UNDERSTANDING THE REGULATION OF PHENOTYPIC PLASTICITY IN PLANTS: INSIGHTS FROM COLD ACCLIMATION AND VERNALIZATION IN BRACHYPODIUM GRASS

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To know, is to know that you know nothing.
That is the meaning of true knowledge.
Socrates

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LIST OF ABBREVIATIONS

amiVRN1	*VRN1* knocked-down transgenic plants
AP1/FUL	*APETALA1/FRUITFULL* family
At*FLC*	*FLC* gene from Arabidopsis thaliana
Bd18-1	*B. distachyon* accession 18-1
Bd21	*B. distachyon* accession 21
Bd21-3	*B. distachyon* accession 21-3
Bd29-1	*B. distachyon* accession 29-1
Bd30-1	*B. distachyon* accession 30-1
Bd*VRN1*	*VRN1* gene from *Brachypodium distachyon*
BhD	subgenome of *B. hybridum* inherited from *B. distachyon*
BhS	subgenome of *B. hybridum* inherited from *B. stacei*
Bsh	warm semi-arid
CBF1-3	*C-REPEAT BINDING FACTOR1-3* genes
CC	constant-chilling
CC7-56	contant-chilling treatment 7 to 56 days long
cDNA	complementary DNA
Cfa/Cfb	humid subtropical/oceanic
ChIP	chromatin immunoprecipitation
COR	COLD-REGULATED
COR410	*COLD-REGULATED410* gene
COR413	*COLD-REGULATED413* gene
Csa	hot-summer Mediterranean
Csb/Bsk	warm-summer Mediterranean/cold semi-arid
CTR	control
D subgenome	subgenome of *B. hybridum* inherited from *B. distachyon*
DF	diurnal-freezing
DF1-4	1^{st} to 4^{th} cycle of exposure to diurnal-freezing
DF7-56	diurnal-freezing treatment 7 to 56 days long
DNA	deoxyribonucleic acid

dtr	diurnal-temperature range
FLC	*FLOWERING LOCUS C* gene
frs	frost frequency (days/month)
FT	FLOWERING LOCUS-T
H1-4	habitats of *B. distachyon* 1 to 4
H2A.Z	histone 2 A variant Z
H3K27me3	histone 3 lysine 27 tri-methylation
H3K4me2/3	histone 3 lysine 4 di-/tri-methylation
Hv*VRN1*	*VRN1* gene from *Hordeum vulgare* (barley)
IRI	*ICE-RECRYSTALLIZATION INHIBITOR* gene
LT_{50}	leathal temperature 50%
mRNA	messenger RNA
N	naïve
NV	non-vernalized
P	primed
P1-2	plant population 1 or 2
PC1-2	principal components 1 and 2
PCA	principal component analysis
pp	photoperiod or daily hours of light
PRC2	Polycomb Repressive Complex 2
qPCR	quantitative polymerase chain reaction
R	recovery time-point of diurnal-freezing
R1-4	recovery time-points of cycles 1 to 4 of DF
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RT-qPCR	reverse-transcriptase qPCR
S subgenome	subgenome of *B. hybridum* inherited from B. stacei
S	stress time-point of diurnal-freezing
S1-4	stress time-points of cycles 1 to 4 of DF
TM	transcriptional memory
tmp	average temperature

TrxG	Trithorax-group proteins
UBC18	*UBIQUITIN-CONJUGATING ENZYME 18* gene
UBI:*VRN1*	*VRN1* overexpressing transgenic plants
VRN1	*VERNALIZATION1* gene

GLOSSARY

Acclimation	Process leading to the ability to survive and/or continue development under suboptimal conditions.
Adaptation	Evolutionary process leading to a better fit in the environment
Chromatin	Complex of DNA and protein involved in the packaging of DNA
Chromatin mark or modification	Chemical modification of chromatin
Cold acclimation	Process that leads to an increase in freezing tolerance
Cold-induced plasticity	Phenotypic change in response to cold
Morphological plasticity	Change in morphology in response to the environment
Naïve	State of an organism or response before priming
Phenological plasticity	Change in the timing of growth, reproduction and senescence in response to the environment
Phenotype	A set of observable characteristics of a living organism
Phenotypic plasticity	Capacity of organisms to change their phenotype (*e.g.* physiological, morphological, phenological

	phenotypes) in response to the environment, without genetic change
Physiological plasticity	Change in physiology in response to the environment
Primed	State of an organism or response after priming
Priming	Process that increases the capacity to respond to a given stimulus
Stress	External conditions that negatively affect growth, development, and productivity
Stress memory	Stress-induced changes that are retained after the stress has passed and that affect subsequent responses to that stress
Temperate-climate adaptive plasticity	Change in phenotype that ensures persistence in temperate climates, notably through cold acclimation and vernalization
Transcriptional memory	Memory event which affects the transcriptional response of a gene
Vernalization	Process that promotes flowering in response to prolonged exposure to cold

ABSTRACT

Agriculture needs to become more resilient to sustain food production through the consequences of global warming. Agricultural systems can benefit from the innate ability of plants to adjust their biology when faced with challenging conditions. Indeed, plants can exhibit phenotypic plasticity, which is the ability to produce different phenotypes in response to the environment, at the level of physiology, morphology or phenology to perform better in their environment. Although stress responses have been extensively studied, the mechanisms that regulate phenotypic plasticity and coordinate phenotypic expression in plants are not well understood. The prevalence of cold and the seasonal conditions found in temperate climates have selected for extensive plasticity in plants, and hence offer a useful system to research the regulation of phenotypic plasticity. *Brachypodium distachyon* is a wild temperate grass used as a model plant and which is closely related to economically important cereals. In its habitat, *B. distachyon* can acclimate to the colder conditions of the fall and subsequently increase its tolerance to winter's freezing through physiological and morphological plasticity, and simultaneously delay its flowering time until spring though a phenological response known as vernalization. The main objective of this thesis was to study the regulation of cold acclimation and vernalization in Brachypodium grass as an effort to understand the mechanisms governing phenotypic plasticity in plants.

The first sub-objective was to identify and reproduce in laboratory settings the cues that induce the expression of temperate-climate adaptive phenotypes in *B. distachyon* and characterize the cold acclimation and vernalization responses induced by these conditions. These simulated seasonal cues, combined into a single treatment which we called diurnal-freezing, induced in *B. distachyon* physiological and morphological responses that led to high freezing tolerance. Diurnal-freezing also conferred flowering competence through vernalization which was characterized by a low expression of the gene *VERNALIZATION1* (*VRN1*). This study revealed that *VRN1* does not only regulate vernalization but is also determinant for the expression of physiological and morphological cold acclimation in *B. distachyon*.

