* (2014) studied the embryogenesis of the species. Guillon *et al.* (2011) observed that grain reach maturity in 36 days after which they appear flattened in cross section, a characteristic proper to wild grasses and not observed in wheat. Laudencia-Chingcuanco *et al.* (2008) and Larré *et al.* (2010) both identified globulins and promalins as the main families of storage proteins for *B. distachyon* seeds. As for starch deposition, Tanackovic *et al.* (2014) determined that *B. distachyon* accumulates low amount of starch (12 %) in its seeds as compared to barley (47 %). Work by Schwartz *et al.* (2010) helped to determine requirements for vernalization in many accessions while Ream *et al.* (2014) and Woods *et al.* (2014) allowed to characterize the phenotypical effect of vernalisation and photoperiod on flowering and the associated molecular response. Other studies determined disease and virus susceptibility for *B. distachyon*. For example, Vogel and Bragg (2009) reported high susceptibility of the species to *Pythium* root disease. Similarly, Catalán *et al.* (2014) reports that *B. distachyon* is susceptible to Rice Blast Disease, *Fusarium* Head Blight and Barley Mosaic Virus (BMV).

In parallel to biological characterization, researchers quickly developed an array of genetic tools for this species that includes high-efficiency transformation protocols, BACs libraries and an

annotated genome (Brkljacic *et al.* 2011). In more details, Christiansen *et al.* (2005) were the firsts to develop a protocol of transformation by bombarding gold micro-pellets coated with the vector of interest onto calluses from different *B. distachyon* accessions. However, this protocol obtained a low transformation efficiency (~ 4%). Consequently, Vogel *et al.* (2006a) later opted for an *Agrobacterium* co-cultivation approach. Their protocol was later optimized and refined by others to eventually obtain transformation efficiencies that range from 35 to 50% (Vogel and Hill, 2008; Vain *et al.*, 2008; Păcurar *et al.*, 2008; Alves *et al.*, 2009). In term of genomic research, Hasterok *et al.* (2006) initially designed an exploitable BAC library for *B. distachyon*. This effort was followed by the design of two additional BACs libraries by Huo *et al.* (2006), the annotation of BAC ends by Huo *et al.* (2008), the sequencing of *B. distachyon* chloroplast genome by Bortiri *et al.* (2008) and finally, the complete sequencing and annotation of the species' genome by The International Brachypodium Initiative (2010) using shotgun sequencing. This latter work in particular allowed deep analyses of *B. distachyon* gene families, syntenies and co-linearities in relation to other models such as *Arabidopsis*, wheat, sorghum and rice. Note that studies by Vogel *et al.* (2006b), Garvin *et al.* (2009) and Qi *et al.* (2010) also contributed to characterizing co-linearity between *B. distachyon*, rice, barley and wheat. Together, those studies revealed considerable genomic similarities between wheat, barley and *B. distachyon*, which suggested that discoveries made in *B. distachyon* may be effectively transferred and applied to the improvement of pooid crops (The International *Brachypodium* Initiative, 2010).

The development of efficient transformation protocols combined to a full characterization of *B. distachyon*'s genome provided scientists with the basic tools they needed to engage into a deeper understanding of the genetic of this model. For instance, genomic information enabled the characterization of standard genes necessary to the performance of real-time, quantitative polymerase chain reaction (RT-qPCR). Indeed, Hong *et al.* (2008) were able to validate the use of nine references genes in *B. distachyon.* Of particular interest to our project, the expression of four genes was more stable under cold and heat condition, that is in ascending order of stability *ACTIN7* (ACT7), *TUBULIN-ALPHA-6 PROTEIN* (*TUA6*), *S-ADENOSYLMETHIONINE DECARBOXYLASE PROENZYME* (*SAMDC*) and *ELONGATION FACTOR-ALPHA 1* (*EF1*). Another example of advanced genetic tools includes the design of multiples, sequenced *B. distachyon* mutant lines collection (The International Brachypodium Initiative, 2010; Thole *et al.*, 2010; Bragg *et al.*, 2012; Dalmais *et al.*, 2013; Catalán *et al.*, 2014; Granier *et al.*, 2015). More

precisely, a group from the John Innes Center generated a collection of 4500 T-DNA mutants from which 741 lines were assessed. In those 741 lines, 660 T-DNA insertions were found at single location in the genome of *B. distachyon*. About 60 % of those loci were covered by Expressed Sequence Tags (ESTs) (Thole *et al.*, 2010). Another group from the United State Department of Agriculture Western Research Center is maintaining an impressive collection of T-DNA mutant lines that now contains 23,649 lines accounting for 26,112 insertions sites (Bragg *et al.*, 2012; Phytozome 12.1.3). The same group developed mutagenesis protocols by treating seeds with ethyl metasulphonate (EMS) and through irradiation by fast neutrons (Granier *et al.*, 2015). In complement to those mutants collections, Targeting Induced Local Lesions In Genomes (TILLING) platforms are being developed and maintained by the Brutnell lab at the Donald Danforth Plant Science Center (~ 5000 mutant) (Vogel *et al.*, 2010), by the Rasmussen lab at the University of Copenhagen (3800 mutants, DNA isolated for 3000 mutants) (Catalán et al, 2014), and by the Institut National de Recherche Agronomique at Evry (~ 6000 mutants for seven lignin biosynthesis genes) (Dalmais *et al.*, 2013). Together, those collections offer thousands of catalogued *B. distachyon* mutant lines that may serve in the study of targeted genes and their associated phenotypes and/or products.

With both a deep knowledge of its basic biology and comprehensive molecular tools, *B. distachyon* is now a preferred model for many researchers who are studying physiological, morphological and molecular processes in grasses. For instance, some researchers are investigating cell wall formation processes in *B. distachyon* with the objective to decrease lignin deposition as such phenomenon impede the deconstruction of biofuel crops (e.g. JY Kim *et al.*, 2015; Yin Wang *et al.*, 2015). *B. distachyon* is also an important model for the study of abiotic stresses. Researchers focused on characterizing its phenotypical and molecular response to drought (e.g. Luo *et al.*, 2011; Verelst *et al.*, 2013), high salt conditions (e.g. Lv *et al.*, 2014) and cold (e.g. Colton-Gagnon *et al.*, 2014; further discussed in section 2.3).

2.2.2 *Brachypodium sylvaticum*

Brachypodium sylvaticum had been proposed as a promising model for perennials biofuel and grain crops, but also for studying differences between perennials and annuals grasses because of its close relationship with *B. distachyon* (Draper *et al.* 2001; Bossolini *et al.* 2007; Steinwand *et*

al. 2013; Fox *et al.* 2013). Because of its model plant attributes, *B. sylvaticum* received increasing attention from grass researchers in the past 15 years.

To date, few studies have investigated the biology of *B. Sylvaticum*. Aarestad (2000) described this weak rhizomatous, shade tolerant perennial grass as a key species of the *Corylus-Brachypodium* forest type in its native range of Norway. American ecologists focussed on defining interactions and related effects between an invasive population of *B. sylvaticum* and other populations of native plants (e.g. Holmes *et al.,* 2010). Khan and Stace (1999) showed that it is highly self-compatible and hybridisable with diploids *B. pinnatum* and *B. glaucovirens*. They also found that this species do not necessarily requires vernalization (facultative) to flower and usually do so within three to four weeks. However, Steinwand *et al.* (2013) observed variations in the flowering requirements of nineteen different accessions. Under a light regime of twenty hours of light and four hours of dark, some accessions flowered in 5 weeks (PI204863, PI440175 and PI564896), 6 weeks (PI251102), 8weeks (PI204865, PI297868, PI318962, PI636630), 9 weeks (PI269842, PI345965, PI380758) or 10 weeks (PI268222) while other never flowered despite 14 weeks of testing (PI172383, PI206546, PI206619, PI287787, PI325218, PI344569, PI610793, W623501, W623524, W623538). Steinwand *et al.* (2013) also provides a single sequence repeats (SSR) map that linked *B. sylvaticum* specimen from Norway to those of Tunisia, and those from Spain to those of China. The same study concluded that *B. sylvaticum* natural population scores high genomic diversity with an average of 11 alleles per SSR markers. Therefore, *B. sylvaticum* is found in a variety of wild accessions that are significantly diverse at both the phenotype and genotype levels.

Although few biological information is available on *B. sylvaticum,* scientists recognized its potential as a model for perennial grasses and started to develop different genetic tools and resources for the species. Hence, Foote *et al.* (2004) first constructed a BAC library that allowed the observation of a highly conserved synteny between wheat, rice and *B. sylvaticum*. Bossolinni *et al.* (2007) used shotgun sequencing to sequence 371 Kbp of *B. sylvaticum*'s genome. They compared orthologous sequences for *B. sylvaticum,* rice and wheat and found high level of co-linearity between wheat and *B. sylvaticum,* and a considerable inversion of 220 Kbp between rice and *B. sylvaticum*. Later, Fox *et al.* (2013) assembled the transcriptome (RNA sequencing) of three *B. sylvaticum* accessions to cover about 90% of the species' exome. Comparison with the *B.*

distachyon Bd21 reference genome highlighted 394,654 single nucleotide polymorphism (SNPs) and over 20,000 SSRs. *B. sylvaticum* transcriptome was annotated by matching homologies with the Bd21 genome as reference and gene expression was compared between *B. distachyon* and *B. sylvaticum*. High correlation was found between both species in terms of genes expression. The draft version of the *B. sylvaticum* transcriptome produced by Fox *et al*. (2013) is available for consultation through the Jaiswal Lab web site page of Oregon State University (http://jaiswallab.cgrb.oregonstate.edu/genomics/brasy). Recently, a first version of the partially annotated genome of *B. sylvaticum* was made available for consultation on JGI platform's Phytozome 12.1.3. To date, *B. sylvaticum* genome size is of approximately 346 Mb arranged in 9 chromosomes, and has 36,927 coding loci and 50,263 protein-coding transcripts. In complement to genomic studies, Steinwand *et al.* (2013) developed an *Agrobacterium* co-cultivation transformation protocol for cultured *B. sylvaticum* calluses. This protocol was mainly adapted from similar protocols developed for *B. distachyon* as presented in Vogel *et al.,* (2006a) and Vogel and Hill (2008). In the end, Steinwand *et al.* (2013) showed that at least one of the tested accession (PI269842) can be successfully transformed with relatively high efficiency.

In light of the discoveries reported under this subsection, one may conclude that *B. sylvaticum* possesses most of the same desirable traits that had contributed to the development of *B. distachyon* as a model. Indeed, *B. sylvaticum* is a self-fertile, diploid and naturally diverse grass that can be reasonably reproduced and transformed under laboratory conditions (Steinwand *et al.*, 2013). In addition, high genomic similarities were observed between *B. sylvaticum,* wheat and *B. distachyon*. Those similarities are consistent with *B. sylvaticum*'s phylogenetic position in 'the crop circle' as it is closely related to oat, wheat and barley (Bossolinni *et al.,* 2007; Fox *et al.*, 2013), but also to perennial wheatgrass and perennial ryegrass (Culman *et al.*, 2013; Lorenzetti *et al.,* 1971). With the recent publication of annotated genomic data, *B. sylvaticum* is now becoming a resourceful model organism that can be used to facilitate perennial grasses researches. Therefore, discoveries made in *B. sylvaticum* could be subsequently applied to improve the productivity of economically important pooid species (Fox *et al.*, 2013). Because our research interests focus on the response to cold stress in cereal crops, we took the opportunity to study such phenomenon in the model *B. sylvaticum*.

2.3 Freezing Tolerance and Cold-acclimation

Freezing stress is a major cause of damage and economic losses for crops in temperate regions (Xin and Browse, 2000). Cellular damage begins when ice nuclearizes on the cell membrane and crystalizes throughout the apoplastic space; a phenomenon encouraged by the lower solute concentration of the apoplastic fluids. Most of the actual damage is dehydration-related as crystalizing ice exerts a dehydrative force on the cellular unfrozen fluid. This force eventually ruptures the cell wall, which ultimately results in cellular death (Guy, 1990; Thomashow, 1999). Alternatively, this same dehydrative force can infer major changes in the ultrastructure of the cell membrane going from a lipid bilayer to a disorganised single layer. As a result, the cell membranes lose their osmotic capability such that the cell cannot rehydrate past freezing stress (Steponkus and Lynch, 1989). Consequently, most temperate plants had evolved cold response mechanisms that prepare their cells to cope with freezing stress. For instance, an exposition to low, non-freezing temperatures prior to freezing stress was found to substantially increase the freezing tolerance of many plants (Guy, 1990). Termed cold-acclimation, this process fosters the production of cryo-protectives proteins and metabolites that either lowers the freezing point of the cytosol, or increases the ability of the cell membrane to cope with freezing related dehydration stress (Guy, 1990; Thomashow, 1999). Therefore, the understanding of freezing tolerance and cold-acclimation mechanisms is crucial for improving plant tolerance to cold and secure their productivity under northern climate. Extensive investigation of those mechanisms is being conducted in models such as *Arabidopsis* and *B. distachyon* as well as in crops like wheat, oat, barley and perennial ryegrass.

2.3.1 Cold-acclimation in *Arabidopsis*

The cold-acclimation process is well documented for the annual model plant *Arabidopsis*. Gilmour *et al.* (1988) tested the cold-acclimation capacity of *Arabidopsis* to find that its freezing tolerance increased from -3 to -6 °C after being cold-acclimated at 4 °C during 24 hours. Freezing tolerance reached as much as -10 °C for plants that were cold-acclimated during 9 days. Following these observations, researchers quickly investigated the genetic regulation of cold-acclimation in *Arabidopsis* to discover a complex network of genes that includes different pathways (Cook *et al.*, 2004). Some of the most described so far are the *C-BINDING REPEAT TRANSCRIPTION FACTORS / DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTORS* (*CBF*s-*DREB*s) dependent pathways. As showed by Cook *et al.* (2004), those pathways could be responsible for

the production of as much of 79% of the cold-related metabolites in *Arabidopsis*. However, a previous transcriptomic study by Fowler and Thomashow (2002) rather associated the *CBFs-DREBs* pathways with 12 % of the response to cold in the same plant. Accordingly, other studies described *CBFs-DREBs* independent genes that play a role in the response to cold in *Arabidopsis* (Xin, 1998; Uno *et al.*, 2000; Zhu *et al.*, 2004; Cao *et al.*, 2005; Kwak *et al.*, 2005; Ciuzan *et al.*, 2015). Nonetheless and because of their direct role in regulating cold-acclimation phenomenon, the *CBFs-DREBs* pathways remain the principal architects of freezing tolerance in plants (Cook *et al.*, 2004).

*CBF/DREB*s proteins (hereafter referred as *CBFs*) bind on *cis* regulatory elements (C-repeat motifs) in the promoter region of cold-responsive genes such as *COLD-REGULATED 15a* (*COR15a*) in *Arabidopsis* or *BRASSICA NAPUS 155* (*BN155*) in *Brassica Napus* (Baker *et al.*, 1994; Jiang *et al.*, 1996). They were also found to binds onto Dehydration-Responsive Element (DRE) (e.g. *rd29a*) in *Arabidopsis* (Stockinger *et al.*, 1997). Although very similar, the C-repeat motif sequence is one nucleotide longer (CCGACC) than for the DRE motif (CCGAC) (Stockinger *et al*., 1997). Combined works in *Arabidopsis* from Yamaguchi-Shinozaki and Shinozaki (1993), Baker *et al.* (1994), Stockinger *et al.* (1997), and in *Brassica napus* by Jiang *et al.* (1996) helped defined the function of *CBFs* as transcription factors. For instance, Stockinger *et al.* (1997) discovered that an *APETALLA2* (*AP2*) domain spanning 60 amino-acids is part of *CBF1* in *Arabidopsis. AP2*, which is also found in the floral homeotic protein *APETALLA2*, is responsible for binding DNA cis-elements in the promoter region of genes. Therefore, the interaction between the *AP2* domain in *CBFs* and the promoter region of cold-responsive genes would seemingly recruits transcription machinery (Stockinger *et al.*, 1997). In other words, *CBFs* genes activate the transcription of cold-responsive genes, thus playing an important role in the orchestration of the response to cold in plants (Chew and Halliday, 2011).

Multiples factors were found to regulate *CBFs* pathway. For instance, inducer genes (Shinwari *et al.*, 1998; Chinnusamy Viswanathan *et al.*, 2003; Zarka *et al.*, 2003; YS Kim *et al.*, 2015), mediator proteins (Wathugala *et al.*, 2012), jasmonate pathway (Hu *et al.*, 2013) and intracellular calcium spiking (Yitting Shi 2012; Finka 2012; Yitting Shi *et al.*, 2014) were all linked to *CBFs* expression in *Arabidopsis*. Of greater relevance to our project, both the circadian rhythm and the photoperiod were also shown to affect *CBFs* expression in *Arabidopsis* (Harmer *et al.* 2000; Fowler *et al*.,

2005; Bieniawska *et al.*, 2008). Indeed, Kidokoro *et al.* (2009) found that the photoreceptor *PHYTOCHROME INTERRACTING FACTOR 7* (*PIF7*) binds on a G-box motif in the promoter of *CBF1* and is required for its constitutive expression during the evening. Dong *et al*. (2011) observed that the transcription factors *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASOCIATED-1* (*CCA1*), both associated to the circadian rhythm, enhance the cold induction of *CBFs*. Under long day regime, the higher expression of the genes *PHYTOCHROME-B* (*PHYB*) (photoreceptor), *PHYTOCHROME INTERRACTING FACTOR 4* (*PIF4*) and *PIF7* together represses the expression of *CBFs* (Lee and Thomashow, 2012).

Furthermore, the *CBF* pathway is seemingly affected by epigenetic modifications. In 2001, Stockinger *et al.* (2001) showed that the expression of *AtCBF1* cloned in yeast was impaired in mutants that were *ADENOSINE AMINASE 2* (*ADA2*) and *3* (*ADA3),* and *GENERAL CONTROL NON-REPRESSIBLE 5* (*GCN5*) deficient, all of which are components of the *SPT-ADA-GCN5 ACETYLTRANSFERASE* complex (*SAGA*). This complex act as an important epigenetic modifier of the open chromatin as it is involved in gene activation (Brownell and Allis, 1996; further discussed in section 2.4). Stockinger *et al.* (2001)'s results suggest that *ADA2*, *ADA3* and *GCN5* are recruited for *AtCBF1* induction in yeast. However, later work by Vlachonasios *et al.* (2003) showed that *CBFs* were still induced in *Arabidopsis* mutants with disrupted *ADA2b* and *GCN5* proteins, but that the resulting expression of *COLD-REGULATEDs* (*CORs*) genes was reduced. Furthermore, non-acclimated *ADA2b* knockout mutants were more resistant to cold than both wild-type and *GCN5* mutant plants suggesting that *ADA2b* act as a repressor of freezing tolerance in *Arabidopsis*.

Some of the main targets of *CBFs* include genes from the *CORs* families (Jaglo-Ottosen *et al.*, 1998). These were first identified in spinach by Guy *et al.* (1985) and subsequently in *Arabidopsis* (e.g. Hajela *et al.*, 1990; Kurkela and Franck, 1990; Gilmour *et al.*, 1992; Lång and Palva, 1992; Lin *et al.*, 1992; Kurkela and Franck, 1992; Nordin *et al.*, 1993; Horvath *et al.*, 1993). *Arabidopsis COR* genes comprises four families (*COR6.*6, *COR78*, *COR15* and *COR47*) of two genes each for which at least one respond to cold stress (Thomashow, 1999). Those genes encode the production of cryoprotectives proteins. For instance, *COR6.6* protein is rich in alanine, glycine and lysine such that it shares structural similarities with fish anti-freeze proteins (Kurkela and Franck, 1990; Kurkela and Franck, 1992). Similarly, Lång and Palva (1992) described a hydrophilic, glycine-

rich protein included in the *COR* family named *RESPONSIVE TO ABA 18* (*RAB18*). This later also had a conserved lysine and alanine-rich domain and was produced in response to low temperature in *Arabidopsis*. The same year, Lin and Thomashow (1992) described the cold-responsive polypeptide *COR15a* as a hydrophilic, glycine-rich protein that is found in the chloroplast. They further predicted that it forms an amphipatic alpha-helix, a structure that may interact with membranes. On that matter, Artus *et al.* (1996) were the first to directly link the *COR15a* protein to the increase of the freezing tolerance in *Arabidopsis.* In their *in vitro* experiment, they observed that *COR15a* lowered on average by 2 °C the freezing tolerance of both protoplasts and chloroplasts. They further observed that *COR15a* interacted with the lipid bilayers, contributing to the view that proteins from the *COR* families act as cell membrane cryoprotectants.

In addition to *COR* genes, another closely related family of genes termed *LATE EMBRYOGENESIS ABUNDANT* (*LEAs*), or *DEHYDRIN* (*DHNs*) respond to drought, high-salt and cold-temperatures stresses (Close, 1997; Thomashow, 1999). Those genes, which are also regulated by *CBFs* (e.g. Kume *et al.,* 2005) are translated into a variety of highly hydrophilic proteins called dehydrins, that protect the membrane from dehydration (Kosová *et al.*, 2014). In an early review on the matter, Close (1997) indicates that dehydrins all have in common an α-helix domain (Y), as well as a phosphorylable serines (S) and a highly conserved N-terminal domain (K). Different permutations or absence of those domains allowed the classification of dehydrins into five families: YSK_n, SK_n, K_n, YK_n, K_nS.

In fact, cold-responsive genes are highly conserved through higher plants (Close, 1997; Jaglo *et al.*, 2001; Chew and Halliday, 2011). Apart from *Arabidopsis*, cold-responsive genes were found in temperate crops such as barley (e.g. Tommasini *et al.,* 2008), wheat (e.g. Badawi *et al.,* 2007) and hemp (e.g. Mayer *et al.*, 2015), but also in crops that can hardly undergo cold stress like maize (e.g. Close *et al.,* 1989), rice (e.g. Jaglo *et al.*, 2001) and tomato (e.g. Zhao *et al.*, 2009). Many cold-responsive genes had evolved taxa-specific characteristics such that they can be differentiated from one taxon to another (Chew and Halliday, 2011). This is markedly observed in the sub-family of pooid grasses which are well known to produce specific cold-acclimation proteins (Li *et al.*, 2012). *B. sylvaticum* being a member of the pooid grasses, a better knowledge of such specificity would serve our project, especially when selecting appropriate genetic markers of cold-acclimation when answering our research objectives.

2.3.2 Cold-acclimation in Annual Pooid Crops

Pooid crops such as wheat and barley are available in a wide range of both cold-susceptible and cold-tolerant cultivars (e.g. Fowler *et al.*, 1981). Eager to take advantage of this diversity in tolerance through the breeding of more cold-tolerant crops (Fowler and Gusta, 1979), cereal scientists seek to unravel the process of cold-acclimation in pooid grasses. Thanks to original discoveries and characterization mostly made in *Arabidospis*, cereal scientists were able to direct their attention onto the orthologous genes involved in cold-acclimation, especially those associated to the *CBFs* pathway. *CBFs* genes in pooid grasses were first localized in einkorn wheat on the loci *FROST TOLERANCE AM 2* (*Fr-Am 2*) by Vágújfalvi *et al*. (2005). Indeed, a cluster of genes encoding for 11 *CBFs* proteins located on *Fr-Am 2* was directly linked to freezing tolerance in einkorn wheat by Miller *et al.* (2006). Similar findings were later made in barley by Francia *et al.* (2007) whom linked the orthologous loci *Fr-H2* to the species' freezing tolerance. As shown in wheat by Pearce *et al.* (2013), the deletion of large sections of *Fr-Ta 2* resulted in a significant reduction of the plant's freezing tolerance. Such findings reinforced the view that this loci and by extension *CBFs* genes play important roles in cereal freezing tolerance.

In 2005, Skinner *et al.* (2005) discovered 20 *CBFs* genes in barley that were separated in four different phylogenetic clades. From those clades, one in particular, Hv*CBFIV* was seemingly specific to pooid grasses as most of the genes it included were unrelated to other non-pooid grasses such as rice and maize. This observation was later corroborated by Badawi *et al.* (2007) whom studied the evolution of *CBFs* in hexaploid wheat and discovered two specific clades of *CBFs* subdivided in six groups: *CBFIIIc* and *IIId,* and *CBFIVa, IVb, IVc* and *IVd.* According to the researchers, all six groups would have originated from gene duplication events followed by gene radiation. Such theory would explain the important evolutionary distance observed between *CBFs* from theses group in pooid as compared to *CBFs* from other non-pooid grasses (Badawi *et al.*, 2007). Later revisited in barley by Knox *et al.* (2010), those two *CBFs* clades as described in Badawi *et al.* (2007) were deemed a particularity of the response to cold in core pooid grasses.

Apart from the specific clades of *CBFIII* and *CBFIV* genes, pooids are also characterized by *ICE RECRYSTALLIZATION* protein (*IRI*) (Li *et al.*, 2012). This type of protein was first discovered and characterized in perennial ryegrass (Sidebottom *et al.*, 2000) and later in wheat (Tremblay *et al.*, 2005) and in the Antarctic pooid grass *Deschampsia antarctica* (John *et al.*, 2009). According

to Tremblay *et al.* (2005), *IRIs* would have evolved from a receptor-like kinase protein (LLR-kinase) as their N-terminal domains resemble that of a LLR-kinase leucine-rich region. Additionally, their C-terminal domains present similarities with ice binding domain from antifreeze proteins. Therefore, *IRIs* probably bind on ice crystals forming at the cell membrane to avoid their full crystallization (Tremblay *et al.*, 2005). Those observations were later corroborated by Sandve *et al.* (2008) which studied the evolution of the protein in wheat, barley and perennial ryegrass.

Another particularity of the pooids response to cold is their production of fructan, a complex sugar polymer composed of fructose molecule (Kawakami and Yoshida, 2002). Indeed, this metabolite was correlated to cold-acclimation in oat (Livingston *et al.*, 1994), wheat (Kawakami and Yoshida, 2002) and *Poa pratensis* (Rao *et al.*, 2011). Fructan is hypothesized to lower the freezing point of the cytosol when it is accumulated in this later. As a result, the formation of ice crystal in the cytosol would occur at lower freezing temperatures than under non-acclimated cellular environment, thus postponing cellular death (Kawakami and Yoshida, 2002). In wheat and barley, fructan synthesis is catalysed by the enzyme *FRUCTOSYLTRANSFERASE* (*FST*) (Sprenger *et al.*, 1995).

In term of dehydrin and *CORs* genes in pooid, Danyluk *et al.* (1994, 1998) discovered a family of dehydrin proteins that are only expressed into frost-tolerant grasses termed *COLD-REGULATED 410* (*COR410*). These proteins were found at higher levels in response to cold, but also during water stress near the cell membrane of wheat cells (Danyluk *et al.*, 1998). Five years later followed the discovery of the transmembrane protein *COLD-REGULATED 413* (*COR413*) in both wheat and *Arabidopsis* by Breton *et al.* (2003). Associated to both the thylakoid and the plasma membrane, *COR413* was found to accumulate in response to cold and water stress as well as light and abscisic acid stimuli. Yuezhi Wang *et al.* (2014) later identified fifty-four dehydrins genes in wheat from a transcriptome database. In barley, Tommasini *et al.* (2008) characterized thirteen dehydrins genes (*DHN1* to *DHN13*) while assembling a drought and cold-stress specific transcriptome. Together, these listings of *COR* proteins enabled research groups such as Kosová *et al.* (2014) or Yokota *et al.* (2015) to study *COR* proteins structural diversity. Both study were able to associate this diversity to differences in freezing tolerance as observed in various tested

cultivar of wheat and barley. Once, again such results stressed the important role that the *CBF* pathway and related components play in the response to cold for pooid grasses.

Although the study of the molecular response to cold in pooid crops had significantly progressed in the last decades, genetic research using those crops remains resource and time consuming because of the inherent complexity of their genome (Mochida *et al.,* 2013). Accordingly, some researchers had decided to investigate the potential of the then, newly developed model organism *B. distachyon* as a model for the study of cold-acclimation in pooid grasses (Li *et al.*, 2012; Colton-Gagnon *et al.,* 2014).

2.3.3 Cold-acclimation in *B. dystachyon*

In 2012, Li *et al.* (2012) tested the potential of *B. distachyon* as a model for freezing tolerance and cold-acclimation studies in pooid grasses. They verified the occurrence in the model organism of genes encoding for pooid-specific proteins of the *CBFs* clades *CBFIII* and *CBFIV* as well as *IRI* and *FST* homologs. Although no homologs from *CBFIV* clade and for *FST* were observed, they were able to localize *CBFIII* homologs and *IRI*-like genes in *B. distachyon*. Furthermore, they were able to observe that fructan metabolically accumulates in response to cold exposure, but at lower levels than core pooids. Therefore, Li *et al.* (2012) determined that *B. distachyon* was an appropriate model for the study of cold-acclimation.

Work by Ryu *et al.* (2014) and Chen *et al.* (2016) followed in which they identified more members of the *CBFIII* and *CBFIV* clades in *B. distachyon* along with providing their expression profile in response to different stresses. Both studies highlighted that *CBFs* from a specific sub-group that includes mostly *CBFIII* clade related genes were quickly induced in response to cold. In fact, their induction occurs within the first hour to finally peak somewhere between 5 and 10 hours of exposition to cold (Ryu *et al.,* 2014; Chen *et al.* 2016). Those observations are consistent with what was monitored for homolog genes in other pooid grasses (Badawi *et al.,* 2007; Tamura and Yamada, 2007). Conveniently, Chen *et al.* (2016) conducted a comparative study of tissues-specific expression to find that most of the *CBFs* in *B. distachyon* are expressed at higher levels in leaves whereas fewer, such as *CBF2.1*, were expressed at higher levels in roots.

Furthermore, in a comprehensive study of *IRIs* function in *B. distachyon*, Bredow *et al.* (2016) showed the direct involvement of those proteins in impeding the formation of cytosolic ice crystals.

Their results further confirm observation made in wheat (Tremblay *et al.*, 2005) and *L. perenne* (Sandve *et al.*, 2008) as well as reinforcing the link between pooid grasses and *IRIs*.

In echo to Li *et al.* (2012)'s work, Colton-Gagnon *et al.* (2014) comprehensively characterized the freezing tolerance and cold-acclimation capacity in seven accessions of *B. distachyon* at the molecular, phenotypic and physiological levels. Indeed, they monitored the relative accumulation of transcripts from the cold-responsive genetic markers *COR413-like, IRI* and *VERNALIZATION 1* (*VRN1*) for both short day and long day photoperiods. The study also included a Whole Plant Freeze Test (WPFT) which is used to determine the freezing tolerance of a population of live plants submitted to freezing stress, as well as an assay of *B. distachyon*'s physiological response to cold (fructan, proline and soluble sugars accumulation). Colton-Gagnon *et al.* (2014) demonstrated that *B. distachyon* can cold-acclimate as a pre-exposure to low, non-freezing temperatures correlates with an increase in *CORs* transcription as well as with an accumulation in fructan and proline. Both phenomenon contributed to an increase in survival when *B. distachyon* was subsequently exposed to freezing temperatures. With slight variations from one accession to another, cold-acclimated *B. distachyon* plants would now survive at freezing temperatures that are approximately 2°C lower than for non-acclimated plants (Colton-Gagnon *et al.*, 2014). Therefore, Colton-Gagnon *et al.* (2014)'s work confirms that *B. distachyon* may be used to study cold-acclimation and freezing tolerance under laboratory conditions.

In light of the studies presented under this section, plant scientists showed that *B. distachyon* possesses pooid-specific traits in respect to cold-acclimation (i.e. *CBFIII* and *IV* clades, *IRI*, fructan). Adding to that its capacity to cold-acclimate and its accessibility for genetic studies, *B. distachyon* makes an ideal model for the study of cold-acclimation in pooid grasses. In such context, one could hypothesized that the closely related *B. sylvaticum* would be equally fit as a cold-acclimation model. However, one must consider the difference in life strategy between both species before making this assumption.

In fact, few studies had compared the response to cold between annual and perennial grasses. In a rare example, Hoffman *et al.* (2010) compared the response to cold between the pooid annual bluegrass and perennial creeping bentgrass. They conducted a WPFT on three months old plants that were acclimated at either 20, 2 or -2 °C during four weeks. Furthermore, they determined soluble sugars and amino acid accumulation just as verifying dehydrin expression. Hoffman *et al.*

(2010) found that the perennials that were cold-acclimated at -2°C had achieved higher freezing tolerance whereas cold-acclimation at warmer temperature rather advantaged the annuals. For all treatments, the perennial had accumulated more fructan than the annual while dehydrin expression patterns were similar in both species. Overall, Hoffman *et al.* (2010) concluded that the perennial creeping bentgrass possesses a greater potential for cold-acclimation than its annual counterpart. Nonetheless, because both compared species were not from the same genus, this difference in response to cold could be attributed to phylogenetic distance rather than life strategy. Therefore, observations by Hoffman *et al.* (2010) would not necessarily apply to a comparison between *B. distachyon* and *B. sylvaticum*.

In order to better direct the design of the current project, we reviewed the literature that treated of the response to cold specifically in perennial grasses.

2.3.4 Cold-acclimation in Perennial Pooids

Perennial ryegrass (*Lolium perenne)*, an economically important forage crop from the subfamily *Pooideae*, clade *Poaea* had been the most studied perennial grass in term of freezing tolerance and cold-acclimation. Indeed, many researchers had attempted to characterize what they termed 'winter hardiness', or the capacity to regrow at spring in *L. perenne* cultivar by verifying their freezing tolerance as a proxy. Lorenzetti *et al.* (1971) were among the first to test cold-acclimation capacity in perennial ryegrass. By conducting a series of WPFT, they determined that plants acclimated for 8 days and grown under 8hrs:16hrs/light:dark regime were most tolerant to freezing as opposed to other acclimation periods and photoperiods. The freezing tolerance for those plants under such condition was increased from -6 to -12 °C. Humphreys and Eagles (1988) conducted an electrolyte leakage test on two years old tissues collected on plants from eighty-six accessions from either Europe, USA or New Zealand. They observed values of Lethal Temperature at which 50% of the population dies (LT50s) that ranged between -3.0 and -12.6 °C. Followed the work of Tcacenco *et al.* (1989) which evaluated winter hardiness in sixteen Romanian accessions observing LT50s values ranging from -9.6 to -11.97 °C. More recently, Ebdon *et al.* (2002) conducted an electrolyte leakage test on tissues collected from five month old plants from different cultivars to compare their freezing tolerance. LT50s values ranged from -2.8 to -12.6 °C for cold-acclimated plants, and from 4.2 to -4.9 °C for non-acclimated plants. Hulke *et al.* (2008) also verified winter hardiness through conducting a WPFT in twenty-one *L. perenne* accessions that mostly originates

from Eurasia. They observed LT50s values ranging from -10.31 to -13.95 °C. Together, those studies report ranges of freezing tolerances that are consistent with what was observed in other pooid crops such as wheat (e.g. Fowler *et al.*, 1981). In the end, those LT50s values observed in *L. perenne* gives us an idea of the extent of the freezing tolerance range in a perennial grass. In this case, the range of LT50s is more extensive than the one observed in the annual grass *B. distachyon*. Therefore and by precaution, one should include a similarly extensive range when first exploring the freezing tolerance capacity of *B. sylvaticum* to rule out the possibility that its perennial attribute is associated to a larger range of survival than its annual counterpart.

As for molecular studies, Xiong and Fei (2006) were the first to identify and characterize a *CBF* ortholog of *L. perenne, LpCBF3.* Later, Tamura and Yamada (2007) found a cluster of five *CBF* genes of common origin with the barley and wheat *CBFIII-CBFIV* clades. Both Xiong and Fei (2006), and Tamura and Yamada (2007) observed that *LpCBFs* are induced in the first hours of exposition to cold in a similar fashion as in *Arabidospsis*, rice, wheat and *B. distachyon*. Also, Tamura and Yonemaru (2010) found a *CBF* regulon in both *L. perenne* and *Festuca pratensis*, a wild perennial pooid while comparing the freezing tolerance of both species. In a mapping study by Yu *et al.* (2015), *LpCBF1* was deemed the best candidate gene to be associated with post-winter regrowth capacity. In term of functional cold-responsive protein, Guerber *et al.* (2007) were able to characterize the expressions of dehydrins in drought-stressed *Lolium arundinaceum*, a close relative of *L. perenne*. Followed work by Zhang *et al.* (2009) which assembled a partial transcriptome for cold and drought stressed *L. perenne*. They observed that *COR*, *DHN* and *IRI* transcripts accumulation increased, whereas photosynthesis and respiration related transcripts accumulation decreased in response to cold-stress. Additionally, combined work from Lasseur *et al.* (2006), Hisano *et al.* (2008) and Abeynayake *et al.* (2015) helped to characterize the genes that are involved in the biosynthesis of fructan in *L. perenne*. In sum, these studies show that perennial pooid possesses some components of the *CBFs* pathway and that they seemingly play the same functions as in annual pooids.

Collectively, the findings presented under this section suggest that the phenotypical, physiological and molecular responses to cold in the perennial pooid are similar to that of their annual counterparts. Furthermore, the occurrence of the *CBFIII* and *CBFIV* clades, *IRI* and fructan production related genes in the aforementioned perennials shows that the pooid-specific response

to cold is conserved in this sub-family regardless of the life strategy. Therefore, we presume that the response to cold in *B. sylvaticum* will be similar to that of other pooid grasses regardless of its perennial status.

2.4 Histone Acetyltransferase Domain *GCN5*

As briefly mentioned in section 2.3.1, *GCN5* is a histone acetyltransferase member of the *SAGA* complex which actively modifies the chromatin epigenetic landscape. More specifically, *GCN5* was linked to gene activation through an acetylation of specific histone lysine residues (Brownell *et al.*, 1996a-b). In fact, the upstream influence of *GCN5* on gene activation was linked to the response to cold in plants (e.g. Stockinger *et al.*, 2001; Vlachonasios *et al.*, 2003; Mayer *et al.*, 2015). Consequently, the study of *GCN5's* role in relation to cold-acclimation in pooid grasses figure among our research interests. The following paragraphs provide background information on *GCN5* as well as on the *SAGA* complex.

Discovered in yeast by Georgakopoulos *et al.* (1992), *GCN5* was first shown to be required for the transcription of *GENERAL CONTROL NON-REPRESSIBLE 4* (*GCN4*), itself an important transcription factor that regulates biosynthesis of amino acid in yeast (Hope and Struhl, 1985). In parallel, Berger *et al.* (1992) and Pińa *et al.* (1993) observed similar interaction between *GCN4* and, respectively, *ADA2* and *ADA3* suggesting a possible link between the latter two and *GCN5*. Indeed, *in vivo* two-hybrid assay as well as *in vitro* co-immunoprecipitation conducted in Marcus *et al.* (1994) confirmed physical interaction between *ADAs, SUPRESSOR of TY* (*SPT*) and *GCN5*. Subsequent work by Grant *et al.* (1997) showed that *GCN5* alone was unable to acetylate nucleosomal histone. This function could only be performed when *GCN5* was part of the *SAGA* complex, which include *SPT* and ADAs. A few month later, Robert and Winston (1997) observed physical interaction between *GCN5* and other component of, or homologous to the *SAGA* complex in an experiment that include yeast mutants, thus further supporting the *SAGA* complex hypothesis. Most importantly, combined work from Brownell *et al.* (1996a-b) and Kuo *et al.* (1996) details how *GCN5* act as a nuclear histone acetyltransferase that affects genes activation. Kuo *et al.* (1996) showed that *GCN5* acetylates lysine residues number 14 and 9 of histone H3 (H3K14 and H3K9) just as lysine residues number 8 and 16 of histone H4 (H4K8 and H4K16). Such epigenetic marking seemingly play a role in enabling genes transcription. A sequence homology analysis completed by Neuwald and Landsman (1997) confirmed that *GCN5* related proteins belong to a

superfamily of *N-acetyltransferases* proteins (*NATs*) based on motif shared with the *MYST* protein family, itself involved in nucleosomal modification. In 2000, Hudson *et al.* (2000) identified and described a bromodomain associated to *GCN5*. Interestingly, this domain allows the protein to recognize acetylation marks of lysine residue on histone such that it is also capable of "reading" the epigenetic landscape of the chromatin.

Highly conserved in prokaryotes and eukaryotes alike (Sterner and Berger, 2000), *GCN5* was first studied in plant by Stockinger *et al.* (2001), which explored its influence on *Arabidopsis*'s response to cold through the *CBF* pathway. Later nuanced by Vlachonasios *et al.* (2003), the influence of *GCN5* on cold response in *Arabidopsis* appear indirect as other member of the *SAGA* complex have different effects on freezing tolerance. In the same publication, the researchers highlighted that disrupted *GCN5* mutants had dwarf growth, elongated hypocotyl and flower tissues deformities. Similar observations were reported in Bertrand *et al.* (2003) whom discovered that *GCN5* is involved in regulating the development of the floral meristem in *Arabidopsis.* Moreover, Cohen *et al.* (2009) observed an up-regulation in *GCN5* over-expressor mutants of the gene *LEAFY* which determine the identity of developing floral tissues. In complement, He *et al*. (2003) showed that *GCN5* influences flowering time in *Arapidopsis* through the regulation of the *FLOWERING LOCUS C* (*FLC*) pathway. Along with work from Benhamed *et al.* (2006) that shows direct interaction between light-regulated genes and *GCN5* or Kornet and Scheres (2009) which links *GCN5* to stem cells maintenance in roots, those observations imply that *GCN5* is both an important regulator of development and environmental response in *Arabidopsis*.

In fact, the *SAGA* complex plays an active role in plant response to environmental cue. In a thorough study of the response to light in germinating *Arabidopsis* seedling, Charron *et al.* (2009) observed a significant appreciation of acetylated H3K9 along with other variations of histone modifications in seedlings transferred to light. These variations of the histones landscape were correlated with the down regulation and upregulation of many genes, notably an upregulation for genes associated to the chloroplast, the cytosol and the ribosome. Observations by Charron *et al.* (2009) suggest that epigenetic modifiers like those of the *SAGA* complex play a primal role in activating the transcription of primary metabolism genes in response to an environmental cue. More in line with our research interests, scientists also linked the action of epigenetic modifier to plants response to abiotic stresses. For instance, JM Kim *et al.* (2008) positively correlated an

enrichment of acetylated H3K9 with drought responsive genes in *Arabidopsis*. Notably, work by Stockinger *et al.* (2001) linked the *SAGA* complex to cold response in *Arabidopsis* while Vlachonasios *et al.* (2003) had observed an overall decrease in the expression of stress related transcript in their disrupted *GCN5 Arabidopsis* mutants. While studying cold-acclimation in hemp, Mayer *et al.* (2015) highlighted a trend in the acetylation of H3K9 at the N-terminal of *COR413pm*: tolerant accessions exposed to cold were found to re-set the amount of acetylation to levels that were equivalent to that of non-acclimated conditions, whereas the acetylation of vulnerable accessions decreased in response to cold exposition. This observation was correlated with a relatively higher accumulation of *COR413* transcripts in cold-tolerant cultivar (Mayer *et al.*, 2015).

Together, findings reported in the previous paragraph suggest that an increase in histones acetylation at stress responsive loci is associated to a stronger response to abiotic stresses in plants. This conclusion led our research group into the study of *GCN5* in *B. distachyon*. Recently, Martel *et al.* (2017) identified three splice variants transcript of the *GCN5* gene in *B. distachyon*. Out of those three, two are interacting with *ADA2* from the *SAGA* complex (*wtGCN5* as translated in higher proportions followed by *Long-GCN5*) while a shorter version (*Short-GCN5*) is being degraded through non-sense mediated decay. Such work provide insight into the higher protein level organisation of the *SAGA* complex in *B. dystachyon* and lays the foundation to initiate the study of the effect of *GCN5* on response to abiotic stresses in the species. It also enabled the study of *GCN5* in *B. sylvaticum* as presented in the current project.

Chapter 3. MATERIALS AND METHODS

3.1 Plant Material and Growth Condition

Nine different accessions of *B. sylvaticum* were selected from the seed catalog of the United State Department of Agriculture's National Genetic Resource Program (USDA-GRIN) according to seed availability and geographical origins. We therefore obtained a selection of accession that covers a wide, diverse range of geographical origin (Figure 2). Background climatic information and PI numbers of the studied accessions are presented in Table 1. Unless stated otherwise, plant material was grown the following way: seeds were soaked in nano-filtered water with gentle agitation for 3 hours, drained and stratified for 14 days at 4°C in the dark. Once stratified, five seeds were planted per 4-inch pots containing Fafard's growing mix G2™ (Fafard et frères Ltée, Saint-Bonaventure, QC) inoculated with inert clay containing the fungal control agent *Trichoderma harzianum* (RootShield WP®, Bioworks, Victor, NY) for the preventive control of Pythium root disease. Pots were then kept under control conditions (22 °C, 20 hr light: 4 hr dark photoperiod, ~ 220 µmol/cm^2) for 35 days in order to develop seedlings with enough aboveground tissues to conduct our experiments (~ 3-5 leaves stage).

3.1.2 Flowering and Reproduction

We conducted an exploratory experiment to determine the flowering and reproduction capacity of the studied accession when using our laboratory resources. Briefly, eighteen weeks-old plants grown under control conditions (n=15 per accessions) were moved at 4°C in the dark during 15 weeks to mimic overwintering. Plants were then moved back to control conditions for another 15 weeks during which flowering time and maximum inflorescence height were recorded when it applied. The germination rate of produced seeds was qualitatively assessed using the following arbitrary classes: very poor (under 20.0% of germination rate), poor (between 20.1% and 40.0% of germination rate), medium (between 40.1% and 70.0%), good (between 70.1% and 90.0%) and very good (over 90.1% of germination rate). Germination rates were informally observed while optimizing our cultivation of *B. sylvaticum* using our laboratory resources.

3.1.3 Long-Term Cold Exposure Kinetic

Thirthy-five days-old seedlings were moved to a low-temperature growing chamber and kept for 14 days (CA14) under cold-acclimating conditions. More specifically, the chamber temperature

Figure 2. Origin of the studied *Brachypodium sylvaticum* accessions. 1: PI564896 from Stavropol, Russia (51.41 N, 87.13 E). **2**: PI384810 Astara county, Iran (38.50 N, 48.20 E). **3**: W6 23524 from Gansu province, China (34.02 N, 102.36 E). **4**: PI268222 nearby Gorgan, Iran (36.71 N, 54.51 E). **5**: PI610793 from Vlore, Albania (40.48 N, 19.58 E). **6**: PI344569 from Slovakia (48.75 N, 18.82 E). **7**: PI297868 from Canberra, Australia (-35.38 N, 148.62 E). **8**: PI440178 from Atyrau, Kazakhstan (47.61 N, 51.92 E). **9**: PI269842 from Ain Draham, Tunisia (36.78 N, 8.78 E). Approximated coordinates based on the collection notes from the United State Department of Agriculture's National Genetic Resource Program (USDA-GRIN).

Table 1. Background climatic information

NPGS accession number	Coordinates[1]	Light : dark photoperiod[2] (hours)	Mean maximum / mean low temperature[3] (°C)	Mean precipitations[3] (mm)
PI564896	51.41 N ; 87.13 E	8 : 16	1 / -5	34
PI384810	38.50 N ; 48.20 E	9.7 : 14.3	11 / 5	24
W6 23524	34.02 N ; 102.36 E	9.3 : 14.7	6.5 / -2.8	9
PI268222	36.71 N ; 54.51 E	9.8 : 14.2	13 / 3	57
PI610793	40.48 N ; 19.58 E	9.6 : 14.4	15 / 6	147
PI344569	48.75 N ; 18.82 E	8.3 : 15.7	2 / -4	54
PI297868	-35.38 N ; 148.62 E	14.5 : 9.5	12 / 1	38
PI440178	47.61 N ; 51.92 E	8.3 : 15.7	- 1 / -7	13
PI269842	36.78 N ; 8.78 E	9.8 : 14.2	13 / 5	227

1. Approximated coordinates based on the collection notes from the United State Department of Agriculture's National Genetic Resource Program (USDA-GRIN).
2. Projected photoperiods calculated for December 10th 2018 in all accessions except PI297868 for which it was calculated for June 10th 2018.
3. For the month of December for all accessions except PI297868 for which the data are for the month of June.
Photoperiod calculator and climatic data were accessed through the web site of the United States National Center for Environmental Information of the National Oceanic and Atmospheric Administration (NCEI-NOAA); www.ncdc.noaa.gov.

was set at 4°C, the photoperiod at 8 hr of light: 16 hr of dark and the amount of light at ~ 220 µmol/cm^2. Thirty-five days-old seedlings grown as described in section 3.1 were used as non-acclimated controls (NA). Tissues for the long-term cold exposure kinetic (*BsCOR413* and *BsCOR410* genes expression) were sampled from the plants that were grown under cold-acclimation conditions as described above. We collected and pooled young tissues from at least five different plants per treatment per accessions by cutting the stems near the soil interface for a total of about 50 mg of tissues per time points. We collected tissues from non-acclimated controls (NA), and from cold-acclimated plants that were kept for 7 days (CA7) and 14 days (CA14) under cold-acclimation conditions. Tissues were systematically collected at 2:00 PM, Eastern Time to avoid circadian rhythm variability. Once collected, tissues were quickly flashed frozen in liquid nitrogen, then stored at -80°C before being processed for later RNA extraction. This experiment was replicated three times.

3.1.4 Short-term Cold Exposure Kinetic

Plant material used for the short-term cold-exposure kinetic (*CBF2.1* gene expression) was grown differently than for other experiments reported in this study. We first soaked, stratified and sowed seeds from the accession PI269842 as described in section 3.1. Then, we placed the pots under non-acclimated conditions (NA, 22°C, 8 hr light: 16 hr dark photoperiod, ~ 140 µmol/cm^2) for a growth period of 14 days. On the 15th day, a set of nine pots containing five plants was kept under NA control conditions while another set nine pots containing five plants was moved under cold-acclimated conditions (CA, 4°C, 8 hr light: 16 hr dark photoperiod, ~ 140 µmol/cm^2) at precisely 9:00 AM. Tissues were collected under NA at time 0, 3, 6 and 9 hour and at times 3, 6 and 9 hour after being moved under CA conditions. At least five plants per times point were collected by cutting the stems near soil interface and pooled to obtain a total of about 25 mg of tissues. Once collected, tissues were quickly flashed frozen in liquid nitrogen, then stored at -80°C before being processed for RNA extraction. This experiment was replicated three times.