The second sub-objective was to identify the molecular mechanisms that regulate the expression of physiological and morphological cold acclimation and of vernalization in *B. distachyon*. Over time in diurnal-freezing, initial cold-stress responses were toned down by transcriptional memories to restore vegetative growth, thereby allowing *B. distachyon* to build a

freezing-tolerant plant morphology. This second study revealed that transcriptional memories regulate, in addition to vernalization, physiological and morphological cold acclimation and are hence central to the expression of temperate-climate adaptive phenotypes in *B. distachyon*.

The third sub-objective was to study cold acclimation and vernalization in an annual Brachypodium line that flowers readily. Results showed that (i) this line belonged to the species *Brachypodium hybridum*, a hybrid and daughter species of *B. distachyon*, (ii) this line was unresponsive to vernalization treatments but (iii) retained the cold acclimation traits of *B. distachyon* and (iv) its development was less restricted by cold and photoperiod compared to *B. distachyon*, which likely contributed the expansion of the species into new environments. This third study provided insights on the evolution of plasticity traits in plants, which in this case, occurred with the dissociation of flowering time from temperate-climate adaptive plasticity.

The fourth sub-objective was to propose possible regulatory mechanisms of phenotypic plasticity in plants. The previous studies revealed the function of transcriptional memories in regulating temperate-climate adaptive plasticity in Brachypodium grass and hence, as mechanisms of transcriptional memory are connected to chromatin, a fourth manuscript focused on the role of chromatin in coordinating phenotypic expression and in linking plasticity to adaptation.

Overall, this work presents detailed studies on the expression, interaction and regulation of cold acclimation and vernalization in Brachypodium grass. Results demonstrate that mechanisms of transcriptional memory are central to the regulation of phenotypic plasticity in Brachypodium and provide useful insights into the processes involved in adaptation.

RÉSUMÉ

Afin que la production alimentaire puisse faire face aux crises environnementales liées aux activités humaines, l'agriculture se doit de devenir plus résiliente. En particulier, les systèmes agricoles pourraient bénéficier de la capacité innée des plantes à ajuster leur biologie lorsqu'elles sont confrontées à des conditions difficiles. En effet, les plantes présentent une plasticité phénotypique, c'est-à-dire une capacité à adopter différentes formes, ou phénotypes, en réponse à l'environnement, tant bien au niveau physiologique, morphologique ou phénologique (relié au cycle de vie) afin d'augmenter leurs chances de survive aux stresses environnementaux. Bien que les réponses au stresse des plantes aient été le sujet de nombreuses études, les mécanismes qui régulent la plasticité phénotypique et qui coordonnent l'expression phénotypique chez les plantes restent encore à être élucidés. La forte présence du froid et d'autres conditions saisonnières issus des climats tempérés ont sélectionnés au fil du temps les plantes capables de démontrer une importante plasticité. Celles-ci offrent donc un système d'étude très utile pour comprendre les mécanismes de régulation de la plasticité phénotypique des plantes. *Brachypodium distachyon* est une graminée sauvage des zones tempérées qui est utilisée comme plante modèle pour les études de laboratoire. Elle est aussi une proche cousine des plantes céréalières telles que le blé, l'orge et le riz. Dans son habitat naturel, *B. distachyon* peut s'acclimater aux conditions plus froides de l'automne et augmenter par la suite sa tolérance au gel grâce à une plasticité physiologique et morphologique, tout en retardant simultanément son temps de floraison jusqu'au printemps, et cela grâce à une réponse phénologique appelée vernalisation. L'objectif principal de cette thèse était d'étudier la régulation de l'acclimatation au froid et de la vernalisation chez Brachypodium dans le but d'acquérir une meilleure compréhension des mécanismes qui gouvernent la plasticité phénotypique des plantes.

Le premier sous-objectif était d'identifier et de reproduire en laboratoire les signaux environnementaux qui induisent l'expression de phénotypes liés à la survie dans les climats tempérés chez *B. distachyon* puis de caractériser les réponses d'acclimatation au froid et de vernalisation qui sont induites par ces conditions. Ces signaux saisonniers, reproduits dans un seul traitement que nous avons appelé « diurnal-freezing », ont induit dans *B. distachyon* des réponses physiologiques et morphologiques qui ont conduit à une tolérance au gel élevée. Ce traitement a également conféré une compétence de floraison par une réponse de vernalisation caractérisée par une faible expression du gène *VERNALIZATION1* (*VRN1*). Cette étude a révélé que *VRN1* ne

régule pas seulement la vernalisation, mais joue également un rôle déterminant lors de l'acclimatation physiologique et morphologique au froid de *B. distachyon*.

Le deuxième sous-objectif était d'identifier les mécanismes moléculaires qui régulent l'expression de l'acclimatation physiologique et morphologique au froid et de la vernalisation chez *B. distachyon*. Au fil des cycles d'exposition au « diurnal-freezing », les réponses initiales au stress induites par le froid ont été atténuées par des mémoires transcriptionnelles pour restaurer la croissance végétative, permettant ainsi à *B. distachyon* d'adopter une morphologie adaptée à la tolérance au gel. Cette deuxième étude a révélé que les mémoires transcriptionnelles régulent, en plus de la vernalisation, l'acclimatation physiologique et morphologique au froid et sont donc au cœur de l'expression des phénotypes liés à la survie dans les climats tempérés chez *B. distachyon*.

Le troisième sous-objectif était d'étudier l'acclimatation au froid et la vernalisation d'une lignée des Brachypodium annuels qui fleurit facilement. Les résultats ont montré (i) que cette lignée appartenait à l'espèce *Brachypodium hybridum*, une espèce hybride issue en partie de *B. distachyon*, (ii) que cette lignée ne répondait aucunement aux traitements de vernalisation, mais (iii) qu'elle possédait toujours les traits d'acclimatation au froid observés chez *B. distachyon* et (iv) qu'elle affichait un développement moins contraint par le froid et la photopériode que *B. distachyon*, ce qui a probablement contribué à l'expansion de l'espèce dans de nouveaux environnements. Cette troisième étude a fourni un exemple de l'évolution des traits de plasticité des plantes qui, dans ce cas, s'est produite avec une dissociation entre la régulation du temps de floraison et le reste des réponses liés à la plasticité dans les climats tempérés.