3.2 Whole-Plant Freeze Test

For each accession, we conducted a series of standard WPFTs on cold-acclimated 14 days (CA14) and non-acclimated (NA) plants as respectively described in sections 3.1 and 3.1.3 to determine their freezing tolerance. NA and CA14 plants were moved into a programmable test chamber specifically designed to conduct freeze tests (Model LT-36V, Percival Scientific Inc., Perry, IA).

To minimize the variability in the cooling rate of the plant samples during the test, pots were saturated with water 5 hours before being moved into the chamber. Then, pots were loaded onto the shelves of the chamber following a random pattern with an equal number of pots per tested accession/treatment per shelf (n=15 per shelf, n=45 in total). Pots were placed 5 centimeter apart from each other and from the chambers' walls to enable adequate air circulation. After a 2 hr equilibration period at -3°C, the temperature in the chamber was lowered at a rate of -1 °C/h. More precisely, the temperature was decreased in the first 5 minutes to then plateau for the remaining 55 minutes. After 55 minutes at -5, -7, -9 or -11 °C, four randomly selected pots for each treatment/accession were removed from the chamber. Those four pots represented a single replicate. See Figure 3 for a graphical representation of the WPFT temperature sequence. The test was conducted in the dark. Once removed from the chamber, plants were quickly moved to 4°C in the dark for 24 hours and then to 22 °C in the dark for 48 hours to reduce thawing stress. Plants were then allowed a recovery period of 14 days under control conditions before counting survival. This experiment was replicated three times for each accessions.

3.3 Molecular Study

3.3.1 Identification of Candidate Genes and Primers Design

Identification of candidate genes was initially done through a data mining strategy. We opted for such strategy because the genomic data for *B. sylvaticum* were still not available to the public at the beginning of our project. Briefly, the coding sequences of candidate genes were identified by comparing the sequence of homologous proteins of *B. distachyon* using Basic Logical Alignment Search Tools (BLAST) as provided by both the National Center for Biotechnology of the National Institute of Health (NCBI-NIH) and the United States Department of Energy's Joint Genome Institute (DOE-JGI). Homologous protein sequences from others species such as wheat (*Triticum aestivum*), maize (*Zea mays*), barley (*Hordeum vulgare*) and perennial ryegrass (*L. perenne*) were also used to confirm the identity of certain candidate genes, especially *SAMDC* and *CBF2.1*. The coding sequences of all our candidate genes were sourced from the database Phytozome v12.1 provided by DOE-JGI to the exception of *SAMDC* and *COR410*. The coding sequences of those two genes were initially obtained by comparing ESTs from the draft transcriptome of *B. sylvaticum* (Fox *et al.*, 2013) and later confirmed using Phytozome v12.1 BLAST tool. We conducted further verification of coding and protein sequences using the multiple sequence analysis tool Clustal-

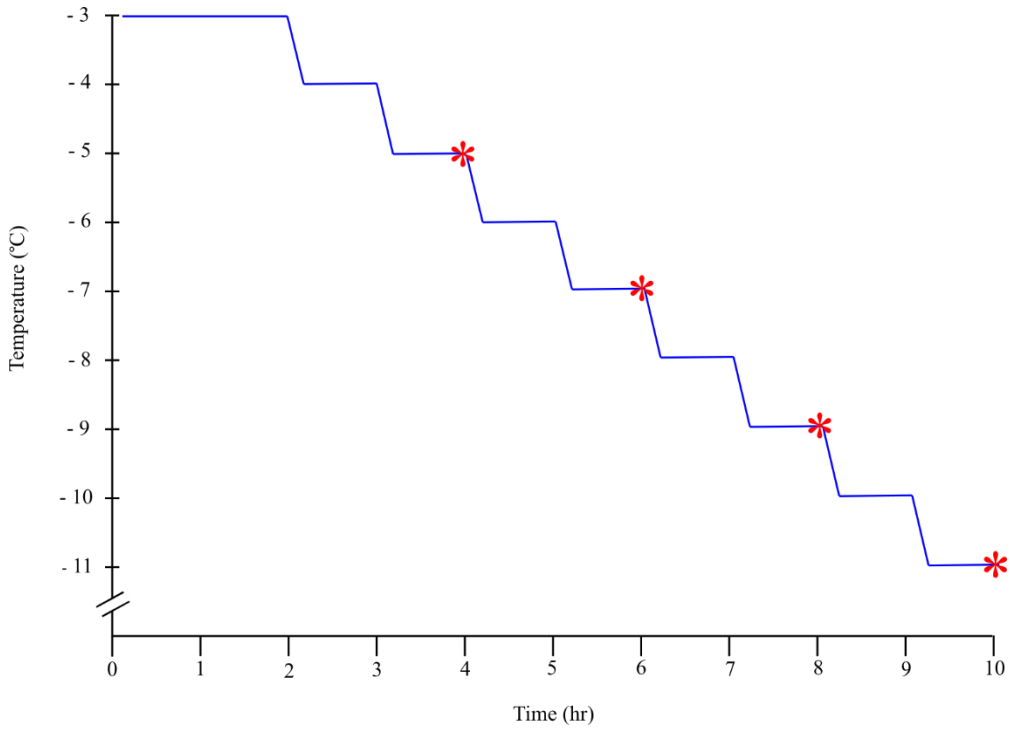

Figure 3. Temperature sequence of the Whole-plant Freeze Test. The blue line show the progress of the temperature (y axis) within the test chamber in function of time (x axis) during our standard WPFT. Note the 2 hr equilibration period at - 3°C at the beginning of the test. The red stars indicate the time at which plants were removed from the chamber after being exposed during 55 min at either -5, -7, -9 or -11 °C.

Omega provided by the European Bioinformatics Institute (EMBL-EBI). The codon translator tool ExPASy Translate available on the Bioinformatics Portal of the Swiss Institute of Bioinformatics (SIB) was also used to confirm the amino-acid sequence of candidate genes. We further used PROSITE from the same portal to determine protein domain of interest as well as tools from the Sequence Manipulation Suite (SMS) to determine sequences identity percent and similarity percent.

Every proposed primer pair was designed using Primer BLAST from NCBI-NHI and Olygo Analyser 3.1 (Integrated DNA Technologies (IDT Inc., San Jose, CA). The complete list of primers used in this study is presented in Table 2. The specificity of the amplicons obtained from each primer pair was confirmed through capillary electrophoresis DNA sequencing (ABI/Life Technologies 3730x system, Life Technologies, Foster City, CA) as ordered to, and proceeded by ACGT Corp. (Toronto, ON).

3.3.2 RNA Extraction and cDNA Synthesis

RNA extraction was performed following the EZ-10 Spin™ Column Plant RNA Miniprep Kit protocol from Bio Basic Inc. (Markham, ON). Briefly, 25 mg of the collected tissues presented in sections 3.1.3 and 3.1.4 were first ground in liquid nitrogen into a fine powder, hydrated with a cell-lysis buffer and incubated at room temperature for five minutes before being centrifuged. Supernatants were mixed with ethanol in a 1:1 volume ratio. The mixture was then loaded on EZ-10 Spin Column™ and spun for 30 seconds. At this step, the column was treated with DNase I (RNase-Free DNase Set™, Bio Basic Inc., Markham, ON) for twenty minutes at room temperature to avoid genomic DNA contamination. The column was then washed with the solutions GT and NT before eluting the RNA into 30 ul of sterile, RNase-free water. Quality of the extracted RNA was assessed visually by loading the samples on a 1% agarose gel as well as by monitoring concentration and purity ratios through spectrophotometry (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA). Samples that failed to meet quality standard, that is a 260:280 ratios above 1.9 and a 260:230 ratios above 1.5 were cleaned-up using the EZ-10 Spin™ Column RNA Cleanup and Concentration Kit from Bio Basic (Markham, ON). About 15% of our samples initially failed to meet quality standards.

Table 2. List of primers used in this study.

Target gene	Sequence	Amplicon size (bp)
EF1	Forward: 5'- CGA CAG GCG ATC TGG TAA GG - 3' Reverse: 5' - ACG ACC AAG AGG AGG GTA CAT - 3'	133
UBC16	Forward: 5'- CGA GTT CCT CGA CTG CTT C - 3' Reverse: 5' - CTT GTA CTT GAA CCC AGC GG - 3'	107
GAPDH	Forward: 5'- GCA GGG CTG CAA GCT TTA AC - 3' Reverse: 5' - AAG GTT TCC CTC AGA TGC CG - 3'	215
SAMDC	Forward: 5'- TTT CGA GCC ATG CGG TTA CT - 3' Reverse: 5' - GAA ATG GAG GCA GGG TCC AA - 3'	78
COR413	Forward: 5'- ACT TCG TCG GCA TCG CCA TA - 3' Reverse: 5' - CCG GGT AGA TGA AGA GCA GGA - 3'	84
COR410	Forward: 5'- GAG GGC GAG CAA AAG ACG A - 3' Reverse: 5' - TTC TTT TCC TCC TCG GGT GC - 3'	149
CBF2.1	Forward: 5'- GGC GTA CGA CGA ATT TGC AT - 3' Reverse: 5' - GCG CCA TAT CCA TAA CAG CC - 3'	162
GCN5	Forward: 5'- GCA ACA TGC CCG AGA TGC TGA TG- 3' Reverse: 5' - CTC TCT GAT CTT CTC ATC AAT AGC CTG CC - 3'	250
GCN5/ AcV5	Forward: 5'- ATG CTC CAC GAG GTT AGA GG - 3' Reverse: 5' - CCG CTC GCA TCT TTC CAA GA - 3'	135
GCN5/ pANIC-6A	Forward: 5'- TTA GCC CTG CCT TCA TAC G - 3' Reverse: 5' - CAA ATA TCA TGC GAT CAT AGG CG - 3'	1818

EF1: *Elongation Factor-α1*. *UBC16*: *UBiquitin Conjugating enzyme-16*. *GAPDH*: *GlycerAldehyde-3-Phosphate-DeHydrogenase*. *SAMDC*: *S-AdenosylMethionine DeCarboxylase proenzyme*. *COR413*: *COld-Regulated 413*. *COR410*: *Cold-Regulated 410*. *CBF2.1*: *C-repeat Binding Factor 2.1*. *GCN5*: *General Control Non-depressible 5*. *AcV5*: *Autographa californica Virus 5* (Epitope). *pANIC-6A*: *Paniculatum virgatum 6A* (*E-Coli* plasmid).

cDNA was synthesized by adapting the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR by Bio-Rad Laboratories Ltd (Hercules, CA) for half-reactions. Input of RNA was adjusted to obtain 495 ng of total cDNA for each sample.

3.3.3 gDNA Extraction

Genomic DNA was extracted with a Chloroform/Phenol/Isoamylalcohol extraction protocol (Mayer *et al.*, 2015). Briefly, 200 mg of young tissues were collected from mutants and control plants (see section 3.4) and then flashed frozen using liquid nitrogen. Tissues were ground into a fine powder, then hydrated with 2 ml of extraction buffer (50 mM Tris HCL pH 8, 50 mM EDTA, 1 % PVP, 3 % SDS) plus 10 ul of beta-mercaptoethanol (Fisher Scientific, Hampton, NH) and 20 ul of proteinase K (Fisher Scientific, Hampton, NH). Samples were then incubated at 65°C in a water bath for an hour with occasional mixing. After incubation, 1: 1 volume of Chloroform: Phenol was added to the sample, vortexed briefly and centrifuged for 20 minutes. The resulting supernatant was kept and mixed with an equal amount of 24: 1 volume of Chloroform: Isoamylalcohol, then vortexed briefly and centrifuged for 10 minutes. The resulting upper phase was mixed with equal amount of Isopropanol and $1/10^{th}$ volume of 3M Sodium Acetate at pH 5.2. The sample was centrifuged for 10 minutes so that a pellet could form. The pellet was drained, resuspended in TE buffer (10:1, pH8) and incubated at 65°C during 10-15 minutes. Following this step, the sample was washed with pure ethanol first, then in a 70% solution of ethanol to be finally resuspended in TE buffer. Quality of the extraction was verified by loading gDNA samples on a 1% agarose gel, and by monitoring concentration and purity ratios through spectrophotometry (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA). Samples that did not meet quality standard (260:280 ratios above 1.8 and below 2.1, 260:230 ratios above 1.5 and concentration below 50 ng/ul) were re-extracted. About 30% of our samples initially failed to meet quality standards.

3.3.4 PCR and RT-qPCR

PCR reactions were conducted on a MyCycler™ thermocycler by Bio-Rad (Hercules, CA). The cycle started with an initial denaturation phase at 95°C during 30 seconds, then a second denaturation phase at 95°C for 30 seconds repeated 30 times, an annealing phase at 60 °C during 30 seconds repeated 30 times, an extension phase at 68°C during 1.5 minutes repeated 30 times, and a final extension phase at 68°C for 5 minutes. PCR mix was adjusted to result in 25 ul of

product and was optimized as such: 14.625 ul of sterile water, 2.5 ul of 10 X Coral Load Buffer (Qiagen, Hilden, DE), 1.25 ul of Dimethyl Sulfoxide (Sigma-Aldrich, St-Louis, MO), 0.5 ul of 10 mM desoxyribonucleotides (dNTPs), 0.5 ul of 100 mM forward and reverse primers, 0.125 ul of Taq DNA polymerase (New-England Biolabs, Ipswich, MA) and 5 ul of gDNA template adjusted for a total input of 250 ng.

We performed RT-qPCR on a CFX Connect™ Real-Time system from Bio-Rad Laboratories Ltd (Hercules, CA) controlled with the Bio-Rad CFX Manager 3.1 software. Reactions were conducted using the Green-2-Go™ qPCR Mastermix by Bio Basic Inc. (Markham, ON). Reactions were adjusted for the input of 3 ul of cDNA template for a total reaction of 10 ul and primers were used at a concentration of 10 pmol/ul. Each set of reactions was optimized by preliminarily conducting a primer temperature gradient as well as a five point standard curve (Figure 4). Assays were performed in technical triplicates into compatible 96 wells plates sealed with clear microfilm. Three biological replicates per gene/treatment/accession as presented under subsections 3.1.3 and 3.1.4 were conducted. Fold-increase results were calculated following the double delta cycle threshold method or Livak method (Livak and Schmittgen, 2001) using non-acclimated controls, or wild-type control as calibrators. Standard genes *BsEF1*, *BsUBC16*, *BsGAPDH* and *BsSAMDC* were tested while verifying cold-regulated transcript accumulation in accession PI564896. Accordingly, we selected *BsEF1* to perform RT-qPCR reactions in the remaining accessions based on the stability of its amplicon (Figure X; further discussed in Chapter 5).

3.4 Plant Transformation

The following section describes a modified version of a high-efficiency transformation protocol (Figure 5) as designed for *B. sylvaticum* by Steinwand *et al.* (2013).

3.4.1 Calluses Culture

Immature seeds of PI269842 were harvested 14 days after inflorescence emergence from parental plants that were grown under NA conditions as described in section 3.1. The lemma and palea were peeled off prior to the surface sterilization of seeds by soaking them in a solution of 5.25% NaOCl and 0.1% Triton™ X-100 (Sigma-Aldrich, St-Louis, MO) during 4 minutes with mild agitation. The seeds were rinsed four times using sterile, double-distilled water before the excision of embryos under sterile environment. About 15 embryos were gently placed on Callus Inducing

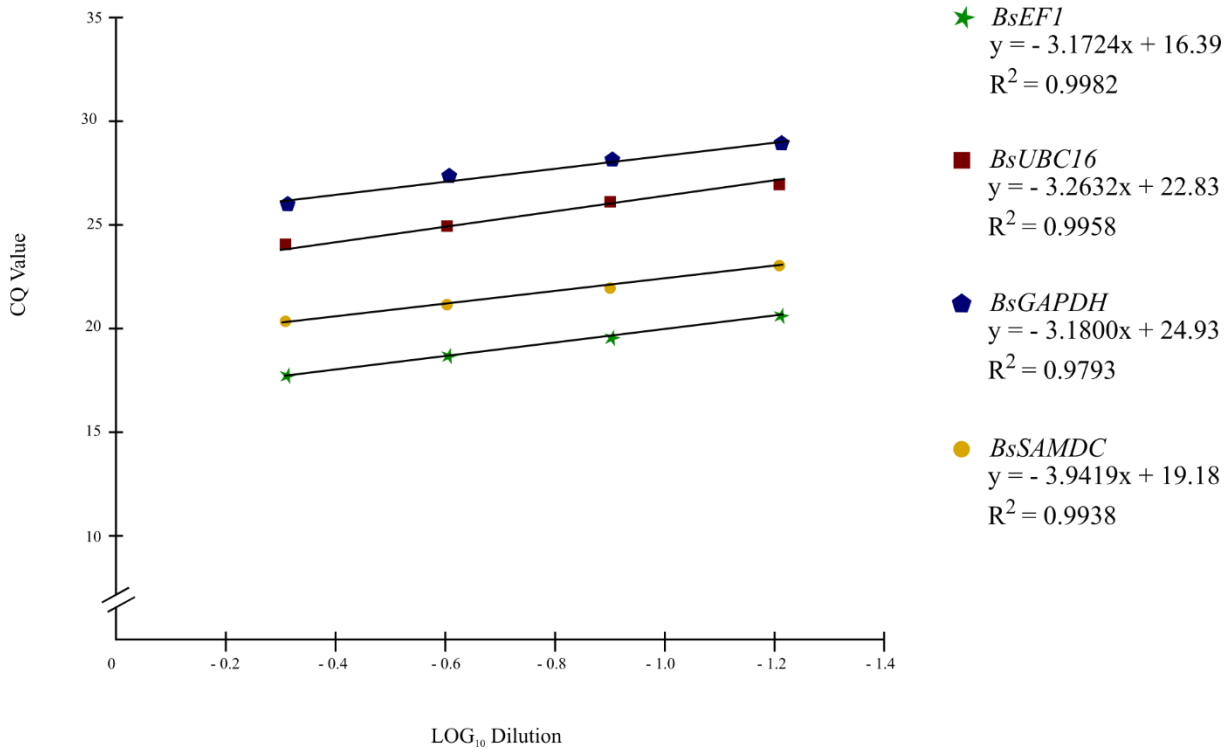

Figure 4. Standard curves of putative standard genes. CQ values of amplification ('y' axis) in function of LOG_{10} dilution ('x' axis) with a dilution factor of 10^{-2}. Initial five points curated down to four to avoid a biased skewing of the curves equations caused by a point using pure, undiluted cDNA template.

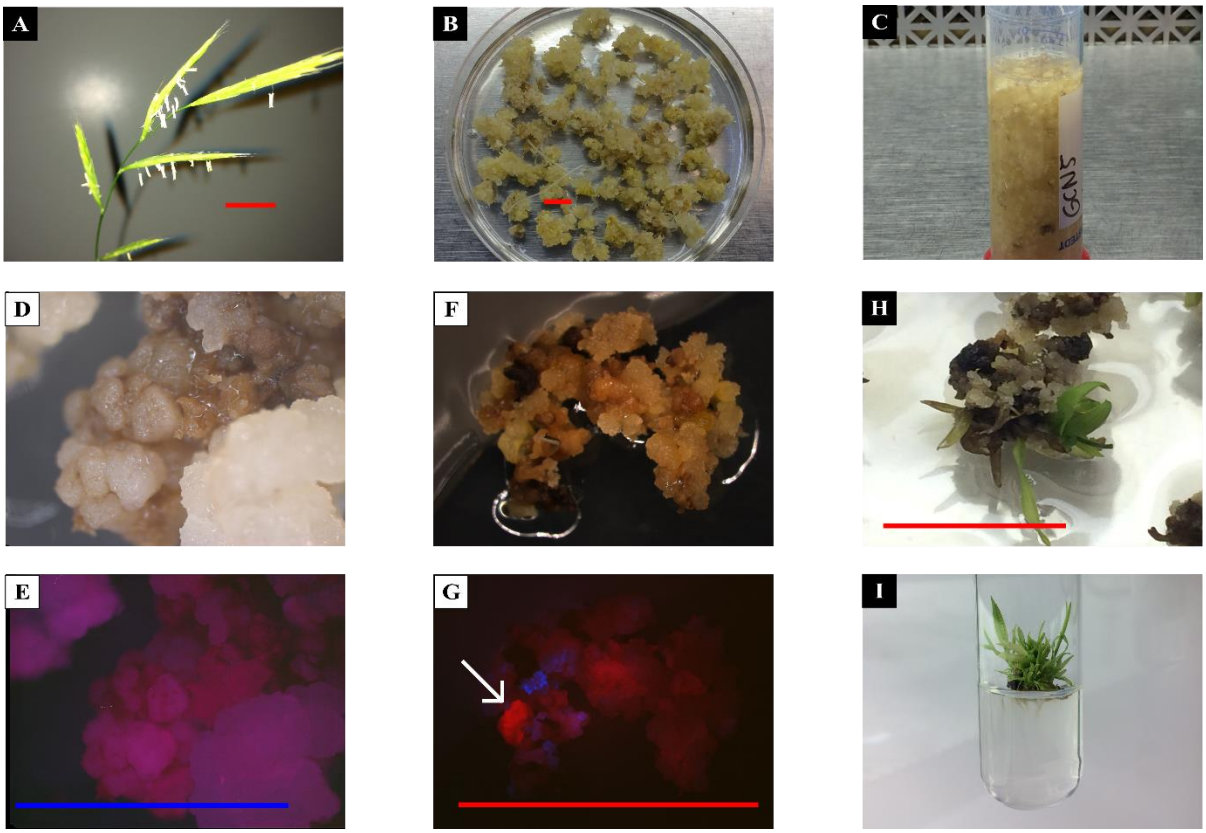

Figure 5. Transformation protocol of *Brachypodium sylvaticum*. A. Spike with mature anthers of *B. sylvaticum* accession PI269842. **B:** 60 days-old calli cultured on LS media enriched with vitamins. Calli shown right before being transformed. **C:** Calli being incubated with *Agrobacterium* strain AGL1. **D:** Untransformed control callus visualized under bright-field. **E:** The same untransformed control callus as in D exposed under UV light and visualized though a Red Fluorescent Protein (RFP) filter. **F:** Bright-field image of a callus transformed with EV during dark incubation. **G:** The same callus as in F exposed under UV and visualized through a RFP filter, the white arrow indicates a transformation foci. **H:** Transformed callus regenerating on LS media. **I:** Transformed plantlets regenerating on regular MS media in a culture tube. Red lines = 1 cm, blue line = 0.5 cm.

3.4.2 *Agrobacterium*-mediated Transformation

Glycerol stock of *Agrobacterium* strain AGL1 that contained either the plasmid pANIC-6A Empty Vector (EV) or the construct pANIC-6A-*BdGCN5*, as selected through a pre-screening of available in-laboratory vectors, were plated on Yeast Extract bacto-Peptone (YEP) medium containing carbenicillin at a concentration of 100 mg/L (GoldBio™, Caisson Laboratories, Smithfield, UT), and kanamycin at a concentration of 50 mg/L (GoldBio™, Caisson Laboratories, Smithfield, UT). YEP plates were then incubated at 28°C during 48 hours. Resulting bacterial growth was streaked with sterile capillary and resuspended in liquid CIM containing 200uM acetosyringone (Sigma-Aldrich, St-Louis, MO) to an Optical Density 600 (OD_{600}) of 0.6. Filtered-sterilized Kolliphor® P 188 (Sigma-Aldrich, St-Louis, MO) which acts as a surfactant was added at a concentration of 0.1%. Calluses were incubated in this bacterial suspension for 5 minutes with gentle mixing. After incubation, calluses were drained as much as possible. Moist calluses were laid on sterile paper towels to absorb excess bacterial suspension and transferred to petri dishes containing sterile filter papers. Petri dishes were left open under the sterile airflow of a laminar hood during a few additional minutes to improve the drying of the calluses. Petri dishes were then sealed using Parafilm™ (Sigma-Aldrich, St-Louis, MO) and incubated in the dark at 22 °C for 72 hours.

After the drying treatment, calluses were spread equally (~15 per plates) onto plates containing CIM media as described in section 3.4.1, but with the selection antibiotics hygromycin at a concentration of 40 mg/L (GoldBio™, Caisson Laboratories, Smithfield, UT), and timentin (control of *Agrobacterium*) at a concentration of 150 mg/L (GoldBio™, Caisson Laboratories, Smithfield, UT). Plates were incubated at 28 °C in the dark. Every 15 days, calluses were subcultured onto fresh plates containing the same media. After 30 days we moved the calluses into regeneration. At this point, we conducted a pre-screening of the transformed calluses using the properties of the pANIC-6A plasmid that includes a Red-Fluorescent Protein reporter gene (RFP). Calluses were exposed to UV light, then visualized with a RFP-adjusted filter using a stereo zoom microscope (Axio Zoom.V16™, Zeiss Microscopy GmbH, Jena, DE). Pictures of the calluses were acquired through the AxioCam Hrm™, and processed using the Z-stack module of the ZEN version 4.16 image acquirer software (Zeiss Microscopy GmbH, Jena, DE). Exported images using Ultra-Violet (UV) filter were color-enhanced using the Adobe® Photoshop CS6 Software (Adobe Systems, San Jose, CA) to faithfully reproduce what was visualized through the microscope.

3.4.3 Plants Regeneration

After the 30 days dark incubation, calluses were spread on Petri dishes containing the following regeneration media: 4.43 g/L of Linsmaier and Skoog basal media (Sigma-Aldrich, St-Louis, MO), 30 g/L of D-maltose (Phyto Technologies Laboratories®, Lenexa, KS) and 2 g/L of Phytagel™ (Sigma-Aldrich, St-Louis, MO). Filtered-sterilized kinetin was added to foster root development at a concentration of 0.2 mg/L as well as selection antibiotics (40 g/L hygromycin and 150g/L timentin). The pH of the mixture was adjusted to 5.8 after autoclaving. Petri dishes were kept in a growth chamber at 28°C with dimed light (~ 65 µmol/cm^2) following a 16 hr light: 8 hr dark photoperiod. Calluses greening occurred within the first 3 days and 10 weeks of exposition. Regenerated plantlets were transferred in culture tubes containing Murashige and Skoog media [4.42 g/L of Murashige and Skoog basal medium with vitamins (Sigma-Aldrich, St-Louis, MO), 30 g/L of sucrose (Phyto Technologies Laboratories®, Lenexa, KS), and 2 g/L of Phytagel™ (Sigma-Aldrich, St-Louis, MO), pH adjusted at 5.7 after autoclaving] and 150 g/L timentin. Culture tubes were kept under the same conditions as described above until the plantlets were big enough to be transferred into soil.

3.4.4 Transgenic Plants Screening

Fully regenerated plants were screened to confirm their transgenic status through a PCR method. This method required a specific pair of primers that amplified the entire *BdGCN5* sequence plus some plasmid sequence beyond the insertion sites (see *GCN5/pANIC-6A* in Table 1) using gDNA as template. Apart from screened transgenics gDNA samples, the PCR included pANIC-6A-*BdGN5* and EV positive control, wild-type *B. sylvaticum* gDNA samples (grown from cultured embryo but were not inoculated with *Agrobacterium*) as well as no-template controls. PCR products were loaded on a 1% agarose gel for weight confirmation and amplicon specificity was confirmed through sequencing. In addition to the PCR method, plants that were confirmed transgenic were submitted to a RT-qPCR to compare their *GCN5* gene expression relatively to that of a wild-type plant. RT-qPCR was proceeded as described in section 3.3.5. The presence for an amplicon that overlaps the epitope AcV5 and the end of the *BdGCN5* insert in the pANIC-6A-*BdGN5* plasmid was also tested. *GCN5* gene expression was reported using the wild-type expression as calibrator, while the product of the AcV5 reaction were loaded and visualized on a 1% agarose gel. All RT-qPCR amplicons were verified by sequencing. Along the transformation

process, used embryos, produced calluses, regenerated plantlets and successfully transformed plant were counted to calculate protocol efficiencies.

3.5 Statistical Analyses

All statistical analyses were performed using the open source software R version 3.3.0 (www.r-project.org) and the software interface R Studio version 0.99.902 (www.rstudio.com). Results from the WPFT were analysed by running an independent one-way ANalysis Of VAriance (ANOVA) with default setting (Null hypothesis, confidence interval = 95 %) for each temperature points. Here, the one-way ANOVA approach was preferred to a two-way assay because it is sufficient to compare the dependent variable "temperature" between two groups (NA vs CA) at each tested temperature points (-5, -7, -9 and -11 °C). In other words, we are not interested in assessing the statistical differences between NA and CA groups across the four tested temperatures as this information is biologically irrelevant to our research questions (Ross and Willson, 2017). Results from the molecular study were first fitted to an ANOVA model with default settings, then analysed using a post-hoc Tukey Honest Significant Different (HSD) test with default settings (confidence interval = 95 %). Furthermore, results were reported as the average of triplicates when applicable. Also, associated standard errors of sampling were calculated. Due to the exploratory and partly qualitative nature of the flowering experiment presented under subsection 3.1.2, we did not conduct statistical analysis on flowering, inflorescence height and germination rate results. This approach is consistent with similar type of results reported in Steinwand *et al.* (2013) for *B. sylvaticum* while preliminarily testing their capacity to reproduce the species using their laboratory resources.

Chapter 4. RESULTS

4.1 Accession PI269842 is easily reproduced using our laboratory resources

As a recent model organism, there is few information available about *B. sylvaticum*'s reproduction requirement under laboratory conditions. Therefore, we investigated *B. sylvaticum*'s capacity to reproduce using our laboratory resources by conducting an exploratory experiment that mimicked overwintering. In Table 2, we report the time in week after which overwintered plants had produced an inflorescence for the studied accessions. We also report the maximum inflorescence height observed as a supplementary source of morphological information as well as a qualitative evaluation of the germination rate in produced seeds. Out of the nine accessions, four flowered after 3 weeks (PI564896, PI384810, PI297868 and PI269842), two after 4 weeks (PI610793, PI440178) and one after 5 weeks (PI268222) of exposition to control conditions. The remaining two accessions (W6 23524 and PI344569) did not flowered after 15 weeks of test. Furthermore, inflorescence height varied between 48.5 and 94 cm. Overall, the germination of seeds that were produced by the flowering accessions was poor to the exception of PI269842. Hence, PI269842 is the only accession that could quickly produce a sufficient amount of good quality seeds that germinate at a rate of over 70.1% to undertake further experiments.

Table 3. Reproduction capacity of *B. sylvaticum* under our laboratory conditions.

NPGS accession number	Inbred line name	Origin	Time to flowering[1] (weeks)	Maximum inflorescence height (cm)	Germination rate[2]
PI564896	na	Stavropol, Russia	3	64	very poor
PI384810	Ast-1	Astara, Iran	3	63	poor
W6 23524	na	Xizang province, China	never	na	na
PI268222	Gor-1	Gorgan, Iran	5	48.5	very poor
PI610793	Vlo-1	Vlore, Albania	4	94	poor
PI344569	na	Slovakia	never	na	na
PI297868	Aus-1	Canberra, Australia	3	50.5	poor
PI440178	na	Atyrau, Kazakhstan	4	45	poor
PI269842	Ain-1	Ain Draham, Tunisia	3	59	high

1. 18 weeks-old plants (n=15 per accession) were kept at 4° C in the dark for 15 weeks to simulate overwintering. Plants were then moved to control conditions (22° C, 20:4 photoperiod) for observation up to 15 weeks after which non-flowering accession were scored "never".

2. Observed for seeds that were produced under our laboratory conditions only. Seeds supplied by the USDA-GRIN had otherwise medium, high or very high germination rates. Very poor (under 20.0% of germination rate), poor (between 20.1% and 40.0% of germination rate), medium (between 40.1% and 70.0%), good (between 70.1% and 90.0%) and very good (over 90.1% of germination rate).

4.2 Cold-acclimation in *B. sylvaticum* increases its freezing tolerance

We conducted a standard WPFT in which live plants are submitted to sub-zero temperatures in order to assess their survival as a proxy to freezing tolerance. Here, we report an increase in *B. sylvaticum*'s survival at freezing temperatures for plants that were cold-acclimated during 14 days (CA14) as compared to non-acclimated control (NA) in the nine tested accession (Figure 6). More precisely, Figure 6 shows significant increases in survival percentage between NA and CA14 plants at either -7 °C or -9°C for all accessions to the exception of PI344569 and PI269842 that lack statistical support. No plants survived at - 11°C regardless of the acclimation treatments and the accessions. Oppositely, all plants survived at -5°C with the exception of a few NA individuals in accessions W6 23524 and PI269842 (Figure 6 C and I). Table 4 summarizes the statistics related to the WPFTs. Overall, our results support our hypothesis that cold-acclimation mechanisms in *B. sylvaticum* increases its freezing tolerance when submitted to sub-zero temperatures.

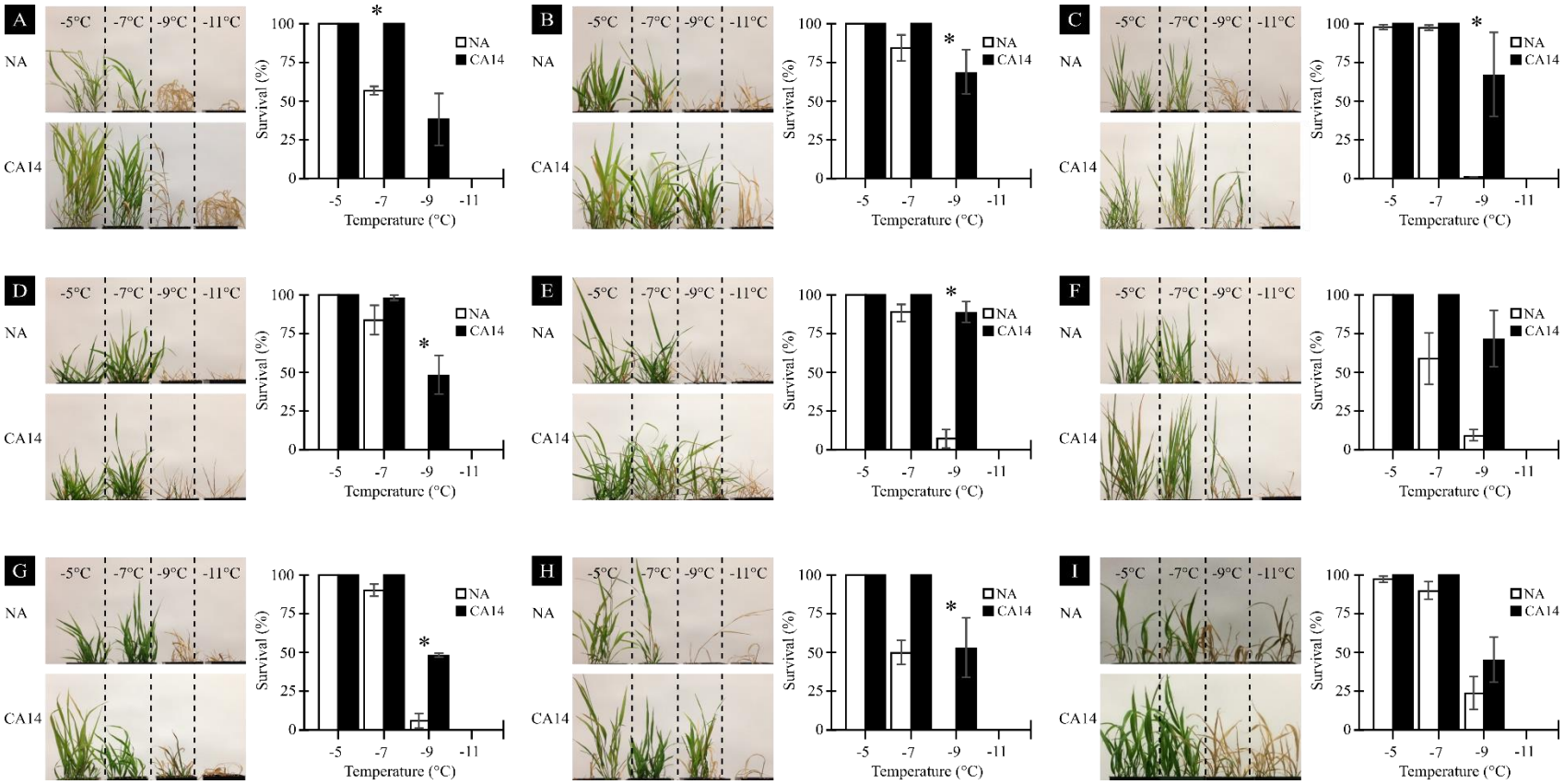

Figure 6. **Cold-acclimation increases survival at sub-zero temperatures in *B. sylvaticum*.** Survival (%) of non-acclimated (NA) and cold-acclimated plants for 14 days (CA14) in the following accessions **A**: PI564896, **B**: PI384810, **C**: W6 23524, **D**: PI268222, **E**: PI610793, **F**: PI344569, **G**: PI297868, **H**: PI440178, **I**: PI269842 as submitted to freezing temperatures of -5, -7, -9 and -11°C during a whole-plants freeze test. Statistical significance from a one-way ANOVA test ($\alpha = 0.05$) is shown by an asterisk. Pictures show representative plants collected at each temperature points for NA and CA14 treatments.

Table 4. One-way analysis of variance conducted on the results of the whole-plant freeze test.

Accession	Temperature (°C)	Degree of Freedom	Sum of square	Mean Square	F-value	p-value
PI564896	- 5	1	0	0	na	na
	- 7 *	1	2783	2783	119.6	0.000397
	- 9	1	2238	2237.8	3.44	0.137
	- 11	1	0	0	na	na
PI384810	- 5	1	0	0	na	na
	- 7	1	366.4	366.4	2.093	0.221
	- 9 *	1	7013	7013	14.78	0.0184
	- 11	1	0	0	na	na
W6 23524	- 5	1	7.407	7.407	1	0.374
	- 7	1	9.86	9.862	1	0.374
	- 9 *	1	7013	7013	14.78	0.0184
	- 11	1	0	0	na	na
PI26822	- 5	1	0	0	na	na
	- 7	1	293.3	293.3	1.284	0.32
	- 9 *	1	3471	3471	9.821	0.0351
	- 11	1	0	0	na	na
PI610793	- 5	1	0	0	na	na
	- 7	1	178.1	178.13	2.173	0.214
	- 9 *	1	9761	9761	47.35	0.00234
	- 11	1	0	0	na	na
PI344569	- 5	1	0	0	na	na
	- 7	1	2518	2518.1	3.916	0.119
	- 9	1	5856	5856	7.497	0.052
	- 11	1	0	0	na	na
PI297868	- 5	1	0	0	na	na
	- 7	1	143	143	3.405	0.139
	- 9 *	1	2679.6	2679.6	46.97	0.00237
	- 11	1	0	0	na	na
PI440178	- 5	1	0	0	na	na
	- 7 *	1	3750	3750	27	0.00653
	- 9	1	5856	5856	7.497	0.052
	- 11	1	0	0	na	na
PI269842	- 5	1	15.43	15.43	1	0.356
	- 7	1	210.9	210.86	2.297	0.18
	- 9	1	870	870	0.982	0.36
	- 11	1	0	0	na	na

* Statistically significant at a confidence interval of 95 %. na= non-applicable.

4.3 *B. sylvaticum* possesses cold-responsive molecular mechanisms

4.3.1 Identification of cold-responsive and standard gene candidates

We conducted a sequence homology search using heterologous sequences to identify the three cold-responsive genes *BsCOR413*, *BsCOR410* and *BsCBF2.1*, and the four standard genes *BsEF1*, *BsUBC16*, *BsGAPDH* and *BsSAMDC*. More specifically, we compared the nucleotide coding sequence of each candidate gene in *B. sylvaticum* to those of heterologous genes from other, better described species such as wheat and *B. distachyon.* In Table 5, we present the identity and similarity percent values associated to such comparison. Identity percent refers to the proportion of perfect matches between two aligned sequences while similarity percent account for both perfect matches and matches based on conserved function (Kanduc, 2012). From Table 5 we gather that *BsCOR413*, *BsCBF2.1*, *BsEF1*, *BsUBC16*, *BsGAPDH* and *BsSAMDC* are the most similar to described homologs in *B. distachyon* with identity and similarity percent values that are both above 96.0 % to the exception of *CBF2.1* which is respectively 83.5% identical, and 83.6% similar between both species. Then follow compared homologs from *T. aestivum* with percent values that range between 78.2% and 92.4% (identity) and 78.2% and 92.6% (similarity). Note that *BsCOR410* could only be compared to *TaCOR410c* (84.3% identity; 84.7% similarity) because it has yet been described in other species. We further compared the sequences of *BsEF1*, *BsUBC16*, *BsGAPDH* and *BsSAMDC* to homologs in *Arabidopsis* to generally observe lower ranges of identity (60.5% to 86.6%) and similarity (60.6% to 86.6%) percent. Our comparison of *CBF2.1* also included the sequence of *LpCBF1a* which was both 60.3% identical and similar to *BsCBF2.1* (Table 5). Overall, we conclude that the nucleotide sequence of each candidate gene in *B. sylvaticum* are similar to that of their compared homolog genes in *B. distachyon*, wheat, *Arabidopsis* and perennial ryegrass.

Table 5. Identity and similarity percent for compared coding nucleotide sequences of homologous genes

Gene in *B. sylvaticum* (gene accession)	Compared homologs (gene accession)	Identity (%)	Similarity (%)
BsCOR413 (Brasy2G354900.1)[1]	*BdCOR413* (Bradi1g07441.1)[1]	96.9	97.0
	TaCOR413 (U73216.1)[2]	83.5	83.7
BsCOR410 (Brasy4G117200.1)[1]	*TaCOR410c* (U73211.1)[2]	84.3	84.7
BsCBF2.1 (Brasy7G035500.1)[1]	*BdCBF2.1* (Bradi1g49570.1)[1]	83.5	83.6
	TaCBFII-5.2 (EF028753.1)[2]	78.2	78.2
	LpCBF1a (AB258392.1)[2]	63.6	63.6
BsEF1 (BrasyJ016500.1)[1]	*BdEF1* (Bradi4g12750.2)[1]	96.7	96.7
	TaEF1 (Traes_2DS_7C52E51F8.1)[1]	89.7	89.8
	AtEF1 (AT5G60390.1)[1]	86.6	86.6
BsUBC16 (Brasy5G007800.1)[1]	*BdUBC18* (Bradi4g00660.2)[1]	97.4	98.6
	TaUBC16 (Traes_5DS_0A5C2D35E.1)[1]	90.9	91.7
	AtUBC18 (AT5G42990.1)[1]	74.9	75.2
BsGAPDH (Brasy3G065900.1)[1]	*BdGAPDH* (Bradi3g14057.1)[1]	97.7	97.8
	TaGAPDH (Traes_7DL_961822B36.1)[1]	92.4	92.6
	AtGAPDH (AT1G13440.1)[1]	79.7	79.8
BsSAMDC (Brasy4G150300.2)[1]	*BdSAMDC* (Bradi3g48490.1)[1]	96.0	96.0
	TaSAMDC (Traes_6AL_FDF023AF6.2)[1]	86.5	86.5
	AtSAMDC (AT3G25570.1)[1]	60.5	60.6

1. From Phytozome v12.1 (DOE-JGI).
2. From the NCBI-NIH protein database.

Bs: Brachypodium sylvaticum. Bd: Brachypodium distachyon. Ta: Triticum aestivum. At: Arabidopsis thaliana. Lp: Lollium perenne. COR413: COld-Regulated 413. COR410: COld-Regulated 410. CBF2.1: C-repeat Binding Factor 2.1. EF1: *Elongation Factor-α1. UBC16*: *UBiquitin Conjugating enzyme-16. GAPDH*: *GlycerAldehyde-3-Phosphate-DeHydrogenase. SAMDC*: *S-AdenosylMethionine DeCarboxylase proenzyme.*

In general, similar nucleotide sequences have a high probability of being phylogenetically related and should therefore code for the translation of homologous proteins across compared organisms (Kanduc, 2012). In further support of the results presented above, we compared the identity and similarity of each gene's associated peptide sequences as presented in Table 6. To the exception of *UBC16* for which the highest similar peptide sequence is from *T. aestivum* and a few comparisons that scored significantly lower identity and similarity percent values (i.e. *LpCBF1a*/*BsCBF2.1* and *AtSAMDC*/*BsSAMDC*), most identity and similarity percent values for compared peptides sequences are consistent with the values reported in Table 5 for compared nucleotide sequences. Therefore, all compared peptides sequences in *B. sylvaticum* are either similar, highly similar or identical in the case of *EF1* to at least one well-described homologous protein produced by either *B. distachyon*, *T. aestivum*, *Arabidopsis* or *L. perenne.*

By proxy, this later observation suggests that the identified genes in *B. sylvaticum* are involved in the function that is described for their closest homolog (Kanduc, 2012). In order to verify if this statement applies to our candidate genes, we further investigated the structural homology of the compared peptide sequences by focusing on domains, active sites and/or amino acid of interest that characterizes protein function. Hence, Figures 7, 8, 9, 10, 11, 12 and 13 display boxshade analyses that visually compare the homologous peptide sequences as well as indicating for each studied genes the equivalent region that we amplified through RT-qPCR using the primers pairs as reported in Table 1.

Table 6. Identity and similarity percent for compared peptide sequences of homologous proteins

Protein in *B. sylvaticum* (protein accession)	Compared homologous protein (protein accession)	Identity (%)	Similarity (%)
BsCOR413 (Brasy2G354900.1)[1]	*BdCOR413* (Bradi1g07441.1)[1]	97.1	98.6
	TaCOR413 (AAB18207)[2]	75.0	85.4
BsCOR410 (Brasy4G117200.1)[1]	*TaCOR410c* (AAB18202.1)[2]	74.7	79.1
BsCBF2.1 (Brasy7G035500.1)[1]	*BdCBF2.1* (Bradi1g49570.1)[1]	74.3	76.6
	TaCBFII-5.2 (ABK55356.1)[2]	64.7	70.6
	LpCBF1a (BAF36837.1)[2]	36.7	42.6
BsEF1 (BrasyJ016500.1)[1]	*BdEF1* (Bradi4g12750.2)[1]	100	100
	TaEF1 (Traes_2DS_7C52E51F8.1)[1]	93.4	94.5
	AtEF1 (AT5G60390.1)[1]	94.7	96.4
BsUBC16 (Brasy5G007800.1)[1]	*BdUBC18* (Bradi4g00660.2)[1]	82.6	82.6
	TaUBC16 (Traes_5DS_0A5C2D35E.1)[1]	98.1	98.1
	AtUBC18 (AT5G42990.1)[1]	86.3	87.6
BsGAPDH (Brasy3G065900.1)[1]	*BdGAPDH* (Bradi3g14057.1)[1]	99.4	99.7
	TaGAPDH (Traes_7DL_961822B36.1)[1]	95.9	97.0
	AtGAPDH (AT1G13440.1)[1]	85.0	89.7
BsSAMDC (Brasy4G150300.2)[1]	*BdSAMDC* (Bradi3g48490.1)[1]	89.6	91.0
	TaSAMDC (Traes_6AL_FDF023AF6.2)[1]	71.4	75.2
	AtSAMDC (AT3G25570.1)[1]	47.5	56.2

1. From Phytozome v12.1 (DOE-JGI).
2. From the NCBI-NIH protein database.

Bs: Brachypodium sylvaticum. Bd: Brachypodium distachyon. Ta: Triticum aestivum. At: Arabidopsis thaliana. Lp: Lollium perenne. COR413: COld-Regulated 413. COR410: COld-Regulated 410. CBF2.1: C-repeat Binding Factor 2.1. EF1: *Elongation Factor-α1. UBC16*: *UBiquitin Conjugating enzyme-16. GAPDH*: *GlycerAldehyde-3-Phosphate-DeHydrogenase. SAMDC*: *S-AdenosylMethionine DeCarboxylase proenzyme.*

More precisely, Figure 7 compares the peptide sequences associated to *COR413pm1* in *B. sylvaticum* (Brasy2G354900.1), *B. distachyon* (Bradi1g07441.1) and *T. aestivum* (AAB18207). In yellow is shown the span of the Alpha-Helix-G-rich secondary structure as described in Breton *et al.* (2003) and predicted to confer *COR413* transmembrane functions (Breton *et al.*, 2003). The levels of identity and similarity for this specific region is of respectively 94.0% and 96.0% comparing *Bs*COR413pm1 to *Bd*COR413pm1, and 76.0% and 88.0% comparing *Bs*COR413pm1 to *Ta*COR413pm. Because we observe both high levels of identity and similarity between the compared sequences of COR413pm1 as well as high levels of similarity for the Alpha-Helix-G-rich specific region, we conclude that Brasy2G354900.1 is homologous to COR413pm1 as described in wheat by Breton *et al.*(2003) and in *B. distachyon* by Colton-Gagnon *et al.*, (2014). The grey bracket labelled *COR413* indicates the region that was amplified through RT-qPCR in *B. sylvaticum* (Figure 7).

Figure 8 compares the aligned peptide sequences associated to *COR410* in *B. sylvaticum* (Brasy4G117200.1) and *T. aestivum* (AAB18202.1). When comparing the regions associated to the functional domains dehydrin 1 marked in yellow, we obtain identity and similarity percent values of both 95.7. Likewise, the dehydrin 2 domains marked in blue are collectively 91.7% identical and 100% similar. Therefore, we conclude that Brasy4G117200.1 is homologous to *COR410* as described in wheat by Danyluk *et al.* (1994) and related to cold-acclimation in the same species by Danyluk *et al.* (1998). Accordingly, we amplified the section delineated by the grey bracket labelled *COR410* through RT-qPCR (Figure 8).