Le quatrième sous-objectif était de proposer d'éventuels mécanismes régulateurs de la plasticité phénotypique des plantes. Les études présentées précédemment ont démontré que des mécanismes de mémoire transcriptionnelle régulent la plasticité liée à l'adaptation au climat tempéré chez Brachypodium et, par conséquent, comme les mécanismes de la mémoire transcriptionnelle sont reliés à la chromatine, un quatrième manuscrit est focalisé sur l'implication de celle-ci dans la coordination de l'expression phénotypique et comme plateforme intermédiaire entre de la plasticité phénotypique et l'adaptation.

Dans l'ensemble, ce travail présente des études détaillées sur l'expression, l'interaction et la régulation de l'acclimatation au froid et de la vernalisation chez la graminée Brachypodium. Ces résultats démontrent que les mécanismes de mémoire transcriptionnelle sont au cœur de la régulation de la plasticité phénotypique chez Brachypodium et fournissent des éléments utiles pour mieux comprendre les procédés qui contribuent à l'adaptation.

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PREFACE AND AUTHOR CONTRIBUTIONS

The following thesis was prepared according to the "Thesis Guidelines" of McGill University. The thesis contains four chapters (Chapters 3-6) representing three separate research manuscripts (Chapters 3-5), and an additional manuscript presenting a perspective (Chapter 6): Chapter 3 was published in *Plant Physiology*, Chapter 4 was published in *New Phytologist*, Chapter 5 and Chapter 6 will be submitted for publication.

Author Contributions

Boris F. Mayer was the primary researcher for each chapter and designed the research, planned, and conducted experiments and analyses with the guidance of Dr. Jean-Benoit Charron, Department of Plant Science of McGill University.

Dr. Jean-Benoit Charron provided supervision, guidance and funding for the research performed in all chapters.

Dr. Annick Bertrand, Agriculture and Agri-food Canada, contributed the soluble carbohydrate analysis performed in Chapter 3, and contributed comments to the manuscript thereof.

Luc Ouellette, Department of Plant Science of McGill University, contributed laboratory work for the qPCR and RNA-seq analyses presented in Chapter 5.

CONTRIBUTION TO KNOWLEDGE

Chapter 3

- This study demonstrates the usefulness of reproducing seasonal cues in laboratory settings to characterize molecular mechanisms, in this case revealing that the gene *VERNALIZATION1* regulates cold acclimation in addition to regulating vernalization
- This study also provides the description of a morphological response connected to cold acclimation and freezing tolerance in grasses

Chapter 4

- This study provides evidence of a connection between physiological and morphological cold acclimation
- This study also describes that transcriptional memories downregulate stress responses to allow plants to grow in stressful conditions

Chapter 5

- This study describes the cold-stress responses of a polyploid annual Brachypodium and highlights its potential use as a model for cold acclimation in temperate grasses
- This study also gives insights on the evolution of temperate-climate adaptive phenotypic plasticity following hybridity and polyploidization

Chapter 6

- This manuscript presents a thinking framework for the regulation of phenotypic plasticity in plants, by proposing that chromatin is a platform that can coordinate phenotypic expression and influence evolution

Overall, the work presented in this thesis demonstrates that mechanisms of transcriptional memory are central to the regulation of phenotypic plasticity in plants and provides useful insights into the processes that govern adaptive evolution in plants.

1. INTRODUCTION

1.1 – Phenotypic plasticity and sustainable agriculture

The alarming rate at which global warming and environmental degradation have been progressing demands the development of resilient and sustainable food systems. Global economies have encouraged the establishment of monocultures of few selected crops and subsequently decreased crop diversity and the resilience of local food systems (Zhu et al. 2000, Hellin et al. 2005, Altieri 2009, Dwivedi et al. 2013, Khoury et al. 2014, Massawe et al. 2016, Renard et al. 2019). Despite providing high precision and productivity, the environmental consequences of industrialized farming practices, such as the application of chemical fertilizers and pesticides, have degraded soils, polluted ecosystems and diminished land fertility over time (Pimentel et al. 1987, Montgomery 2007, Köhler et al. 2013, Lehman et al. 2015, Maxwell et al. 2016). Moreover, the demand for food will continue to rise with the expansion of the human population, which comes in addition to the environmental crises and the fragility of the global food system that already menace the access to sufficient nutritious food, or food security (Boussard et al. 2009, Puma et al. 2015, Depenbusch et al. 2019). Historically, increasing productivity was done at the cost of environmental degradation (Beddington 2010, Thiaw et al. 2011, Tomlinson 2013). Therefore, increasing food security while decreasing emissions of greenhouse gas and conserving biodiversity is a considerable challenge that requires innovative approaches.

Researchers have previously mentioned the necessity of new approaches to increase food security including agroforestry, breeding using group selection to increase yields, and promoting the recruitment of local crops (Nair 2014, Shelef et al. 2017, Weiner et al. 2017, Weiner 2019). It was also argued that, to meet the challenges of food security, agriculture should undergo sustainable, ecological and agroecological intensifications to increase agricultural outputs and mitigate environmental costs (Baulcombe et al. 2009, Godfray et al. 2014, Tittonell 2014, Wezel et al. 2015, Mockshell et al. 2018). Environmentally friendly management practices are being increasingly adopted to improve the sustainability of agriculture and include techniques such as no-tilling, the use of cover crops, a reasoned usage of agrochemicals, as well as the development of perennial crop varieties (Langdale et al. 1979, Dabney et al. 2001, Glover et al. 2010). However, these are likely not sufficient to increase food security over the next decades as the development of sustainable and resilient food systems is also dependent on reinstating biodiversity and local

food economies (Altieri 2009, Massawe et al. 2016, Schipanski et al. 2016). Indeed, food systems are built through complex networks that encompass economic activities surrounding ecology, production, preservation, delivery, nutrition and food culture, requiring a comprehensive approach and multidisciplinary efforts (Ericksen 2008, Thiaw et al. 2011). Hence, effective approaches must be multidimensional and incorporate local environments (Poppy et al. 2014, Lurie et al. 2017, Veldhuizen et al. 2020). In fact, there are many economical and ecological benefits of adopting locally adapted production systems which, in addition to producing food, can conserve biodiversity, strengthen local economies and increase food sovereignty (Altieri 2009, Kay et al. 2019, van der Ploeg et al. 2019, van der Ploeg 2020).