Figure 7. Boxshade analysis comparing homologous peptide sequences of *Cold-Regulated 413 plasma membrane 1* (*COR413pm1*) in *Brachypodium sylvaticum* (BsCOR413pm1 or Brasy2G354900.1), *Brachypodium distachyon* (BdCOR413pm1 or Bradi1g07441.1) and *Triticum aestivum* (TaCOR413pm1 or AAB18207). The yellow line spans the Alpha-Helix-G-rich domain that is characteristic to *COR413*. The grey bracket represents the *COR413* amplicon obtained through RT-qPCR method in *B. sylvaticum*. Multiple alignment and Boxshade analysis conducted using tools available at the European Molecular Biology Network (EMBNet) portal.

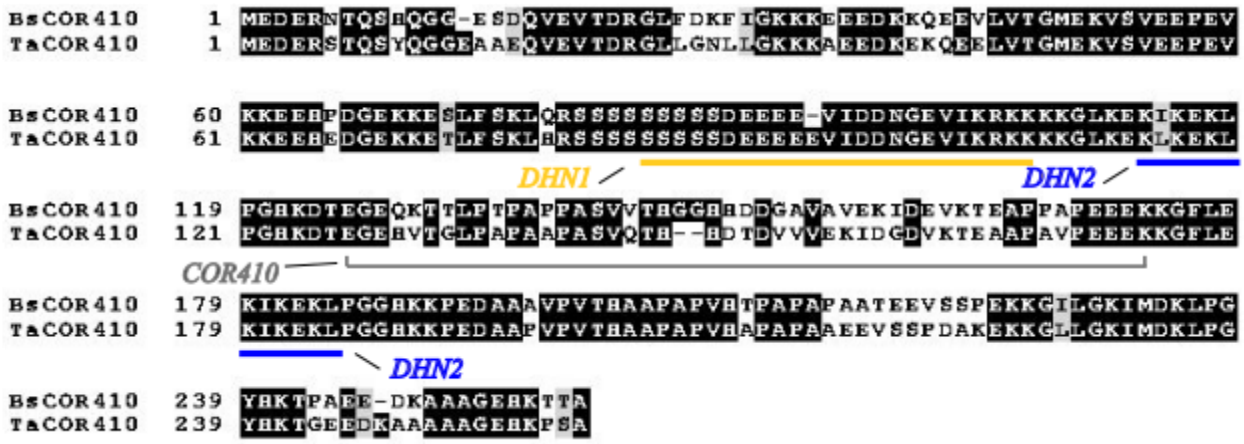

Figure 8. Boxshade analysis comparing homologous peptide sequences of *Cold-Regulated 410* (*COR410*) in *Brachypodium sylvaticum* (BsCOR410 or Brasy4G117200.1) and *Triticum aestivum* (TaCOR410 or AAB18202.1). The yellow and blue lines show respectively the Dehydrin 1 and 2 domains which are characteristics of the function of this gene. The grey bracket represents the *COR410* amplicon obtained through RT-qPCR method in *B. sylvaticum.* Multiple alignment and Boxshade analysis conducted using tools available at the European Molecular Biology Network (EMBNet) portal.

In Figure 9 panel A, we compare the peptide sequences of *CBFIII*-related proteins in *B. sylvaticum* (*CBF2.1* – Brasy7G035500.1), *B. distachyon* (*CBF2.1*- Bradi1g49570.1), *T. aestivum* (*TaCBFII-5.2* – ABK55356.1) and *L. perenne* (*LpCBFIa* – BAF36837.1). When analysing the CBF-specific motif described in Tamura and Yamada (2007) and represented by the blue dashed line in panel A, we observe that it is identical in *B. sylvaticum, B. distachyon* and *T. aestivum*, and 93.8% identical in *L. perenne*. Another *CBF* signature motif, the LSWY motif (Tamura and Yamada, 2007) indicated by green stars is identical in all four species. Furthermore, in red is highlighted the *AP2* domain which is involved in *CBFs*'s function (Stockinger *et al.*, 1997). The *AP2* domain of *CBF2.1* in *B. sylvaticum* is 93.1% identical and 94.8% similar to the same domains of *CBF2.1* in *B. distachyon* and *CBFII-5.2* in *T. aestivum*, as well as 67.2% identical and 73.8% similar to that of *CBFIa* in *L. perenne*. In light of the observed similarities in *CBFs*-specific motifs and the *AP2* domain sequences, we conclude that Brasy7G035500.1 is homologous to *CBF2.1* in *B. distachyon* as identified by Chen *et al.* (2016), *CBFII-5.2* in wheat as described in Badawi *et al.* (2007) and *CBFIa* in perennial ryegrass as identified by Tamura and Yamada (2007). Accordingly, the span of the amplicon that we obtain through RT-qPCR for *CBF2.1* in *B. sylvaticum* is indicated by the grey bracket (Figure 9 A).

Complementarily, in Figure 9 panel B we present a distance tree obtained from the multiple sequence alignment reported in panel A. This tree supply an estimation of the phylogenetic distance that links the compared *CBFIII*-related protein sequences. Here, we observe that *CBF2.1* peptide sequences in *B. sylvaticum* and *B. distachyon* are more closely related when considering all distances. Those two form a pair that is related to first *CBFII-5.2* in wheat and then *CBFIa* in perennial ryegrass, the farthest related sequence of all.

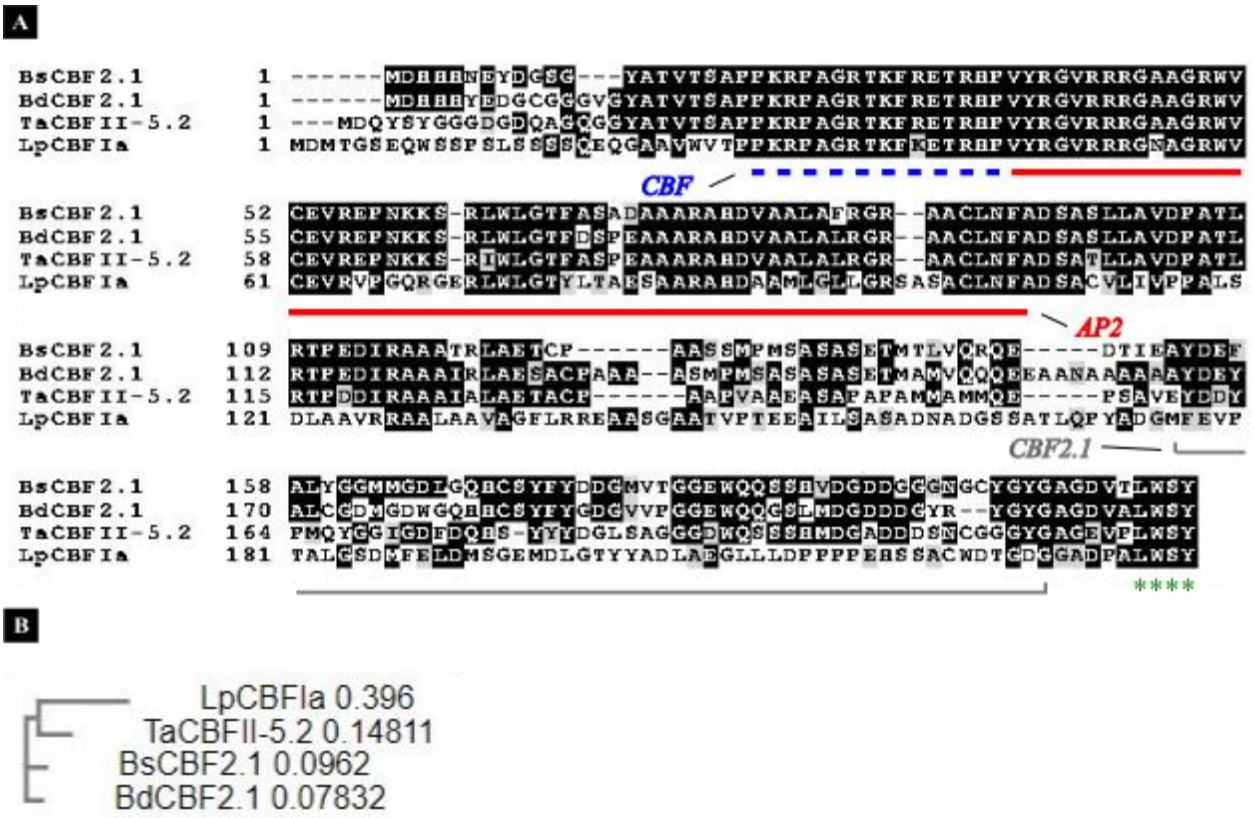

Figure 9. A: **Boxshade analysis comparing homologous peptide sequences of the *C-repeat Binding Factor* class *III*** in *Brachypodium sylvaticum* (BsCBF2.1 or Brasy7G035500.1), *Brachypodium distachyon* (BdCBF2.1 or Bradi1g49570.1), *Triticum aestivum* (TaCBFII-5.2 or ABK55356.1) and *Lolium perenne* (LpCBFIa or BAF36837.1). The dotted blue line indicate the *CBF*s-specific motif, the red line spans the *APetalla-2* (*AP2*) domain and the green stars highlight the *CBFs*-specific LWSY motif. The grey bracket represents the *CBF2.1* amplicon obtained through RT-qPCR method in *B. sylvaticum* **B:** Neighbour-joining tree without distance corrections resulting from the alignment in A. Multiple alignment and tree conducted using defaults settings from the tool CLUSTAL OMEGA and CLUSTAL W as provided by the European Bioinformatics Institute (EMBL-EBI). Boxshade analysis conducted using tools available at the European Molecular Biology Network (EMBNet) portal.

Figure 10 compares the peptide sequences associated to *EF1* in *B. sylvaticum* (BrasyJ016500.1), *B. distachyon* (Bradi4g12750.2), *T. aestivum* (Traes_2DS_7C52E51F8.1) and *A. thaliana* (AT5G60390.1). The red line indicates the *Translational-type guanine nucleotide-binding domain 2* (*G_Tr_2*), a domain that catalyses ribosomal subunit involved in cellular elongation, a characteristic function of *EF1* (Eiler *et al.*, 2013). As for the complete sequences, the peptide sequence for the G_Tr_2 domain of *EF1* in *B. sylvaticum* is identical in *B. distachyon*, whereas 93.4% identical and 94.5% similar to *EF1* in wheat, and 94.7% identical and 96.4% similar to *EF1* in *Arabidopsis*. In conclusion, we observe that BrasyJ016500.1 is homologous to *EF1* in *B. distachyon* as identified in Hong *et al.* (2008), in wheat as described in Metz *et al.* (1992) and in *Arabidopsis* as reported by Kazusa DNA Research Institute *et al.* (2000). The grey bracket labelled *EF1* represent the equivalent span of the *EFI* amplicon that we isolated through RT-qPCR (Figure 10).

Figure 11 shows the multiple peptide sequences alignment for *UBCs* protein in *B. sylvaticum* (*UBC16* - Brasy5G007800.1), *B. distachyon* (*UBC18* - Bradi4g00660.2), *T. aestivum* (*UBC16* - Traes_5DS_0A5C2D35E.1) and *A. thaliana* (*UBC18* - AT5G42990.1). In green is represented the Ubiquitin-conjugating enzymes domain which is characteristic to *UBC* family proteins (Jentsch *et al.*, 1990). The blue star indicates the domain enzymatic active site. The *UBC* domain of *UBC16* in *B. sylvaticum* is identical to the one in *B. distachyon*, 98.3% identical as in *T. aestivum*, and 85.0% identical and 86.7% similar as in *A. thaliana*. Consequently, we observe that Brasy5G007800.1 in *B. sylvaticum* is homologous to *UBC18* in *B. distachyon* as described in Hong *et al.* (2008), *UBCs* in wheat as described by Girod and Vierstra (1993) and in *Arabidopsis* as described by Kazusa DNA Research Institute *et al.* (2000). The grey bracket labelled *UBC16* spans the equivalent region of the amplicon as obtained through RT-qPCR for *UBC16* in *B. sylvaticum* (Figure 11).

Figure 10. Boxshade analysis comparing homologous peptide sequences of *Elongation Factor-α 1* (*EF1*) in *Brachypodium sylvaticum* (BsEF1 or BrasyJ016500.1), *Brachypodium distachyon* (BdEF1 or Bradi4g12750.2), *Triticum aestivum* (TaEF1 or Traes_2DS_7C52E51F8.1) and *Arabidopsis thaliana* (AtEF1 or AT5G60390.1). The red line indicates the *Translational-type guanine nucleotide-binding domain 2* (*G_TR_2*) that is characteristic to the function of *EF1s* genes. The grey bracket represents the *EF1* amplicon obtained through RT-qPCR method in *B. sylvaticum.* Multiple alignment and Boxshade analysis conducted using tools available at the European Molecular Biology Network (EMBNet) portal.

Figure 11. Boxshade analysis comparing homologous peptide sequences of *UBiquitin Conjugating enzyme* (*UBC*) in *Brachypodium sylvaticum* (BsUBC16 or Brasy5G007800.1), *Brachypodium distachyon* (BdUBC18 or Bradi4g00660.2), *Triticum aestivum* (TaUBC16 or Traes_5DS_0A5C2D35E.1) and *Arabidopsis thaliana* (AtUBC18 or AT5G42990.1). The green line spans the *Ubiquitin-conjugating enzymes* family profile (*UBC*) that is characteristic to the function of *UBCs* genes. The blue star indicates the domain's active site. The grey bracket represents the *BsUBC16* amplicon as obtained through RT-qPCR method in *B. sylvaticum.* Multiple alignment and Boxshade conducted using tools provided on the European Molecular Biology Network (EMBNet) portal.

In Figure 12 we compare the peptide sequences of *GAPDH* in *B. sylvaticum* (Brasy3G065900.1), *B. distachyon* (Bradi3g14057.1), *T. aestivum* (Traes_7DL_961822B36.1) and *A. thaliana* (AT1G13440.1). The red line stretches along the *GAPDH* domain which is crucial to *GAPDHs* protein enzymatic function (Fabry *et al.*, 1989). The blue star indicates the enzyme active site. The region corresponding to the *GAPDH* domain is identical across all four compared sequences. Considering the high level of similarity between the four peptide sequences, we conclude that Brasy3G065900.1 is homologous to *GAPDH* in *B. distachyon* as described in Hong *et al.* (2008), in *T. aestivum* as reported in Sander *et al.* (2011) and in *A. thaliana* as described by Theologis *et al.* (2000). The grey bracket indicate the equivalent region that we amplified for *GAPDH* through RT-qPCR in *B. sylvaticum* (Figure 12).

Figure 13 compares the peptide sequences associated to *SAMDC* in *B. sylvaticum* (Brasy4G150300.2), *B. distachyon* (Bradi3g48490.1), *T. aestivum* (Traes_6AL_FDF023AF6.2) and *A. thaliana* (AT3G25570.1). When comparing the sequences that encompasses the *ADOMET* motif as indicated in red, the peptide alignment is identical for *B. sylvaticum*, *B. distachyon* and *A. thaliana*, and 90.0% identical and 100% similar for *T. aestivum*. The *ADOMET* motif is a signature of the *SAMDC* protein family (Ekstrom *et al.*, 1999). Accordingly, we observe that Brasy4G150300.2 is homologous to *SAMDC* in *B. distachyon* as reported by Hong *et al.* (2008), in wheat as characterized by Franceschetti *et al.* (2001) and in *Arabidopsis* as reported by the European Union Chromosome 3 Arabidopsis Sequencing Consortium *et al.* (2000). The grey bracket labelled *SAMDC* represent the equivalent span of the amplicon that we obtained through RT-qPCR for *SAMDC* in *B. sylvaticum* (Figure 13).

Figure 12. Boxshade analysis comparing homologous peptide sequences of cytosolic *GlycerAldehyde-3-Phosphate-DeHydrogenase* (*GAPDH*) in *Brachypodium sylvaticum* (BsGAPDH or Brasy3G065900.1), *Brachypodium distachyon* (BdGAPDH orBradi3g14057.1), *Triticum aestivum* (TaGAPDH or Traes_7DL_961822B36.1) and *Arabidopsis thaliana* (AtGAPDH or AT1G13440.1). The red line shows the *Glyceraldehyde 3-phosphate dehydrogenase* domain (*GAPDH*) that is characteristic to the function of *GAPDHs* genes. The blue start indicates the protein active site. The grey bracket represents the *GAPDH* amplicon as obtained through RT-qPCR method in *B. sylvaticum.* Multiple alignment and Boxshade conducted using tools provided on the European Molecular Biology Network (EMBNet) portal.

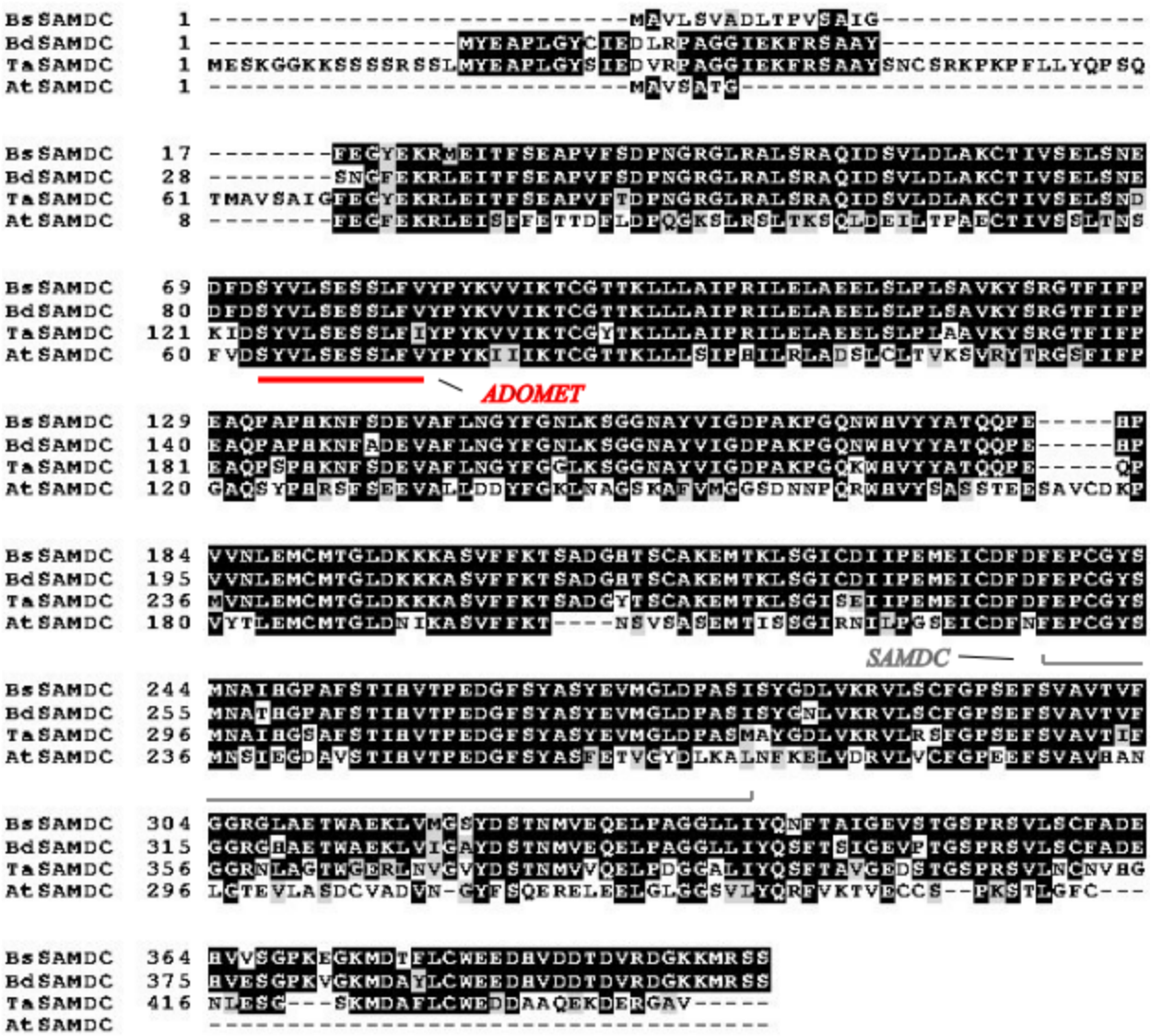

Figure 13. Boxshade analysis comparing homologous peptide sequences of of *S-AdenosylMethionine DeCarboxylase proenzyme* (*SAMDC*) in *Brachypodium sylvaticum* (BsSAMDC or Brasy4G150300.2), *Brachypodium distachyon* (BdSAMDC or Bradi3g48490.1), *Triticum aestivum* (TaSAMDC or Traes_6AL_FDF023AF6.2) and *Arabidopsis thaliana* (AtSAMDC or AT3G25570.1). The red line indicates the *S-adenosylmethionine decarboxylase* (*ADOMET*) signature motif that is characteristic to the function of *SAMDC* genes. The grey bracket represents the *SAMDC* amplicon as obtained through RT-qPCR method in *B. sylvaticum.* Multiple alignment and Boxshade conducted using tools provided on the European Molecular Biology Network (EMBNet) portal.

In light of the results presented under this subsection, we were confident enough to move forward with testing *in planta BsCOR413, BsCOR410* and *BsCBF2.1* as cold-responsive genetic markers and *BsEF1*, *BsUBC16*, *BsGAPDH* and *BsSAMDC* as RT-qPCR standard genes. The next section reports our observations on that matter.

4.3.2 The expression of *CORs* genes increases in response to cold-acclimation in *B. sylvaticum*

Although the use of *EF1, UBC16, GAPDH* and *SAMDC* as standard genes had been investigated in *B. distachyon* by Hong *et al.* (2008), their reliability for RT-qPCR method in *B. sylvaticum* had yet to be tested. Therefore, we first compared the transcript accumulation profiles for *BsCOR413* and *BsCOR410* in accession PI564896 using *BsEF1, BsUBC16*, *BsGAPDH* and *BsSAMDC* to determine which of those standard genes should be selected to complete the proposed work with the remaining accessions. Note that we specifically chose PI564896 to conduct this test because of the ample amount of tissues at our disposal. We thus determined the relative transcript accumulation of *BsCOR413* and *BsCOR410* using *BsEF1, BsUBC16, BsGAPDH* and *BsSAMDC* as standard genes in tissues that were non-acclimated (NA) as well as cold-acclimated for 7 days (CA7) and 14 days (CA14) using RT-qPCR (Figure 14). We observe that *BsCOR413* and *BsCOR410* relative transcript accumulation are significantly higher at CA7 and remains elevated at CA14 in comparison to NA control plants when using *BsEF1, BsUBC16* and *BsGAPDH* as standard genes, which agrees well with the profiles observed for these genes in response to cold in other species (Danyluk *et al.*, 1994, 1998; Breton *et al.*, 2003; Colton-Gagnon *et al.*, 2014). Oppositely, little to no change in *BsCOR413* and *BsCOR410* relative transcripts accumulation could be observed when standardized with *BsSAMDC* (Figure 10). Table 7 summarizes the Tukey HSD statistical analysis performed for this assay.