Agroecological practices which in broad terms are based on using ecology to study, design and manage agriculture, led to successful restoration of land fertility, increased water availability, soil health and ecosystem services (Altieri et al. 2008, Altieri 2009, MacLaren et al. 2020). These have in turn shown the potential to increase biodiversity and revitalize local economies (Zhu et al. 2000, Smith et al. 2008, Kremen et al. 2018, van der Ploeg et al. 2019, MacLaren et al. 2020, van der Ploeg 2020). However, agroecological systems add a relatively new context to the recent developments in agricultural technologies, require intensive management as well as policy and institutional changes (Mockshell et al. 2019). It was argued that a hybrid of conventional and organic farming, such as allowing external input into organic systems, was likely to offer the best outcomes but combining conventional and sustainable agriculture seems to have limits, as genetically modified organisms may not be compatible to an agroecological system because transgenes could escape easily (Altieri 2005, Meemken et al. 2018). Developing a basic understanding of how agroecosystems work can help fast-tracking the building of resilient food production systems. Indeed, much remains to be researched on the functioning of plant communities in the context of agriculture, including the effect of competition on production, the ecology of soil microbes in polycultures and the control of diseases in plant communities (Martin et al. 2018). Interestingly, there have been recent shifts in the way agronomy is conducted, promoting local innovation and breeding with farmers rather than following a strict research-and-extension channel (Altieri et al. 2008, Weltzien et al. 2017, Sinclair et al. 2019). This is important as agriculture will have to quickly bounce back in the event of abrupt changes in environmental conditions that will continue to intensify with climate change (Collins et al. 2013). Hence, the adaptability of agroecological systems is an essential quality but depends on their structure (e.g.

landscapes, microclimates, species-species interactions), and on the response diversity and plasticity of its crops (Fischer et al. 2006, Mori et al. 2013).

Phenotypic plasticity is essentially the capacity of organisms to undergo phenotypic changes in response to their environment (Schlichting 1986). This fundamental quality of living systems regroups physiological, morphological and phenological responses that occur without genetic mutation (Sultan 2000, Nicotra et al. 2010). Encompassing developmental and stress responses, plasticity provides the means for organisms to adjust their biology and continue performing when faced with changes in their environment. There is ongoing research to evaluate the contribution of phenotypic plasticity to adaptation; studies report that phenotypic plasticity has an influence on adaptation, notably by facilitating the evolution of gene networks and the re-adaptation to ancestral environments, while other studies indicate that in some cases genetic changes may reverse the effects of phenotypic plasticity and that the adaptive nature of phenotypic plasticity is difficult to predict (Ghalambor et al. 2007, Espinosa-Soto et al. 2011, Ghalambor et al. 2015, Rodríguez-Verdugo et al. 2016, Bonamour et al. 2019, Ho et al. 2020). Nevertheless, it was also reported that phenotypic plasticity has been the main mechanism behind the response to climate change, along with species migration and standing genetic variation (Gienapp et al. 2008, Alberto et al. 2013, Merilä et al. 2014, Arnold et al. 2019). In addition to the mechanistic specificities to each stress or developmental response, phenotypic plasticity may be regulated by fundamental mechanisms which are not well known, although some have proposed links with developmental mechanisms, epigenetics, and transposable elements (Fortes et al. 2017, Pimpinelli et al. 2020) The regulatory mechanisms of phenotypic plasticity as well as whether phenotypic plasticity will aid adaptation to climate change or be counter-productive are still actively researched (Fox et al. 2019, Levis et al. 2020). In order to have a deeper understanding of the mechanisms regulating phenotypic plasticity in plants, this thesis focuses on studying the main mechanisms that regulate complementary phenotypic responses, namely physiological, morphological and phenological responses which occur simultaneously and are considered adaptive in temperate environments.

Brachypodium distachyon is an annual undomesticated temperate grass, closely related to economically important cereals, that has been used for the past decade as a model organism for genetics, genomics, ecology and evolution studies (Scholthof et al. 2018). In addition to possessing the desirable characteristics of a model plant like small stature, ease of propagation, short

generation time and small diploid genome, *B. distachyon* has a high synteny with wheat, barley and rice which facilitates the applicability of scientific knowledge to cereal crops compared to the model *Arabidopsis* (Scholthof et al. 2018). Being undomesticated, and with a collection of hundreds of accessions from different environments, *B. distachyon* can reveal the mechanisms and traits that could have been lost with breeding in cereal crops. In addition, the trio of Brachypodium annuals, namely *B. distachyon*, *B. stacei* and *B. hybridum*, have also been studied for evolution studies, as *B. hybridum* is a hybrid and polyploid that resulted from a cross between the diploids *B. distachyon* and *B. stacei* (Catalan et al. 2012). As a temperate plant, *B. distachyon* has adapted to continue performing through seasons by being plastic. Notably, *B. distachyon* can increase its tolerance to freezing when exposed to low temperature, which typically occurs in the fall, through a response called cold acclimation (Colton-Gagnon et al. 2014, Ryu et al. 2014). Moreover, cold also accelerates flowering in the species, through a process known as vernalization (Colton-Gagnon et al. 2014, Ream et al. 2014). Cold acclimation and vernalization are examples of physiological and phenological plasticity respectively. Hence, studying the regulation and the interaction between cold acclimation and vernalization in *Brachypodium distachyon* can reveal mechanisms that regulate phenotypic plasticity in plants, which can hopefully be utilized for sustainable intensification and for increasing food security.

1.2 – Hypothesis and objectives

Understanding the mechanisms that regulate phenotypic plasticity in plants requires to understand the expression, regulation and connection between physiological, morphological and phenological responses. Temperate-climate adaptive plasticity, which is important for the persistence of temperate plants in their native climates, regroups responses leading to freezing tolerance and flowering competence, respectively known as cold acclimation and vernalization. *B. distachyon* is a temperate grass used as a model for ecology, evolution and genomics, and provides a useful system to study the regulation of phenotypic plasticity in plants.

Main hypothesis

Cold acclimation and vernalization are connected and controlled by common mechanisms which regulate phenotypic plasticity in Brachypodium

Main objective

Study the regulation of cold acclimation and vernalization in Brachypodium grass as an effort to understand the mechanisms governing phenotypic plasticity in plants.

Sub-objectives

Chapter 3

- Identify and reproduce in laboratory settings the cues that induce the expression of temperate-climate adaptive phenotypes in *B. distachyon* and characterize the cold acclimation and vernalization responses induced by these conditions.

Chapter 4

- Identify the molecular mechanisms that regulate the expression of physiological and morphological cold acclimation and of vernalization in *B. distachyon*.