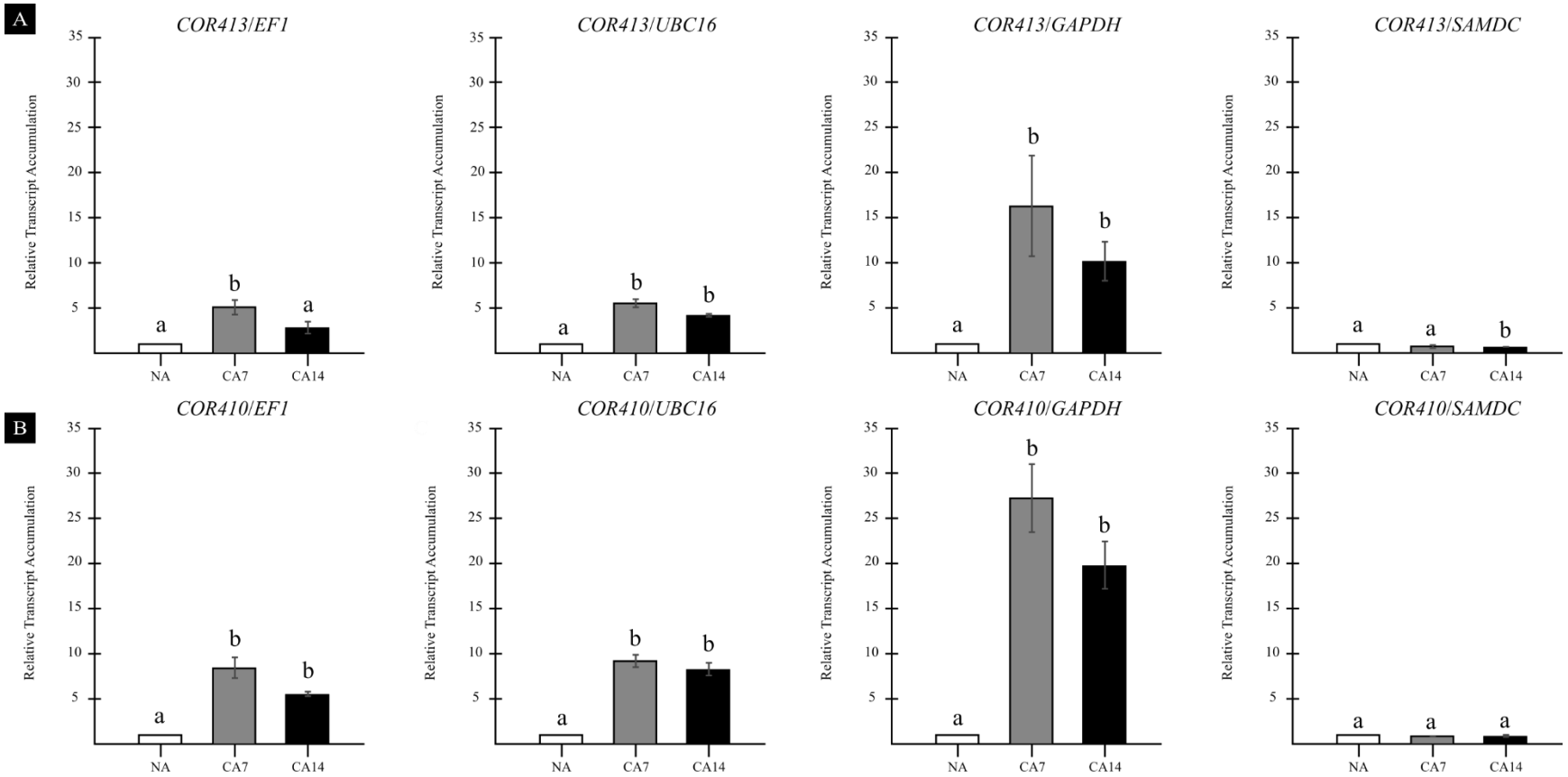

Figure 14. Transcript accumulation profiles of two *BsCOR* genes in response to cold exposure using four standard genes in accession PI564896. Relative transcript accumulation of **A:** *COR413* and **B:** *COR410* in tissues collected (n= 5) from control plants and plants exposed to seven (CA7) and 14 days (CA14) of cold (4 °C). RT-qPCR results were analyzed using *Elongation Factor-α1* (*EF1*), *UBiquitin Conjugating enzyme-16* (*UBC16*) and *GlycerAldehyde-3-Phosphate-DeHydrogenase* (*GAPDH*) as standard genes. The experiment was replicated three times. Letters indicate statistical significance as determined through a one-way ANOVA test (α= 0.05) followed by a Tukey HSD procedure.

Table 7. Tukey HSD test conducted on the results of *COld-Regulated 413* (*COR413*) and *COld-Regulated 410* (*COR410*) relative transcripts accumulation in PI564896.

Genes	Group compared	Degree of Freedom	Difference	Lower boundary	Upper boundary	p-value adjusted
COR413/ EF1	NA-CA7 *	2	- 4.086997	- 6.7997116	- 1.3742821	0.0085887
	NA-CA14	2	- 1.765114	- 4.4778290	0.9476005	0.1938143
	CA7-CA14	2	2.321883	- 0.3908321	5.0345974	0.0869452
COR413/ UBC16	NA-CA7 *	2	- 4.521153	- 6.2071613	- 2.835145	0.0004269
	NA-CA14 *	2	- 3.132410	- 4.8184175	- 1.446402	0.0030437
	CA7-CA14	2	1.388744	- 0.2972642	3.074752	0.0985903
COR413/ GAPDH	NA-CA7 *	2	- 15.246069	- 23.845825	- 6.6463139	0.0038628
	NA-CA14 *	2	- 9.104738	- 17.704493	- 0.5049822	0.0401243
	CA7-CA14	2	6.141332	- 2.458424	14.7410873	0.1513881
COR413/ SAMDC	NA-CA7	2	0.45405789	- 0.01665394	0.9247697	0.0571846
	NA-CA14 *	2	0.55004225	0.07933042	1.0207541	0.0268672
	CA7-CA14	2	0.09598436	- 0.37472747	0.5666962	0.8120415
COR410/ EF1	NA-CA7 *	2	- 11.364209	7.4261455	- 3.4880804	0.0028199
	NA-CA14 *	2	- 4.480646	- 8.4187106	- 0.5425815	0.0300178
	CA7-CA14	2	2.945499	- 0.9925657	6.8835635	0.1326118
COR410/ UBC16	NA-CA7 *	2	- 8.2152721	- 11.48351	- 4.947030	0.0006098
	NA-CA14 *	2	- 7.2210793	- 10.48932	- 3.952837	0.0012271
	CA7-CA14	2	0.9941928	- 2.27405	4.262435	0.6410453
COR410/ GAPDH	NA-CA7 *	2	- 26.2434	- 40.736223	- 11.750582	0.0034698
	NA-CA14 *	2	- 18.7066	-33.199424	- 4.213782	0.0174944
	CA7-CA14	2	7.5368	- 6.956021	22.029620	0.3175988
COR410/ SAMDC	NA-CA7	2	0.06677534	- 0.6840882	0.8176389	0.9600567
	NA-CA14	2	0.15786495	- 0.5929986	0.9087285	0.8018378
	CA7-CA14	2	3.011144	- 4.781240	10.803528	0.5028334

* Statistically significant at a confidence level of 95 %. *EF1* : *Elongation Factor-α1*. *UBC16*: *UBiquitin Conjugating enzyme-16*. *GAPDH*: *GlycerAldehyde-3-Phosphate-DeHydrogenase*. *SAMDC*: *S-AdenosylMethionineDeCarboxylase proenzyme*.

For reasons that will be later addressed in the discussion (Chapter 5, section 5.2), the standard gene *BsEF1* was selected to determine the relative transcript accumulation of *BsCOR413* and *BsCOR410* in the remaining eight accessions. Hence, Figure 15 reports the results of relative transcript accumulation of *BsCOR413* for CA7 and CA14 treatments as compare to NA in all studied accessions. Differences in relative transcript accumulation in response to cold ranged from 0.65 fold (PI269842, CA14; Figure 15 panel I) to 14.55 fold (W6 23524, CA7; Figure 15 panel C). A summary of the associated Tukey HSD test is reported in Table 8. Overall, we observe that Bs*COR413* transcripts tend to accumulate in response to cold in all studied accessions.

Furthermore, the relative transcript accumulation profiles for *BsCOR410* in NA, CA7 and CA14 treated plants were also compared in nine *B. sylvaticum* accessions (Figure 16). Relative transcript accumulation range from 0.46 fold (PI269842, CA7; Figure 16 panel I) to 9.98 fold (PI297868, CA7; Figure 16 panel G). We also noticed that *BsCOR410* relative transcript accumulation is usually higher at CA7 than at CA14 for all accessions with the exception of PI269842 in which *BsCOR410* expression is higher at CA14 (Figure 16 panel I). Associated statistical results from a Tukey HSD test are reported in Table 9. Altogether, we observe that *BsCOR410* transcripts accumulate in response to cold in all studied accessions.

Although most of the observed differential expression profiles were not significantly different from one another, the results presented under subsection 4.3.2 indicates that transcripts for both *BsCOR413* and *BsCOR410* tend to accumulate in tissues exposed to 7 and 14 days of cold in the majority of the tested accessions. Therefore, we observe an increase of the expression of those tested *COR* genes in response to cold in *B. sylvaticum*, which support that cold acclimation mechanisms potentially exist in this perennial. Our results are consistent with other observations reported in *B. distachyon* and wheat (Danyluk *et al.*, 1994, 1998; Breton *et al.*, 2003; Colton-Gagnon *et al.*, 2014).

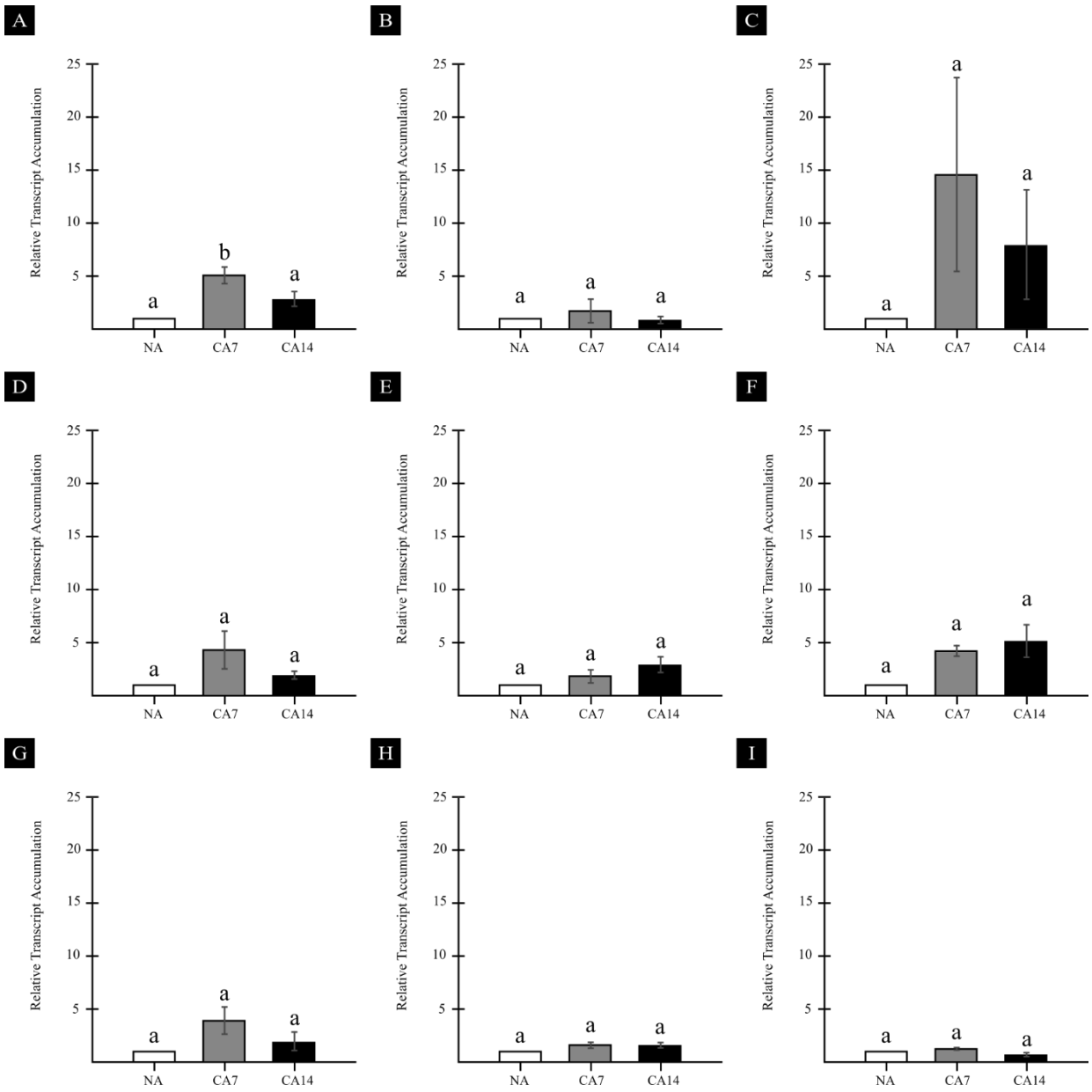

Figure 15. Transcript accumulation profiles of *BsCOR413* gene in response to cold exposure in nine *B. sylvaticum* accessions. Relative transcript accumulation in tissues collected (n= 5) from control plants and plants exposed to seven (CA7) and 14 days (CA14) of cold (4 °C) of accessions A: PI564896, B: PI384810, C: W6 23524, D: PI268222, E: PI610793, F: PI344569, G: PI297868, H: PI440178, I: PI269842. RT-qPCR results were analyzed using *Elongation Factor-α1* (*EF1*) as standard gene. The experiment was replicated three times. Letters indicate statistical significance as determined through a one-way ANOVA test (α= 0.05) followed by a Tukey HSD procedure.

Table 8. Tukey HSD test conducted on the results of *COld-Regulated 413* (*COR413*) relative transcripts accumulation in nine *B. sylvaticum* accessions.

Accession	Group compared	Degree of Freedom	Difference	Lower boundary	Upper boundary	p-value adjusted
PI564896	NA-CA7 *	2	- 4.086997	- 6.7997116	- 1.3742821	0.0085887
	NA-CA14	2	- 1.765114	- 4.4778290	0.9476005	0.1938143
	CA7-CA14	2	2.321883	- 0.3908321	5.0345974	0.0869452
PI384810	NA-CA7	2	- 0.7299605	- 4.379093	2.919172	0.2549151
	NA-CA14	2	0.1868239	- 3.462308	3.835956	0.9865188
	CA7-CA14	2	0.9167844	- 2.732348	4.565917	0.7330926
W6 23524	NA-CA7	2	- 13.555073	- 45.90926	18.79912	0.4524191
	NA-CA14	2	- 6.850025	- 39.20422	25.50417	0.7994406
	CA7-CA14	2	6.705047	- 25.64914	39.05924	0.8066990
PI26822	NA-CA7	2	- 3.30098	- 3.256248	2.398908	0.2549151
	NA-CA14	2	- 0.85734	- 6.557227	4.842547	0.8912562
	CA7-CA14	2	2.44364	- 0.3908321	8.143527	0.4379173
PI610793	NA-CA7	2	- 0.8452207	- 3.979459	2.289017	0.7011937
	NA-CA14	2	- 1.8505228	- 4.984761	1.283715	0.2442416
	CA7-CA14	2	- 1.0053020	- 4.139540	2.128936	0.6123766
PI344569	NA-CA7	2	- 3.2095517	- 8.363273	1.944170	0.2158515
	NA-CA14	2	- 4.0826775	- 9.236399	1.071044	0.1115185
	CA7-CA14	2	- 0.8731258	- 8.363273	4.280596	0.8647707
PI297868	NA-CA7	2	- 2.9139096	- 8.086787	2.258968	0.2515294
	NA-CA14	2	- 0.8451937	- 6.628647	4.938259	0.8855155
	CA7-CA14	2	- 2.0687158	- 8.086787	7.852169	0.5211214
PI440178	NA-CA7	2	- 0.6131839	- 1.994439	0.7680711	0.4159133
	NA-CA14	2	- 0.5469478	- 1.928203	0.8343072	0.4877109
	CA7-CA14	2	0.0662361	- 1.315019	1.4474911	0.9881595
PI269842	NA-CA7	2	- 0.2570912	-1.0087678	0.4945855	0.5759955
	NA-CA14	2	0.3464066	- 0.4052701	1.0980832	0.3923513
	CA7-CA14	2	0.6034977	-0.1481789	1.3551744	0.1069498

* Statistically significant at a confidence level of 95 %.

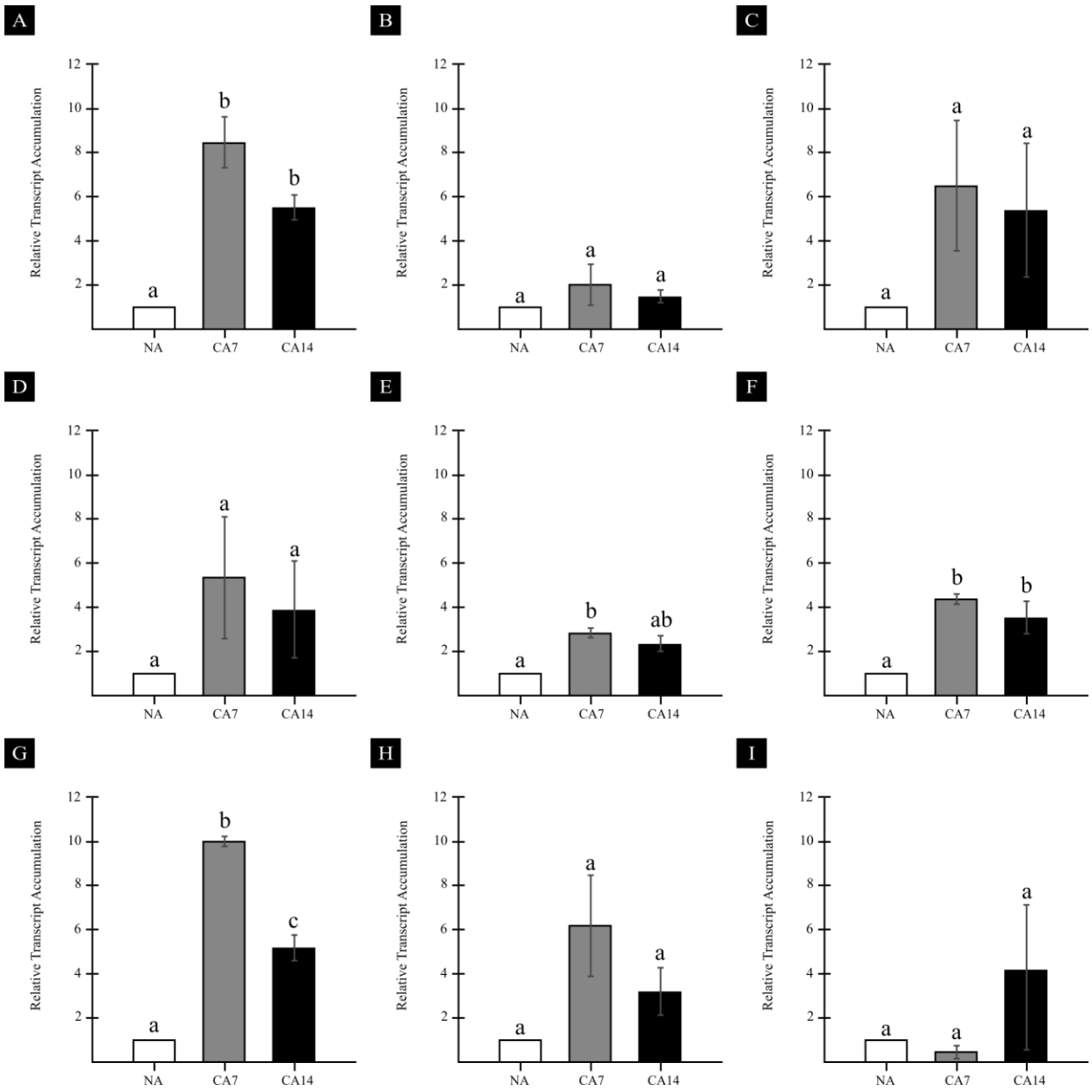

Figure 16. Transcript accumulation profiles of *BsCOR410* gene in response to cold exposure in nine *B. sylvaticum* accessions. Relative transcript accumulation in tissues collected (n= 5) from control plants and plants exposed to seven (CA7) and 14 days (CA14) of cold (4 °C) of accessions A: PI564896, B: PI384810, C: W6 23524, D: PI268222, E: PI610793, F: PI344569, G: PI297868, H: PI440178, I: PI269842. RT-qPCR results were analyzed using *Elongation Factor-α1* (*EF1*) as standard gene. The experiment was replicated three times. Letters indicate statistical significance as determined through a one-way ANOVA test (α= 0.05) followed by a Tukey HSD procedure.

Table 9. Tukey HSD test conducted on the results of *COld-Regulated 410* (*COR410*) relative transcripts accumulation in nine *B. sylvaticum* accessions.

Accession	Group compared	Degree of Freedom	Difference	Lower boundary	Upper boundary	p-value adjusted
PI564896	NA-CA7 *	2	- 11.364209	- 74261455	- 3.4880804	0.0028199
	NA-CA14 *	2	- 4.480646	- 8.4187106	- 0.5425815	0.0300178
	CA7-CA14	2	2.945499	- 0.9925657	6.8835635	0.1326118
PI384810	NA-CA7	2	- 0.8452207	- 3.979459	2.289017	0.7011937
	NA-CA14	2	- 1.8505228	- 4.984761	1.283715	0.2442416
	CA7-CA14	2	- 1.0053020	- 4.139540	2.289017	0.6123766
W6 23524	NA-CA7	2	- 5.474539	- 18.49040	7.541317	0.4499512
	NA-CA14	2	- 4.354594	- 17.37045	8.661262	0.5886817
	CA7-CA14	2	1.119945	- 11.89591	14.135801	0.9625430
PI26822	NA-CA7	2	- 4.342950	- 15.217057	6.531157	0.4823834
	NA-CA14	2	- 2.847385	- 13.721492	8.026722	0.7147699
	CA7-CA14	2	1.495565	- 9.378542	12.369672	0.9079540
PI610793	NA-CA7 *	2	- 1.816465	- 3.1913582	- 0.4415722	0.0157689
	NA-CA14	2	- 1.307536	- 2.6842291	0.06735676	0.060243
	CA7-CA14	2	0.508929	- 0.8659639	1.88382197	0.5291128
PI344569	NA-CA7 *	2	- 3.3594215	- 5.747406	-0.9714369	0.0118469
	NA-CA14 *	2	- 2.5012716	- 4.889256	-0.1132870	0.0418447
	CA7-CA14	2	0.8581499	- 1.529835	3.2461345	0.5469101
PI297868	NA-CA7 *	2	- 8.979522	- 11.009408	- 6.949637	0.0000243
	NA-CA14 *	2	- 4.150254	- 6.180139	- 2.120368	0.0018516
	CA7-CA14 *	2	4.829268	2.799383	6.859154	0.0008235
PI440178	NA-CA7	2	- 5.178711	- 12.971095	2.613673	0.1836420
	NA-CA14	2	- 2.167567	- 9.959951	5.624817	0.6864129
	CA7-CA14	2	3.011144	- 4.781240	10.803528	0.5028334
PI269842	NA-CA7	2	0.5394893	- 9.605022	10.684001	0.9854644
	NA-CA14	2	- 3.1506189	- 13.295130	6.993893	0.6299735
	CA7-CA14	2	-3.6901082	- 13.834620	6.454403	0.5396383

* Statistically significant at a confidence level of 95 %.

4.3.3 The transcription factor *CBF2.1* is rapidly induced in response to cold in *B. sylvaticum*

As discussed in Chapter 2, the *CBFs* transcription factors are important orchestrators of the molecular response to cold in plants as they activate the transcription of downstream *COR* genes. To be effective, *CBFs* are usually induced within the first few hours that follow an exposition to cold (Chew and Halliday, 2011). Therefore, to test if the candidate gene *BsCBF2.1* previously identified in section 4.3.1 also respond rapidly to low temperatures, we designed a short kinetic experiment to determine the profile of relative transcript accumulation of this gene during the first 9 hours of exposition to cold. In Figure 17, we observe a gradual increase in the relative accumulation of the *BsCBF2.1* transcript for cold-acclimated plants as compared to the 0h time point with a statistically significant differential transcripts accumulation after 9h of cold exposure. On the other hand, we observed that *BsCBF2.1* transcript levels remained constant in non-acclimated plants at time points 3 h, 6 h and 9 h. A summary of associated statistic is reported in Table 10.

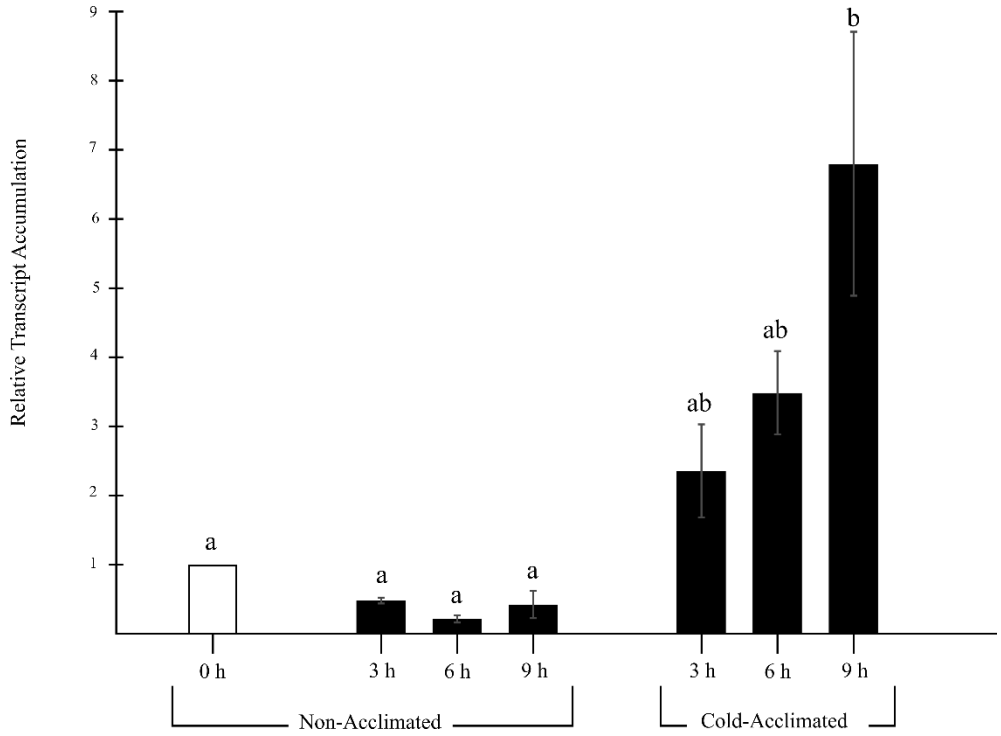

Figure 17. Transcript accumulation profiles of *BsCBF2.1* gene in response to cold exposure in accession PI269842. Relative transcript accumulation in tissues collected (n= 5) from plants exposed to 3, 6 and 9 hours of cold (4 °C) as well as from matching controls. RT-qPCR results were analyzed using *Elongation Factor-α1* (*EF1*) as standard gene. The experiment was replicated three times. Letters indicate statistical significance as determined through a one-way ANOVA test (α= 0.05) followed by a Tukey HSD procedure.