Chapter 5

- Study cold acclimation and vernalization in an annual Brachypodium line that flowers readily.

Chapter 6

- Propose possible regulatory mechanisms of phenotypic plasticity in plants.

2. LITERATURE REVIEW

2.1 - Phenotypic plasticity: from response to adaptation

2.1.1 Growing in a changing environment

Plants occupy a wide range of environments. The basic requirements for plant life are light, carbon and oxygen gases, water, macronutrients and trace minerals. Their availability and delivery in the environment, such as the quality and quantity of light, the balance of nutrients and water levels in soils, are crucial for plant growth and development. Temperature is another important factor that can have profound effects on the environments and the biology of plants. Variations in these parameters constitute the abiotic (or non-living) conditions that, in addition to biotic (living) factors, (i) describe the different environments that support plant life and (ii), in addition to other potential stressors like high salinity, constitute the constraints plants have adapted to in their native habitat.

Sudden environmental fluctuations can create stressful conditions that perturb the growth and development of plants. Generally, plants can overcome stress to varying degrees depending on their adaptability and the severity of the conditions they face. When stresses are not so extreme as to cause death, plants can generally enter a state of dormancy until conditions improve, or instead, can continue growing. They continue growing either because plants can be inherently resilient to the stress in question, or because they can successfully adjust their phenotype to overcome it. Indeed, plants have a remarkable ability to adjust their physiology, metabolism and structure to continue performing in response to environmental stress. Importantly, this capacity to change, called phenotypic plasticity, occurs within a shorter timescale than adaptation (Ghalambor et al. 2007). Specifically, adaptation involves characteristics that are developed over multiple generations with changes in DNA, whereas phenotypes produced by plasticity occur without DNA change, although the ability to be plastic is genotypic and heritable (Bradshaw 2006, Lande 2009).

2.1.2 Phenotypic plasticity and stress response

Phenotypic plasticity is defined as the ability to express different phenotypes in response to the environment without changes in genotype (Schlichting 1986, Sultan 2000, Nicotra et al. 2010). The responses to various abiotic stresses are relatively well studied in plants, especially in the context of dehydration, flood, salt, cold and heat stresses. Although there have been significant

advances in elucidating the mechanisms that characterize these responses, there are still knowledge gaps to be filled. Generally, stress responses are complex and comprise multiple actors and mechanisms that are stress-specific, but that are also general (Lamers et al. 2020). Typically, stress responses are initiated by the perception of signals *via* sensors usually present in the cell membranes or cytoplasm. For example, protein denaturation is involved in sensing heat stress and low oxygen levels are implicated in signalling flooding, while to this day, the sensors of drought, salt and cold are still unknown (Schlesinger 1990, Åkerfelt et al. 2010, Gibbs et al. 2011, Gasch et al. 2016, Lamers et al. 2020). The perception of stress is then followed by signaling cascades which, although leading to different stress-specific responses, tend to largely overlap between stresses – how plants can accomplish this remains unknown (Lamers et al. 2020). Signaling leads to responses at several levels often through changes in gene expression (Ingram et al. 1996, Thomashow 1999, Zhang et al. 2006).

In natural environments, plants are subjected to multiple signals and environmental perturbations. Thus, specific stress signals rarely occur in isolation. The experiments that led to understanding stress responses in plants were mostly performed in controlled conditions and often focused on a unique stress signals (Mahalingam 2015). This approach has been successful in building a basic knowledge of stress response pathways through detailed experimental designs. However, studies that investigate the responses to combinations of stress and that reproduce conditions closer to the complexity of natural environments are lacking (Suzuki et al. 2014, Crisp et al. 2016, Yeung et al. 2018). Stress responses, although usually defined as the immediate reactions to stress, can stretch over time (e.g. response chronic stress), change following further perturbations in the environment, and even evolve when plants are exposed to repeated stress episodes. The effects of such chronic or repetitive stresses on plants are especially visible at the morphological and structural levels as seen, for example, in tall and thin plants growing in low light settings (Smith et al. 1997, Franklin 2008).

Phenotypic plasticity can generally be connected to two processes that are not mutually exclusive: acclimation (or acclimatization) and learning (Kelly et al. 2011). Acclimation is a gradual process through which organisms become accustomed to new conditions. Typically involving physiological and structural adjustments, acclimation builds stress tolerance over time, from the first few hours to weeks of stress exposure. Moreover, the term *learning* applied to plants is a relatively new one for plant biologists, although learning experiments performed on *Mimosa*

pudica (touch-me-not or the sensitive plant), mostly by comparative psychologists, were first reported almost 150 years ago (Abramson et al. 2016). Although still controversial, the community is increasingly accepting that plants can learn and show signs of intelligence (Gagliano et al. 2014, Trewavas 2016, Gagliano et al. 2018), and an increasing number of studies demonstrate that plants can form stress memories, and hence build experience (Ding et al. 2012, Mayer et al. 2014, Feng et al. 2016, Lamke et al. 2016, Ling et al. 2018, Bäurle et al. 2020).

2.1.3 Transcriptional memories

Through a process known as priming, stress memories can lead to improved plant responses to subsequent stress exposures. Specifically, stress memories allow plants to retain part of the response to a given stress stimuli, usually following an initial stress response said to be naïve, in order to be more successful or more tolerant in response to a subsequent stress episode. Studies show that primed plants perform better in the face of stress in both biotic and abiotic stress contexts (Ding et al. 2012, Pastor et al. 2013, Lamke et al. 2016, Savvides et al. 2016). Being the primary mechanism behind priming, stress memories hence provide plants the means to learn, build experience and increase the changes of survival to stress (Crisp et al. 2016, Yeung et al. 2018). As stress-responses are often linked to extensive changes in gene expression, studies have reported cases of stress memories linked to transcription. Called transcriptional memories, these affect the transcriptional responses to a stress stimulus (Avramova 2015, Lämke et al. 2017). Although studies tend to report that transcriptional memories induce stronger and faster transcriptional responses and are hence hyperactivating (Ding et al. 2012, Lamke et al. 2016, D'Urso et al. 2017, Liu et al. 2018), different types of memory are possible (Ding et al. 2013, Bäurle et al. 2020). Examples of transcriptional memory types are shown in Figure 2.1: these can be hyperactivating or hypoactivating and can maintain gene activation after the stress event has passed either temporarily (lingering response memory) or permanently (Fig. 2.1).

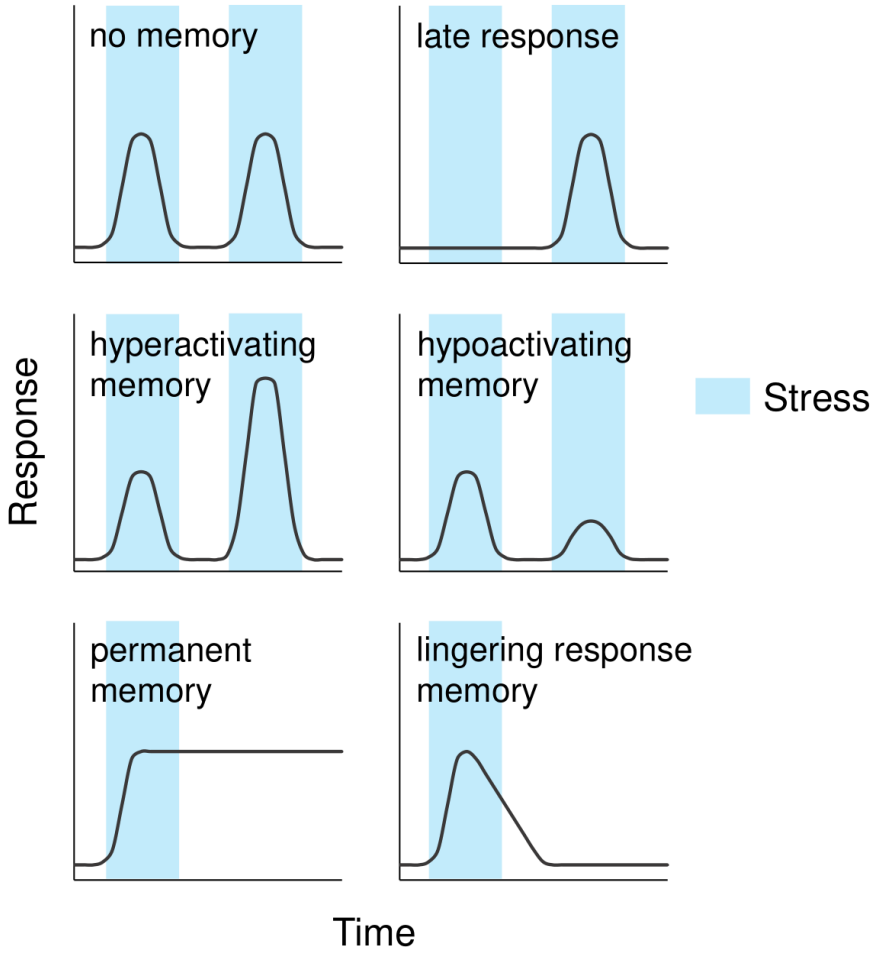

Figure 2.1: Patterns of transcriptional response and memory.
Depending on their expression pattern in response to stress and repeated stress, genes can show no memory and respond similarly to repeated stress, or previously unresponsive genes can become responsive (late response or acquired responsive genes, not considered memory). Genes can also display different types of memory such as a hyperactivating or hypoactivating memory to repeated stress, and a maintained expression after stress is removed either temporarily (lingering response) or permanently.

Vernalization is perhaps one of the best studied phenotypic response that is regulated by a transcriptional memory. In response to cold exposure, vernalization can accelerate flowering or enable the transition to the reproductive stage. Hence conferring flowering competence, vernalization is characterized by the gradual establishment of a permanent transcriptional memory

in response to cold (Fig. 2,1), which, maintained after cold exposure, allows plants to flower (Danyluk et al. 2003, Amasino 2004, Bastow et al. 2004). Well studied in *Arabidopsis*, vernalization is characterized by silencing the gene *FLOWERING LOCUS C* (*FLC*), which codes for a repressor of flowering (Michaels et al. 1999). In temperate cereals, vernalization occurs with the activation of *VERNALIZATION1* (*VRN1*) which codes for a flowering inducer (Danyluk et al. 2003). Transcriptional memories established on *FLC* and *VRN1* are maintained long after they are established in cold conditions to induce flowering but are removed at reproduction to ensure that a requirement for vernalization is maintained in the plant population (Pien et al. 2008, Chen et al. 2012, Huan et al. 2018). This long maintenance, however, is not always observed, as transcriptional memories of stress-response genes may be lost after a few days outside of stress conditions (Ding et al. 2012, Crisp et al. 2016). Indeed, the consequences of maintaining stress memories could inhibit growth (Karasov et al. 2017, Kudo et al. 2019).

Studies that investigated stress recovery, responses to repetitive stress and stress priming revealed that transcriptional memories are regulated by chromatin (Ding et al. 2012, Crisp et al. 2016, Lamke et al. 2016, Ling et al. 2018, Yeung et al. 2018, Bäurle et al. 2020). Chromatin is a complex of DNA and protein whose primary function is to package DNA molecules in eukaryotes. Made of DNA wrapped around histone proteins, the structure and composition of chromatin can regulate gene transcription by affecting the accessibility of genes through looser or tighter binding of DNA to histones, and by regulating the binding of transcriptional regulators which are affected by its composition (Hendrich et al. 1995, Felsenfeld et al. 1996, Shilatifard 2006). The regulation of transcriptional memories was associated to chromatin modifications (Ding et al. 2012, Lamke et al. 2016). For instance, the di- and tri-methylation of histone 3 lysine 4 (H3K4me2/3) and the tri-methylation of lysine 27 (H3K27me3) regulate transcriptional memories in response to stress, including cold, heat, salt and dehydration stress (Bastow et al. 2004, Oliver et al. 2009, Liu et al. 2014, Shen et al. 2014, Feng et al. 2016, Lamke et al. 2016, Liu et al. 2018, Zeng et al. 2019). Moreover, DNA methylation is involved in regulating stress-responses, the establishment of transcriptional memories and adaptation to environmental stress (Verhoeven et al. 2010, Jiang et al. 2014, Mayer et al. 2014, Sanchez et al. 2014, Wibowo et al. 2016). Overall, transcriptional memories appear to be a primary mechanism of learning in plants, allowing them to remember past events to perform better the next time they face the same conditions or, in the case of

vernalization, remember passed events to continue their life cycle. Therefore, memories provide plasticity to stress responses and can also influence phenotypic plasticity.