Table 10. Tukey HSD test conducted on the results of *C-repeat Binding Factor 2.1* (*CBF2.1*) relative transcript accumulation in PI269842.

Group compared	Degree of Freedom	Difference	Lower boundary	Upper boundary	p-value adjusted
NA0-NA3	6	- 0.52000000	- 5.960095	4.9200947	0.9998624
NA0-NA6	6	- 0.79333333	- 6.233428	4.6467614	0.9984696
NA0-NA9	6	- 0.58666667	- 6.026761	4.8534280	0.9997236
NA0-CA3	6	- 1.35333333	- 6.793428	4.0867614	0.9746460
NA0-CA6	6	- 2.48333333	- 7.923428	2.9567614	0.7081699
NA0-CA9 *	6	- 5.79333333	- 11.233428	- 0.3532386	0.0335961
NA3-NA6	6	- 0.27333333	- 5.713428	5.1667614	0.9999969
NA3-NA9	6	- 0.06666667	- 5.506761	5.3734280	1.0000000
NA3-CA3	6	- 1.87333333	- 7.313428	3.5667614	0.8920854
NA3-CA6	6	- 3.00333333	- 8.443428	2.4367614	0.5192634
NA3-CA9 *	6	- 6.31333333	- 11.753428	- 0.8732386	0.0185791
NA6-NA9	6	0.20666667	- 5.233428	5.6467614	0.9999994
NA6-CA3	6	- 2.14666667	- 7.586761	3.2934280	0.8192249
NA6-CA6	6	- 3.27666667	- 8.716761	2.1634280	0.4250280
NA6-CA9 *	6	- 6.58666667	- 12.026761	- 1.1465720	0.0135893
NA9-CA3	6	- 1.94000000	- 7.380095	3.5000947	0.8760802
NA9-CA6	6	- 3.07000000	- 8.510095	2.3700947	0.4955908
NA9-CA9 *	6	- 6.38000000	- 11.820095	- 0.9399053	0.0172151
CA3-CA6	6	1.13000000	- 4.310095	6.5700947	0.9897241
CA3-CA9	6	4.44000000	- 1.000095	9.8800947	0.1468352
CA6-CA9	6	3.31000000	- 2.130095	8.7500947	0.4141272

* Statistically significant at a confidence level of 95 %. NA0-3-6-9 = Non-acclimated 0, 3, 6 or 9 hours. CA3-6-9 = Cold-Acclimated 3, 6 or 9 hours.

4.4 *Agrobacterium*-mediated transformation of *B. sylvaticum* accession PI269842

Resourceful model grasses such as rice (Sallaud *et al.*, 2003), *B. distachyon* (Vogel and Hill, 2008) and recently *B. sylvaticum* (Steinwand *et al.*, 2013) all have described protocol of transformation available to researchers. The following section details the adaptation of the Steinwand *et al.* (2013)'s transformation protocol to our laboratory conditions as a complementary objective to the current project.

Steinwand *et al.* (2013)'s high-efficiency transformation protocol relies onto the co-cultivation of *B. sylvaticum* calluses and *Agrobacterium* as a gateway transformation vector. The sequence of such protocol is conveyed in Figure 5, which includes images showing the aspect of PI269842's spikelet (panel A) from which embryos are excised for later calluses culture (panel B). It also shows the incubation step (panel C) and highlights the presence of a transformation foci in a callus transformed with EV through Ultra-Violet microscopy (panels D, E, F and G) as well as the aspect of calluses regenerating onto Linsmaier and Skoog media (panel H) and onto regular Murashige and Skoog media (panel I) before being transferred into soil. In all, to proceed from embryo production to fully grown, regenerated plant took between 7 to 15 months.

Our goal for this objective was to overexpress *GCN5*, an epigenetic modifier that is known to influence the response to cold stresses in plants (Stockinger *et al.*, 2001; Vlachonasios *et al.*, 2003; Mayer *et al.*, 2015). We therefore used *Agrobacterium* suspensions that contained the plasmids of interest pANIC-6A-*BdGCN5* (Figure 18) and the plasmid pANIC-6A-Empty Vector (EV) as a control. The pANIC-6A-*BdGCN5* construct contained the wild-type *Brachypodium distachyon GCN5* (*BdGCN5*). In fact, when comparing the aligned nucleotide sequences for *GCN5* in *B. sylvaticum* (Brasy4G240700.1) and *B. distachyon* (Bradi3g26800.1), we observe a high level of similarity between both genes (98.2% identical and similar). A similar trend (98.6% identical and similar) is observed when comparing the associated peptide sequences as visually shown in Figure 19. Furthermore, the peptide sequences corresponding to two functional domains of *GCN5*, that is the Acetyltransferase domain (*GNAT*, marked in magenta in Figure 19) and *Bromodomain-2* (*BROMO*, marked in turquoise in Figure 19) are identical in both species. Hence and in light of its high level of conservation throughout eukaryotes (Sterner and Berger, 2000), we understand that *BdGCN5* is homologous to *BsGCN5*. Therefore, the exogenous *BdGCN5* should play the same epigenetic role as the endogenous *BsGCN5* when expressed *in planta*.

Note that because of the high similarity between *BdGCN5* and *BsGCN5*, we were unsuccessful in separating the expression of endogenous and exogenous *GCN5* in mutant lines. The blue stars in Figure 19 indicates regions where we designed specific RT-qPCR primers that had failed to amplify in both *BdGCN5* and *BsGCN5*. Instead, we verified the expression of *GCN5 in planta* using a primer pair that equally amplifies *BdGCN5* and *BsGCN5* (indicated by the grey bracket in Figure 19). Further details concerning mutant line characterization are provided in subsection 4.4.2 and are discussed in chapter 5, subsection 5.3.

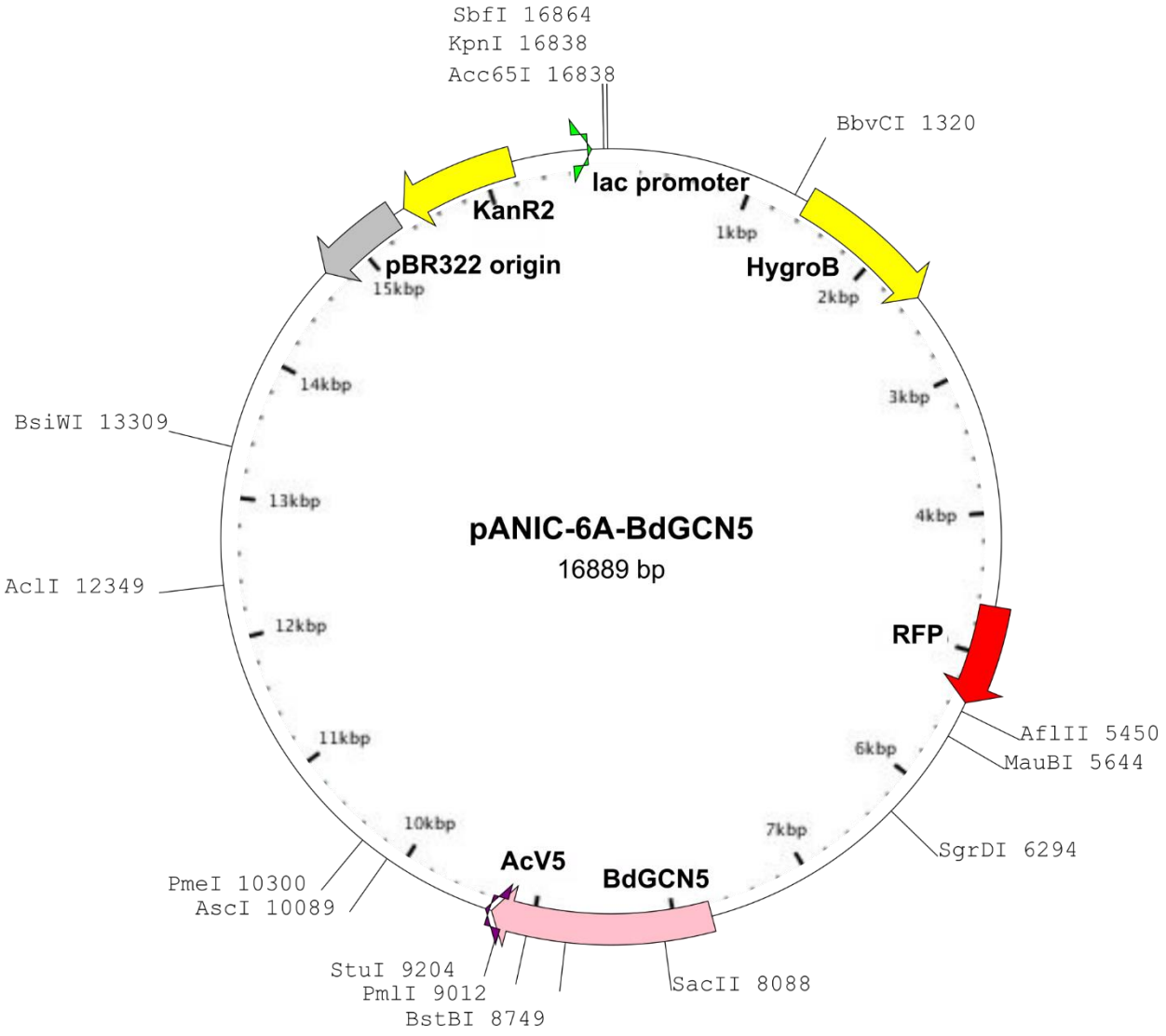

Figure 18. Vector map of the pANIC-6A-*BdGCN5* construct used in transformation. Forward strand: Lactose-operon promoter (Lac promoter) in green, Hygromycin B (HygroB) resistance gene in yellow, Red Fluorescent Protein (RFP) reporter gene in red, *Brachypodium distachyon General Control Non-repressible 5* (*BdGCN5*) gene of interest in pink and *AcV5* epitope in purple. Reverse strand: Kanamycin (KanR2) resistance gene in yellow and pBR322 origin of replication in grey. Lines indicates various enzymatic restriction sites.

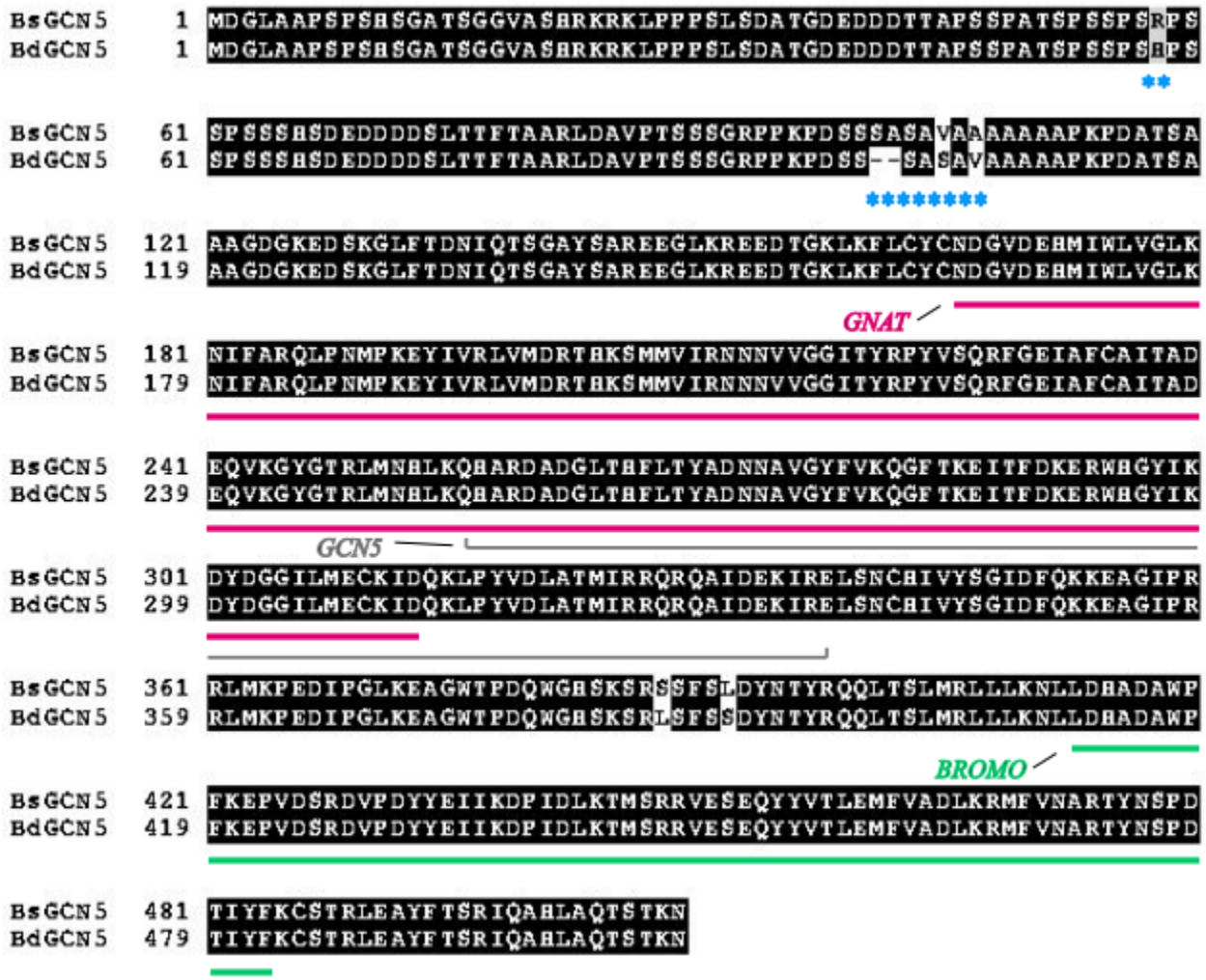

Figure 19. Boxshade analysis comparing homologous peptide sequences of *General Control Non-repressible 5* (*GCN5*) in *B. sylvaticum* (BsGCN5 or Brasy4G240700.1) and in *B. distachyon* (BdGCN5 or Bradi3g26800.1). The magenta line spans the *GCN5-related ACETYLTRANSFERASE* (*GNAT*) domain while the turquoise line show the *BROMODOMAIN-2* (*BROMO*), both characteristic to *GCN5* function. The grey bracket indicates the *GCN5* amplicon as obtained through RT-qPCR. Blue stars highlights region of interest at which species-specific primers were designed, but failed to amplify. Multiple alignment and Boxshade conducted using tools provided on the European Molecular Biology Network (EMBNet) portal.

As for the protocol conduct, we started with 40 and 30 excised embryos respectively for pANIC-6A (EV) and pANIC-6A-*BdGCN5*. Those embryos were cultured and sub-cultured into, respectively 250 and 150 calluses that met the quality requirements for transformation. After the transformation and the following drying treatment and subculture step, 103 (EV) and 138 (*GCN5*) calluses were kept for regeneration under light conditions. From those numbers, 30 (EV) and 62 (*GCN5*) regenerated plantlets were healthy enough to be transferred into soil, which represents efficiency values of 29.1 % and 44.9%. From there, only one of the *GCN5* plantlets transferred into soil was successfully confirmed as a transgenic plant by PCR, thus confirming that Steinwand *et al.* (2013) transformation protocol is transferrable to our specific laboratory conditions. Following this first pass at the transformation protocol, we calculated the final efficiency as the number of confirmed transgenic plants over the number of transformation events. Therefore, we obtain a transformation efficiency of 0% for the empty vector control and 0.007% for the pANIC-6A-*BdGCN5*. In other words, we were unable to transform a plant with the empty vector control. However, we transformed at least one specimen of *B. sylvaticum* with the pANIC6A-*BdGCN5* construct. Table 1 summarizes the efficiency observed while conducting the transformation protocol from one step to another.

Table 11. Transformation efficiency.

Construct	Embryos used	Transformation events (ef. %)[1]	Regenerated calli (ef. %)[1]	Calli transferred to soil (ef. %)[1]	Confirmed transgenic (ef. %)[1]	Final efficiency (%)[2]
pANIC-6A Empty Vector	40	250 (625)	103 (41.2)	30 (29.1)	0 (0)	0
pANIC-6A *BdGCN5*	30	150 (500)	138 (92)	62 (44.9)	1 (0.016)	0.007

1. Efficiency percentage of a given step is calculated considering the count of the preceding step.
2. Final efficiency percentage was calculated considering the number of confirmed transgenic plants over the initial number of transformation events.
ef: efficiency. pANIC-6A: *Paniculatum virgatum* (*E-coli* plasmid). *BdGCN5*: *Brachypodium distachyon General Control Non-repressible 5*.

4.4.1 Transgenic line *BsGCN5*-OX over-expresses *GCN5* by thirty fold

The transgenic line that we obtained was further characterized. At the plantlet stage, no morphological difference was observed (Figure 20 panel A). A PCR analysis revealed the presence of a 1818 bp amplicon specific to the *BdGCN5* insert in the *BsGCN5*-OX as well as with the plasmid pANIC6A-*BdGCN5* used as a positive control. No amplicon was observed in the negative controls *B. sylvaticum* wild-type plants and no-template controls (Figure 20 panel B). Moreover, a 135bp *AcV5-BdGCN5* overlapping, specific amplicon was only amplified in the *BsGCN5*-OX plants (Figure 20 panel C), and not in the *B. sylvaticum* wild-type nor no-template controls (Figure 20 panel C). Together, results presented under panels B and C indicates that *BsGCN5*-OX positively contains the insert of interest. In addition, a RT-qPCR analysis showed that the *BsGCN5*-OX plants accumulate 30 times more *GCN5* transcript when compared to wild-type *B. sylvaticum* plants (Figure 20 panel D). Consequently, we have successfully created a *B. sylvaticum* transgenic line that overexpresses the epigenetic modifier *GCN5*.

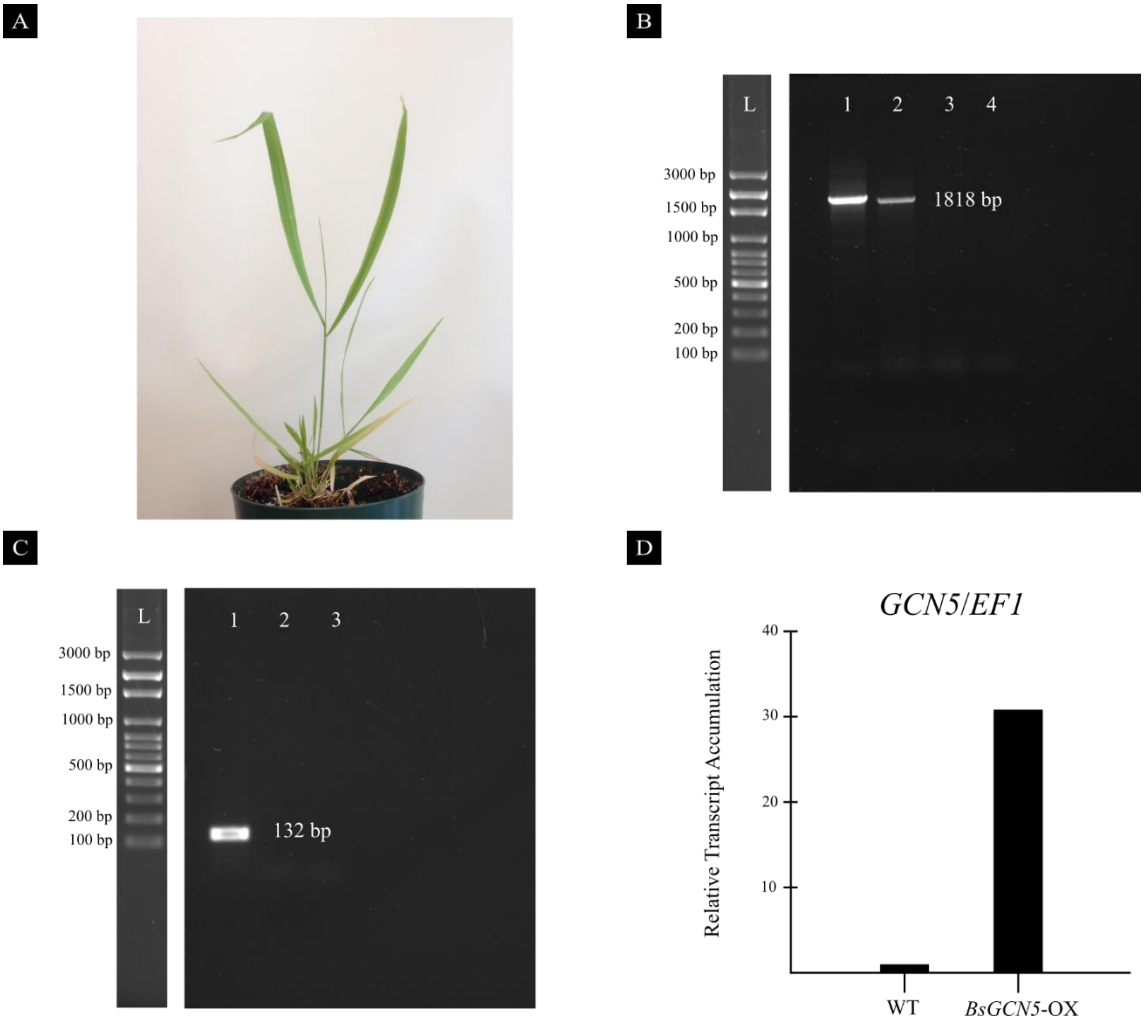

Figure 20. Characterization of a *Brachypodium sylvaticum* overexpressing *General Control Non-repressible 5* (*GCN5*). A: *B. sylvaticum* plant overexpressing *Brachypodium distachyon GCN5* (*BdGCN5*-OX). **B:** Amplification of the entire *GCN5* insert contained in the pANIC-6A vector plus a section of both the right and left borders for a total weight of 1818 bp. L: 100 bp ladder. Lane 1: plasmid DNA from pANIC-6A vector containing the *GCN5* insert (positive control). Lane 2: Genomic DNA extracted from *BdGCN5*-OX *B. sylvaticum* tissues. Lane 3: Genomic DNA extracted from wild-type *B. sylvaticum* tissues (Wild-type negative control). Lane 4: No-template control. **C:** Amplification of a 135 bp section that overlaps the AcV5 epitope located near the right border of pANIC-6A, and the *GCN5* insert. L: 100 bp ladder. Lane 1: *BsGCN5*-OX. Lane 2: *B. sylvaticum* wild-type. Lane 3: No-template control. **D:** Relative transcript accumulation for *GCN5* observed after conducting a Real-Time quantitative Polymerase-Chain Reaction (RT-qPCR).

Chapter 5. DISCUSSION

5.1 Growing *B. sylvaticum* Under Laboratory Conditions

The ability to reproduce a model organism under laboratory conditions is crucial to enable its use in research (Mochida and Shinozaki, 2013). Indeed, annual models such as *Arabidopsis* or *B. distachyon* owe their popularity in part to their small stature, short generation time and self-fertility which all facilitate their reproduction under laboratory conditions (Gilmour *et al*, 1988; Vogel and Bragg, 2009; The International Brachypodium Initiative, 2010; Brkljacic *et al.* 2011). Although *B. sylvaticum*'s stature and generation time are respectively larger and longer than in the closely related *B. distachyon* (Khan and Stace, 1999; Steinwand *et al.*, 2013; this study), we were able to reproduce adequatly at least one accession of *B. sylvaticum* (PI269842) under our laboratory conditions thanks to its high self-fertility (Khan and Stace, 1999) and high germination rate (Table 2).

First year perennial grasses allocate most of their energy to producing overwintering organs rather than investing it in reproduction (Clifton-Brown and Lewandowski, 2000). However, none of the *B. sylvaticum* plants that we have grown under our laboratory conditions were able to produce rhizomes as described in Aarestad (2000). We understand that certain measures, such as the outdoor reproduction of *B. sylvaticum*, could have increase seeds productivity of accessions other than PI269842 (Steinwand *et al.*, 2013). As opposed to indoor growth chambers, outdoor facilities have the advantage to accommodate the larger morphology of *B. sylvaticum.* Therefore, they foster the development of more biomass and rhizomes so that the plant can allocate more energy to its reproduction (Steinwand *et al.*, 2013, Clifton-Brown and Lewandowski, 2000). Overall, we are satisfied with the reproduction capacity of *B. sylvaticum* under laboratory conditions, especially the accession PI269842. However, limitations in seeds availability for the remaining studied accessions cautioned us into making conservative choices in term of experimental design.