2.1.4 Linking behavior to evolution

Phenotypic plasticity is a valuable trait in variable environments. Although not always adaptive (i.e. does not always lead to better reproduction), phenotypic plasticity allows plants to survive and reproduce through fluctuating conditions (Gienapp et al. 2008, Alberto et al. 2013, Merilä et al. 2014, Arnold et al. 2019). In seasonal climates, because the change of conditions is recurrent and predictable, plants tend to express acquired phenotypes that were the product of adaptation, affecting stress response and phenological traits (i.e. affecting the life cycle), and that are dependent on seasonal cues (Bonamour et al. 2019, Ho et al. 2020). For example, the daily of hours of darkness have to cross a specific threshold to induce flowering in the summer (in long-day plants) or in the fall (in short-day plants) (Borthwick et al. 1960). The cooler temperatures of the fall initiate cold acclimation, while longer episodes of cold encode the memory of vernalization (Chouard 1960, Thomashow 1999). In arid climates, sparser rains signal the arrival of the drought season, to which plants respond by flowering and transitioning to the seed stage to escape drought (Shavrukov et al. 2017). Although escaping severe conditions in the form of seed is a strategy observed in many annual plants, the link between phenotypic plasticity, adaptation and perenniality is not well studied (Sultan 2000, Nicotra et al. 2010).

The interplay between phenotypic plasticity and adaptation is complex and not well understood (Ghalambor et al. 2007, Ho et al. 2020). The capacity to adopt a given phenotype is a trait encoded in genetics, and is hence likely shaped through adaptation, however, the acquisition of adaptive traits can also be influenced by behavior, including responses and phenotypic plasticity (Ghalambor et al. 2007, Acasuso-Rivero et al. 2019, Bonamour et al. 2019). Indeed, research suggests that organisms, including plants, play an active role in their evolution by influencing adaptation through their behavior (Gienapp et al. 2008, Alberto et al. 2013, Merilä et al. 2014, Ghalambor et al. 2015, Bateson 2017, Arnold et al. 2019). Hence, phenotypes that organisms express in response to given situations likely contribute to driving adaptation. Such mechanisms may be in play during the early transition from stress response to phenological dependence on environmental cues, which would need further investigation. When attempting to study the expression of complex phenotypes, it is not always evident to attribute specific responses to

environmental factors, and determine whether specific traits were acquired through adaptation, resulted from a genetic predisposition or from general plasticity (Merilä et al. 2014). Moreover, because these responses often rely on complex signals, the phenotypes observed in controlled environment may be quite different from the naturally occurring ones, or not show their full extent (Kampichler et al. 2001, Kohler 2002, Gusta et al. 2013). Although replicating the natural environment in laboratory settings is challenging, reproducing as best as possible the natural environment of the experimental subject is often informative (Kohler 2002, Crisp et al. 2016, Yeung et al. 2018).

When plants face new conditions, their capacity to respond and develop to new phenotypes influences their survival and persistence in the environment (Bateson 2017). Over time, natural selection favors the fittest individuals, which then contribute to shifting the genetics of the population towards a new equilibrium. Research that focuses on deciphering how these two mechanisms connect, and how responses drive or occur concurrently with genetic change, will likely shed light on the connection between behavior and evolution (Bateson 2017). Mechanisms regulating phenotypic plasticity likely play a role in this process (Levis et al. 2020). Temperate plants offer useful systems to study the mechanisms that regulate phenotypic plasticity.

2.2 – Cold acclimation and winter survival

2.2.1 Phenotypic plasticity in temperate climates: a case study

The occurrence of cold poses major constraints to plant life. To agriculture, cold temperatures can cause high economic losses in various climates, including temperate and sub-tropical climates (Aggarwal 2008, Kutcher et al. 2010). For instance, late frost damaged citrus trees and caused a 700$ million loss in USA in 1998, and early frost caused a 270$ million loss in the production of tea leaves in China in 2010 (Snyder et al. 2005, Lou et al. 2013, Papagiannaki et al. 2014). Frost is considered one, if not the, most devastating weather condition for agriculture (Snyder et al. 2005). Climate change has destabilized the return of high temperatures in spring, and in some instances have increased the risk of late spring frosts (Meier et al. 2018, Unterberger et al. 2018). Hence, it is highly unlikely that the negative consequences of low temperature will subside, even with global warming.

In northernmost latitudes or highest altitudes, only the most cold-hardy species can persist by employing various coping strategies. Some species escape cold by overwintering as seeds,

while others can withstand freezing temperatures below -50ºC (Wisniewski et al. 1999, Körner 2012, Vitasse et al. 2014). Although plants living in extreme environments must constantly maintain a tolerance to cold (e.g. in arctic environments), most cold-adapted plants only seasonally express their tolerance traits by cold acclimating (Thomashow 1999, Körner 2012). Hence, most temperate plants can seasonally change their phenotype.

The control of flowering time is an important adaptive strategy in temperate climates for plants to escape the freezing of flowers. Hence, vernalization, or the acquisition of flowering competence by cold exposure, is often deeply encoded in a temperate plant's genome. Lysenko, the scientist who coined the term "vernalization", showed that winter wheat seeds could be sown in spring (instead of the fall) after being treated with moisture and long periods of artificial cold (Chouard 1960). This helped palliate for harsh winters with no snow that was killing winter wheat seedlings at the time (Lysenko 1928). This Russian scientist also tried to adapt wheat to grow in Siberia but failed, which showed that high cold-tolerance traits could not be acquired readily and hence evolved over relatively long evolutionary times (Soyfer 1989, Amasino 2004). In fact, adapting to cold was an important trait that led to the radiation of several groups of plants out of the tropics such as temperate grasses, *e.g.* wheat, barley, rye and Brachypodium (Sandve et al. 2008, Sandve et al. 2010, McKeown et al. 2016, Zhong et al. 2018).

Many plant species that persist in temperate regions have hence evolved to be plastic and seasonally modify their phenotype. Indeed, they have adapted their life cycle to follow seasons (*i.e.* have adapted their phenology), express cold-adaptive traits before the onset of winter through cold acclimation to withstand freezing and delay their flowering through vernalization until the more clement conditions of spring. As such, they represent a useful study system for researching the mechanisms that regulate phenotypic plasticity.