5.2 Freezing Tolerance and Cold-Acclimation in *B. sylvaticum*

The induction of freezing tolerance through cold-acclimation is a well described phenomenon in temperate plants (Thomashow, 1999). It was extensively observed in both annual and perennial grasses alike such as in wheat (Fowler and Gusta, 1979; Fowler *et al.*, 1981; Badawi *et al.*, 2007; Pearce *et al.*, 2013), barley (Francia *et al.*, 2007), *B. distachyon* (Li *et al.*, 2012; Colton-Gagnon *et al.* 2014) and perennial ryegrass (Lorenzetti *et al*, 1971; Fuller and Eagles, 1978; Humphreys and

Eagles, 1988; Tcacenco *et al.*, 1989; Ebdon *et al.*, 2002; Hulke *et al.*, 2008). Our study suggests that *B. sylvaticum* is no exception to this phenomenon. Namely, we observe that *B. sylvaticum*'s freezing tolerance is increased in response to cold-acclimation.

More specifically, survival results from our WPFT show that a pre-exposition to low, non-freezing temperatures increases *B. sylvaticum*'s freezing tolerance at the temperature points -7°C and -9°C (Figure 6). Hence, we estimate that cold-acclimated plants from most accessions are able to survive to freezing temperatures that are 2°C lower than for non-acclimated plants (Figure 6). Despite this moderate increase in freezing tolerance, we consider the series of WPFT that we conducted biologically relevant. Indeed, the use of whole, live plants while investigating freezing tolerance still better reflects the plant's natural response than by using excised tissues (Gusta and Wisnieski, 2013). There also exist a substantial body of literature that successfully correlates the winter-hardiness in the field of perennial grass crops such as *L. perenne* to results obtained from a WPFT (Lorenzetti *et al*, 1971; Fuller and Eagles, 1978; Waldron *et al*, 1998; Hulke *et al*., 2008). In those studies just like in ours, the survival of live plants is used as a proxy to their freezing tolerance, but is additionally related to direct applications in the field. These studies also used an experimental design that is similar to the one described in this project, which is to grow plants under a long day photoperiod at room temperatures and then cold-acclimate them under a short day photoperiod at low, non-freezing temperatures for several days. Such design was originally inspired by work the like of Lorenzetti *et al.* (1971) which determined that out of many possible combinations, *L. perenne* achieves its higher freezing tolerance when cold-acclimated for 8 days under a 8 hr of light : 16 hr of dark photoperiod.

In support to our WPFT results, we observe an increase in the transcription of two cold-responsive genes (*BsCOR413*; Figure 15 and *BsCOR410*; Figure 16) during the first days of cold-acclimation in all studied accessions. Such results are consistent with observations reported in wheat for *COR413* (Breton *et al.*, 2003) and *COR410* (Danyluk *et al.*, 1994), and in *B. distachyon* for *COR413* (Colton-Gagnon *et al*, 2014). In light of the homology demonstrated between those genes in *B. sylvaticum* and in wheat (Tables 5 and 6; Figure 7 and 8), our results suggest that *BsCOR413* and *BsCOR410* play an active role in *B. sylvaticum*'s cold-acclimation process just as their homologs do in wheat. Indeed, Breton *et al.* (2003) suggest that the induction of *TaCOR413pm1* in response to cold in wheat is associated to the preparation of the cell membrane to cope with

freezing stress. Such suggestion was advanced after discovering the Alpha-helix structure which confer *COR413pm1* transmembrane property (Breton *et al.* 2003). In the case of *COR410*, Danyluk *et al.* (1998) directly observed the accumulation of this dehydrin near the cell membrane of previously cold-acclimated wheat tissues. Therefore, we suggest that when exposed to low, non-freezing temperatures, *B. sylvaticum* increases its transcription of *BsCOR413* and *BsCOR410* to prevent its cell membranes from undergoing freezing stress damages.

In addition, we observe the rapid induction in response to cold of a *CBFIII*-related transcription factor (*BsCBF2.1*) in PI269842 (Figure 17). The observed expression profile is similar to those observed for closely related *CBF* genes which were linked to cold-response in *B. distachyon* (Chen *et al.*, 2016), wheat (Badawi *et al.*, 2007) and perennial ryegrass (Tamura and Yamada, 2007). When also considering the highlighted homology between *BsCBF2.1* and homologs from the aforementioned species (Tables 5 and 6, Figure 9), we suggest that *BsCBF2.1* acts as a transcription factor that participates in orchestrating the molecular response to cold in *B. sylvaticum*. In other words, *BsCBF2.1* should play a role in activating the transcription of downstream *COR* genes the like of *BsCOR413* and *BsCOR410*.

According to the theory of cold-acclimation (Guy, 1990; Guy and Thomashow, 1999; Chew and Halliday, 2011; Li *et al.*, 2012), this increase in *COR* and *CBF* genes transcription should directly contributes to an increase in *B. sylvaticum*'s freezing tolerance. Hence, the expression profiles that we observe for *BsCOR413, BsCOR410* and *BsCBF2.1* in response to cold is inherently linked to the increase in survival at -7°C and -9°C that we observe for cold-acclimated *B. sylvaticum*. However, temperate grasses are known to possess numerous *CBFs* and *COR* genes which collectively contribute to enable freezing tolerance at the organism level (Badawi *et al.*, 2007; Tommasini *et al.*, 2008; Ryu *et al.* , 2014; Yuezhi Wang *et al.* 2014; Chen *et al.*, 2016). Therefore, *BsCOR413, BsCOR410* and *BsCBF2.1* are only isolated actors of the molecular response to cold in *B. sylvaticum*. Nevetheless, in the current experimental context we consider *BsCOR413, BsCOR410* and *BsCBF2.1* as genetic markers that inform us about the existence of cold-response molecular mechanisms in *B. sylvaticum*. Henceforth, the fact that they increase their transcription in response to cold confirms that *B. sylvaticum* possesses the molecular mechanisms that enable an increase in its freezing tolerance (Figure 6).

Consequently, our molecular study do not allow to determine at which extent *BsCOR413*, *BsCOR410* and *BsCBF2.1* are responsible for *B. sylvaticum*'s increase in survival at freezing temperatures. This responsibility could be isolated while studying the response to cold in mutant plants that are deficient in the genes of interest such as in Vlachonasios *et al.* (2003) for *ADA2* or Bredow *et al.* (2016) for *IRIs*. Moreover, the obtained expression profiles in the case of *BsCBF2.1* could have been biased by our tissue collection method. That is, Chen *et al.* 2016 showed that *CBF2.1* in *B. distachyon* is expressed at higher levels in roots tissues as opposed to crown and leaves tissues. Because we used leaves tissues only during collection, the results that we report in Figure 17 might not render the full extent at which *BsCBF2.1* is expressed in *B. sylvaticum*. Note that in the case of *COR413* and *COR410*, Breton *et al.* (2003) and Danyluk *et al.* (1994) respectively showed that each genes is expressed at higher levels in leaves tissues.

On a side note, the study of *BsCOR413, BsCOR410* and *BsCBF2.1* could not have been performed without the selection and use of an internal standard gene that is adapted to our experimental design. Because very little information concerning the molecular study of *B. sylvaticum* had been published at the beginning of the current project, we had to identify (Tables 5 and 6; Figures 10-13) and test (Figure 14) the proficiency of *BsEF1*, *BsUB16*, *BsGAPDH* and *BsSAMDC* as standard genes. Homologs of those genes are commonly used as internal standards for molecular studies conducted in *B. distachyon* (Hong *et al.*, 2008; Li *et al.*, 2012; Ream *et al.*, 2014; Woods *et al.*, 2014; Martel *et al.* 2017). Notably, Hong *et al.* (2008) tested the use of the aforementioned genes and more under various experimental conditions to observe that *EF1* and *SAMDC* are more stably expressed than others under contrasting temperature conditions. Although we could not afford to individually test *BsEF1*, *BsUB16*, *BsGAPDH* and *BsSAMDC* in all accessions due to tissues limitation, we minimally performed a series of standard curves with pooled cDNA from all accessions and treatments to verify the stability of their amplification under our experimental conditions. In Figure 4, we provide an example of such standard curves. Note that the standard curve for *BsEF1* scored the R^2 value (0.9982) that is the closest to 1. Followed *BsUBC16* (0.9958), *BsSAMDC* (0.9938) and *BsGAPDH* (0.9793). Here, the closer the R^2 value is to 1, the more the amplification is influenced by the dilution factor only and the less it is biased by other factors or a combination of other factors. Due to the high sensitivity of the RT-qPCR method, amplicons that are associated to a R^2 value under 0.9999 or over 1.1000 should be rejected, which is the case here with *BsGAPDH* (Livak and Schmittgen, 2001). Therefore, of all the genes that we

tested, the amplicon of *BsEF1* is the one that is the most stably amplified under our set of conditions and across all accessions. When combined to the results that we observe in PI564896 (Figure 14) and to the observations by Hong *et al.* (2008) in *B. distachyon* in relation to expression stability, such results made us choose *BsEF1* as internal standard to complete our molecular study.

Although the resolutions of both our WPFT and molecular study are not optimal, they are powerful enough to highlight a gradient in cold-acclimation capacity among studied accessions. Indeed, the nine accessions may be grouped according to their survival profiles and supporting *COR* genes expression profiles. As an example, the accessions PI564896, PI344569 and PI440178 all display a more pronounced survival profile at the temperature points -7 °C and -9°C which suggest a greater cold-acclimation potential (Figure 6). Consequently and to the exception of *BsCOR410* in PI440178, the associated expression profile of the *CORs* genes in those accessions are well responsive to cold and especially significant in the case of PI564896. In light of the climatic backgrounds reported in Table 1, we can draw an interesting parallel with the geographic origin of the studied accessions and their survival profile. This is particularly visible for PI564896, PI344569 and PI440178 as they all originates from the northernmost locations (Figure 2). Therefore, they are submitted to a colder climate and shorter photoperiods during late autumn and early winter (Table 1), that is the period at which cold-acclimation occurs in a natural environment (Xin and Browse, 2000). Moreover, the photoperiod in early December at the latitudes corresponding to the original site of collection for those accessions is similar to the one from our cold-acclimation conditions and could be related to an induction of the observed greater cold-acclimation potential (Figure 6). Consequently, it is possible that the local climate of origin act as a driver of the intra-specific diversity that we observe in *B. sylvaticum*'s response to cold under our laboratory condition.

In fact, several cereal breeders have noticed that geographic origin is an important contributor to intra-species molecular diversity (Autrique *et al.*, 1996; Malysheva-Otto *et al.*, 2006; Eivazi *et al.*, 2008; Chaudhary *et al.*, 2011; Najaphy *et al.*, 2012). For instance, in a proteomic study of wheat intraspecific diversity, Eivazi *et al.* (2008) isolated a dozen of proteins that where genotype-specific. Out of those, two of them where *COR* proteins and one was a transcription factor induced by cold, thus showing that cold-related proteins can be genotype-specific in wheat (Eivazi *et al.*, 2008). In wild grasses, the influence of geographic origin on intra-species molecular diversity is

potentially more pronounced (Gordon *et al.*, 2014). For instance, Lubbers *et al.* (1991) studied the intra-species molecular diversity of *Triticum tauschii*, a parental species of wheat, over a geographical range that closely resemble the one encompassed by our studied *B. sylvaticum* accessions (Caspian Sea, Balkans and Mediterranean crescent). They observed that each accession had a unique genotype (Lubbers *et al.* 1991). In addition, Gordon *et al.* (2014) found that variance in sequences for phenotypically divergent *B. distachyon* accessions from a similar geographic distribution affected the expression of drought-responsive genes. Such variances could hypothetically drive diversity in *B. distachyon*'s drought tolerance, a trait that is intrinsically related to freezing tolerance in plants (Sandve *et al.*, 2011). Therefore, it is likely that the genomic diversity observed in geographically diverse accessions of *B. sylvaticum* by Steinwand *et al.* (2013) affects the species' molecular response to cold, thus diversifying its intra-specific cold-acclimation capacity as we observe it.

Many other studies that focuses on temperate grasses had observed an intra-species diversity in freezing tolerances. For instance, in Fowler *et al.* (1981) the value of LT50s for 36 cultivars of wheat was found to range from -11.6 °C to -19.6 °C. Similarly, studies by Tcacenco *et al.* (1989), Ebdon *et al.* (2002) and Hulke *et al.* (2008) collectively determined the freezing tolerances of 46 different cultivars of *L. perenne* and had observed LT50s values that range from -2.8 °C to -13.95 °C for cold-acclimated specimens. As exemplified by those studies, the ranges of possible intra-species freezing tolerances for domesticated crops is usually extensive. Such range may be explained by the consecutive breeding efforts to obtain specific agronomic traits, hence directly or indirectly affecting freezing tolerance and cold-acclimation capacity (Xin and Browse, 2000). In wild species of temperate grasses such as *B. sylvaticum* or *B. distachyon*, the range of possible intra-species freezing tolerance was not voluntarily skewed by humans. Therefore, those model organisms offer the opportunity to better understand what naturally drives intra-specific diversity in response to cold. Findings on that matter could turn useful when selectively breeding economically important pooid grasses in order to achieve a higher freezing tolerance (Mickelbart *et al.*, 2015).

In addition to the observed increase in survival supported by molecular mechanisms, our WPFT approach informs us on mechanisms of freezing stress avoidance, namely, mechanisms by which plants focus their resources in specific organs to the detriment of others while undergoing freezing

stress (Gusta and Wisnieski, 2013). Here, a link may be made between freezing stress avoidance and the overwintering capacity of perennial grasses, thanks to their persistent root systems. Accordingly, we had observed that during the recovery phase after the WPFT, a few individual plants for which the aerial tissues were completely dead had regrown from the roots and crown even though our tested plants were too young to be considered established perennials. Such observation was never made in the annual *B. distachyon* while conducting WPFTs similar as in Colton-Gagnon *et al.* (2014). The nature of our WPFT approach allow us to access this additional layer of information pertaining to *B. sylvaticum'*s response to cold.

Interestingly enough and despite this difference in response between *B. sylvaticum* and *B. distachyon*, the cold-acclimation potential of the first is similar to that of the second as reported in Colton-Gagnon *et al.* (2014). In the later study, the response to cold of seven different accessions with similar geographical background as the ones used in the current study was tested. Overall, the observed difference in freezing tolerance between NA and CA plants is of about 2 °C in both species regardless of their life strategies. This observation might be explained by the fact that young, first year perennial plants like the one we tested may be more vulnerable to freezing stress than older, established perennial plants (Clifton-Brown and Lewandowski, 2000; Yu *et al.*, 2015). Considering that both species are closely related (Catalán *et al.*, 2012), we speculate that an important similarity between the cold-response mechanisms of young *B. sylvaticum* plants and *B. distachyon* causes this convergence between the two species' freezing tolerance.

However, the speculation stated in the previous paragraph could only be verifyed by conducting a proper comparative test of the two species' response to cold. Typically, researchers that studied freezing tolerance capacities of established perennials in other species are opting for a field study approach (Clifton-Brown and Lewandowski, 2000; Yu *et al.*, 2015) or an electrolyte leakage test approach (Humphreys and Eagles, 1988; Tcacenco *et al.*, 1989; Ebdon *et al.*, 2002). Therefore, an experimental design where the freezing tolerance of annual *B. distachyon* plants would be compared to the one of young, first year *B. sylvaticum* and established, second year *B. sylvaticum* plants with supporting molecular studies could further inform us on the winter hardiness of both species. To our knowledge, Hoffman *et al.* (2010) is the sole example in the literature where the freezing tolerance of an annual grass (annual blue grass) is quantitatively compared to that of a perennial grass (creeping bentgrass) (see Chapter 2, section 2.3.3 for more details). Even though

the relationship of the two compared species goes back to the tribe level (*Poeae*), they are not as closely related as *B. sylvaticum* and *B. distachyon* are (Catalán *et al.*, 2012). Furthermore, annual blue grass and creeping bentgrass do not benefit of extensive genetic tools and resources (Hoffman *et al.*, 2010). Therefore, the proposed comparative assay between *B. sylvaticum* and *B. distachyon* has the potential to better circumscribe the response to cold in relation to life strategy in pooid grasses. In the eventuality where an advantageous traits would be identified from this comparison, findings made in the *Brachypodium* genus could be transferred and applied to improve the freezing tolerance of economically important pooid grasses (Xin and Browse, 2000; Mochida and Shinozaki, 2013; Mickelbart *et al.*, 2015).

To sum up, our results suggest that *B. sylvaticum* is capable of cold-acclimation and to consequently increase its survival in response to freezing stress. They also suggest that a phenotypical gradient in *B. sylvaticum*'s cold-acclimation capacity differentiates the nine accessions that we studied. Finally, our results allow to qualitatively observe that the response to freezing stress in *B. sylvaticum* is similar to that of *B. distachyon* regardless of their difference in life strategy.

5.3 Transformation of *BsGCN5*-OX

In light of our recent effort to better characterize *GCN5's* role into the *Brachypodium* genus (Martel *et al.*, 2017), we chose to test the Steinwand *et al.* (2013)'s high-efficiency transformation protocol for *B. sylvaticum* with a pANIC-6A-*BdGCN5* construct (Figure 18). The following section discusses of the generation of the transgenic line *BsGCN5*-OX and suggests some improvements to increase the overall protocol efficiency under our laboratory conditions.

We successfully generated a line of *B. sylvaticum* PI269842 that over-expresses by thirtyfold *GCN5* as compared to a wild-type control. At this stage of the project, it was impossible for us to determine the expression ratio of the endogenous versus exogenous *GCN5* in *BsGCN5*-OX. Because of the high level of conservation between *BdGCN5* and *BsGCN5* (Figure 19), we tried to exploit two areas for primers design with slight differences in sequences indicated by the blue stars in Figure 19, that is one site with alternating substitutions and another with an insertion-deletion. However, this strategy failed as the obtained qPCR primers did not reliably amplified the specific regions. More efforts will have to be deployed so that specific primer pairs could be generate for future studies. Instead, we were able to amplify a section of the pANIC-6A-*BdGCN5* that overlaps

both the end of the exogenous protein's sequence and the epitope *AcV5* (Figure 18). Because *BsGCN5*-OX is the only transgenic line that we could test, we obtained no other CQ threshold value to relativize it with. To give an impression of the expression of the recombinant gene, we observed that the CQ value of three technical replicates of *BdGCN5-AcV5* averaged 17.47 while the average CQ value for all technical replicates of the standard gene *BsEF1* in both *BsGCN5*-OX and the wild-type control was at 17.90. Although we cannot supply an absolute quantification of the expression of the exogenous *GCN5* in *BsGCN5*-OX, our approach at least confirms the transgenic status of this plant.

Furthermore and as reported in Table 10, we could not transform *B. sylvaticum* with the pANIC-6A empty vector. Therefore, we are unable to control for the effect of the inserted vector alone in *BsGCN5*-OX. In Figure 5, panels F and G we show through Ultra-Violet microscopy an active transformation foci in one of the callus that has been co-cultivated with *Agrobacterium* containing the empty vector. Such observation suggests that we successfully inserted the empty vector in at least a few calluses (transformation foci were observed in a dozen of transformed calluses, data not shown) before losing all of the lines along the remaining steps of the protocol. When looking at the values of efficiency reported in Table 10, we see that the efficiency of going from one step to another for empty vector drastically drops between the transformation and the regeneration steps. This drop in efficiency is explained by a high rate of contamination with *Agrobacterium* AGL1 during the incubation/subculture period that follows transformation. In an attempt to preserve healthy calluses, we increased our frequency of subculture as suggested in Alves *et al.* (2009). We also tried to rescue many calluses by rinsing them with 1000x timentin (*Agrobacterium*-specific antibiotic) as suggested in Nishimura *et al.* (2006) but obtained mitigated results using this strategy.

Agrobacterium contamination occurs when the bacterial suspension used during transformation remains active long enough on the surface of the calluses to take advantage of the LS media Sallaud *et al.* (2003). Desiccating adequately the calluses after the transformation step is crucial to avoid contamination and increase transformation efficiency (Vogel and Hill, 2008; Nishimura *et al.*, 2006, Alves *et al.*, 2009; Steinwand *et al.*, 2013). As detailed in chapter 3, section 3.4.2, we furthered that step by carefully absorbing the excess of bacterial suspension with sterile paper towel and exposing the calluses to sterile airflow in hope to increase their drying before dark

incubation. Considering that two of our initial transformation efforts had failed, those slight protocol improvements ultimately enabled us to produce one *B. sylvaticum* transgenic plant.

Additional parameters that may have caused a low transformation efficiency include poor regeneration of calluses or suboptimal selection and timing when carrying a protocol. While the first possibility did not seem to be a problem for us (some calluses would start greening only 2 days after being exposed to light), we hypothesize that the second had impeded our final efficiency. For instance, in rice Sallaud *et al.,* (2003) achieved high transformation efficiencies of 75 to 98% by meticulously picking the right calluses at the right time. Although this statement seems obvious at first, one must take into account all the optimization work that is required to design such a high efficiency protocol. Indeed, Sallaud *et al.* (2003) had to try many different protocol variations before proposing an elaborate protocol.

Similarly, many researchers had contribute to develop high-throughput transformation protocols in the *Brachypodium* genus as initial values of transformation efficiency were increased from 4-13% (Christiansen *et al.*, 2005; Vogel *et al.,* 2006; Vain *et al.*, 2008) to current values of 35-50 % (Vogel and Hill, 2008; Păcurar *et al.,* 2008). In *B. sylvaticum*, Steinwand *et al.*, (2013) reports a transformation efficiency value as high as 168% with PI269842. However, this high value was observed because of the fragmentation of their calluses after the transformation step. Hence, it is difficult to weigh the representativeness of this value as it is inflated by the multiplication of an initially lower value of transformation efficiency. Although we lost most of our transformed calluses to *Agrobacterium* contamination, we observed similar ratios of calluses division in *B. sylvaticum* as in Steinwand *et al.* (2013).

With reported efficiencies value as low as null and 0.007% (Table 10), we recognize that our adaptation of Steinwand *et al.* (2013)'s protocol needs to be optimized to appreciable levels of efficiency before engaging into successful high-throughput transformation of *B. sylvaticum.* To do so, we suggest the fine-tuning of the post-transformation drying treatment to avoid *Agrobacterium* contamination, as well as to reduce the friability of the calluses. Although we were unable to transform *B. sylvaticum* more than once under our laboratory condition, we are confident that the suggested improvements of our protocol adaptation will lead to reproducible results.

Chapter 6. GENERAL CONCLUSION

6.1 Conclusion

Altogether, our results support our general hypothesis which states that "*B. sylvaticum* can cold-acclimate in response to low temperatures and show an increase in its freezing tolerance". To our knowledge, our study is the first to characterize the response to cold in *B. sylvaticum*, both at the phenotypic and molecular level. Additionally, our production of the single transgenic line *BsGCN5*-OX shows that we can transform *B. sylvaticum* plants for research purposes under our laboratory conditions given that a proper optimization of the transformation protocol is undertaken to achieve high throughput capabilities. Therefore, by having completed our research objectives, we acknowledge the accessibility and usefulness of the model *B. sylvaticum* for the study of response to abiotic stress and related components in perennial grasses. Coupled with its proximity to the already important grass model *B. distachyon*, *B. sylvaticum* has the potential to become a powerful genetic model for temperate, perennial grasses such that discoveries made with this species may later apply in other economically important grasses.

6.2 Contribution to Science

- First report of the cold-acclimation capacity and a related increase in the freezing tolerance of live plants from 9 accessions in *B. sylvaticum*.
- Isolation of the cold-responsive genes *BsCBF2.1, BsCOR413* and *BsCOR410* and relative transcripts accumulation profiles in response to cold in *B. sylvaticum*.
- Production of a transgenic *B. sylvaticum* plant overexpressing by thirtyfold the histone acetyltransferase *GCN5*.

6.3 Future Directions

Futures studies investigating *B. sylvaticum*'s response to cold should focus on expanding both the phenotypical, molecular and metabolic characterization of the species' cold-acclimation response. For instance, the study of cold-acclimation in second-year, established *B. sylvaticum* would reveal whether its life strategy is having an effect or not on its freezing tolerance. Such study would also enable an intra-genus comparison with *B. distachyon*, which itself would inform us on perennial-specific cold-acclimation mechanisms. Furthermore, the identification and isolation of other pooid-specific *IRI* genes as well as *CBFs* and *CORs* genes in *B. sylvaticum* would reinforce our

observation of cold-acclimation mechanisms in the species. Similarly, the study of cold-related metabolites such as the pooid-specific fructan or free-sugars would provide novel information about the species capacity to cold-acclimate at the metabolic level, an aspect that we did not explore here. Also, the genome sequencing of phenotypically divergent accessions would reveals interesting molecular diversity in the expression of *CBFs* and *CORs* genes, or in the function of related proteins. The availability of such genomic data would allow us to characterize molecularly the diversity in response to cold that we observed in *B. sylvaticum* and possibly take advantage of this diversity to apply it to the targeted breeding of freezing tolerant pooid crops. Finally, if enhanced with an empty vector control and more independent over-expressing lines, our over-expressor of *BdGCN5* could turn useful while studying the effect of epigenetic modifications, in particular histone acetylation, on the response to abiotic stresses in *B. sylvaticum*.– Discoveries made in that field could be applied and transferred to the improvement of the response to abiotic stresses in economically important grasses.

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