2.2.2 Responses to cold and freezing

Chilling and freezing temperatures can have strong physical and physiological effects on plants. Chilling (temperatures that are low but non-freezing) affect molecular motion, kinetic energy which decrease the rate of biochemical reactions and can cause cell membranes to become rigid and collapse, producing visible water-soaked spots on stems, leaves and fruits (Levitt 1980). Chilling also disrupts the electron transport chain in chloroplasts, leading to the accumulation of reactive oxygen species that are toxic to proteins, lipids and nucleic acids and which affect

photosynthesis and metabolism (Allen et al. 2001, Apel et al. 2004). Freezing temperatures can induce the formation of ice crystals on the outside or on the inside of plant tissues and cause physical damage (Levitt 1980). Ice that forms externally on plants can lead to encasement and, in turn, hypoxia (Andrews 1996). When forming extracellularly or intracellularly, sharp ice crystals can puncture membranes and lyse cells, while ice built inside the vascular system and through freeze-thaw episodes cause the accumulation of air bubbles that lead to embolism, which was especially studied in trees (Sperry et al. 1988, Davis et al. 1999). Interestingly, because ice crystals grow at the expense of liquid water, freezing also induces a dehydration stress (Steponkus et al. 1992). Hence, surviving cold and freezing requires a varied set of physiological and structural characteristics to palliate for a decrease in molecular kinetics and physical damage.

The capacity of plants to cold acclimate and tolerate varies widely between species. For example, tropical species have a higher sensitivity to low temperatures and a tolerance threshold which can be as high as 15°C, while temperate species are generally affected when temperatures fall below 8-10°C (Levitt 1980). In addition, tropical species hardly survive episodes of frost, while the more tolerant temperate plants can better withstand these (Guy 1990). The cold-adaptive traits that plants display were grouped into three categories (Körner 2016). First, these can be genotypic: they are part of the normal structure and physiology and are irreversible within one life cycle. Second, cold tolerance traits can be acquired-structural: some plants are able to change their structure and morphology to adapt to low temperatures. Third, plants respond by cold acclimating through reversible physiological adjustments, which result in an increase in freezing tolerance. Therefore, except for the plants that constitutively express tolerance traits, phenotypic plasticity allows plants to change their morphology and physiology to tolerate cold and freezing.

Most winter-hardy plants only seasonally express their cold tolerance through cold acclimation. Cold acclimation likely evolved because of the trade-offs associated with the constant expression of cold tolerance, including energy costs and the inhibition of normal plant growth. It is interesting that, although cold acclimation and its associated physiological responses are well studied, little is known about the growth and morphological responses that lead to acquired cold-adaptive structures. Whether the acquisition of such a structure is connected to cold acclimation has not been addressed in detail. Cold acclimation is described as the major process that increases freezing tolerance, which occurs early on (the first few hours) in response to cold exposure (Bond et al. 2011). However, if temperatures are not too low as to prevent growth, the structures and

morphology gained under the influence of cold may also influence freezing tolerance (Equiza et al. 2001, Patel et al. 2009). This, specifically, has not been clearly addressed.

2.2.3 Cold acclimation and physiology

Cold acclimation regroups the early responses to low temperature that limit the negative effects of cold on plant physiology and survival. Because cold decreases the rate of biochemical reactions, plants deploy several compensating adjustments. For example, protein synthesis and the number of ribosomal RNA increase to maintain normal protein synthesis rate (Guy 1990). Moreover, plants respond to the rapid accumulation of reactive oxygen species by producing antioxidant enzymes and compounds (Dreyer et al. 2018). Plants accumulate other protective molecules (e.g. carbohydrates, chaperones) and osmoprotectants (e.g. amino-acids such as proline, glycine betaine), alter the composition of membranes (plasma and thylakoid), show increased levels of the hormone abscisic acid (ABA) and changes in gene expression (Welling et al. 2002, Breton et al. 2003, Uemura et al. 2006). This response ensures that, on multiple fronts, the negative effects of cold on physiology are countered.

The accumulation of soluble carbohydrates limits cellular dehydration, helps protecting macromolecules by lowering the freezing temperature of cells and help scavenge reactive oxygen species (Guy et al. 1992, Ruelland et al. 2009, Van den Ende et al. 2009). Although carbohydrates are important protective molecules, plants also accumulate amino-acids and proteins to limit the effects of cold and freezing (Dionne et al. 2001, Rai 2002). The accumulation of solutes contributes to a state of supercooling water in plants, during which sap that is well inside the freezing range does not turn solid. This state is especially observed in trees that can withstand extremely low temperatures (Wisniewski et al. 2004).

Cold-protective proteins range from chaperones that ensure proper protein folding to anti-freeze proteins that prevent the expansion of ice. Anti-freeze proteins, such as *ICE-RECRYSTALLIZATION INHIBITORS* (*IRI*), prevent the growth of ice crystals by inhibiting their recrystallization, and often accumulate in the intercellular spaces of plant tissues (Griffith et al. 2004, Zhang et al. 2010). This group of proteins is not restricted to plants and is found, for example, in arctic fish. Anti-freeze proteins have evolved from unrelated protein families into a convergent function: prevent the expansion of ice into living tissue. Anti-freeze proteins are indeed efficient in avoiding the accumulation of damaging ice crystals. Their primary role is hence to maintain the

integrity of living tissues under freezing conditions. Dehydration-protective proteins are also synthesized during cold acclimation, in conjecture with increased ABA levels (Close 1997). These proteins include dehydrins, such as the wheat protein *COLD-REGULATED410* (*WCOR410*), *LATE-EMBRYOGENESIS ABUNDANT* and *RESPONSE TO ABSCISIC ACID* that are highly stable and extremely hydrophilic (Danyluk et al. 1998, Wisniewski et al. 1999). For instance, dehydrins are small hydrophilic proteins that play a role in both cold and drought stress response pathways (Barrett 2001, Liu et al. 2017). First identified in drought-stressed barley and corn, these diffuse throughout cells but tend, nonetheless, to localize to the nucleus, the cytoplasm and chloroplasts (Ingram et al. 1996, Close 1997). Dehydrins have various functions in plants, but in the context of low temperature stress, these are thought to protect the integrity of proteins, membranes and DNA, while some dehydrins display anti-freeze activity (Wisniewski et al. 1999, Liu et al. 2017)

Cold exposure ultimately throws off the balance of energy flow in the cell (Puhakainen et al. 2004). Plants respond partly to this imbalance by adjusting the expression of enzymes involved in photosynthesis (Liu et al. 2012), by relocating and altering chloroplast membranes (Ogasawara et al. 2013) and by increasing thylakoid membrane fluidity (Shinozaki et al. 1996). As membranes are among the structures most affected by cold, the fluidity of plasma membranes is also increased during cold acclimation. Plants express desaturase enzymes that introduce double bonds in the fatty-acid chains of membrane phospholipids and synthesize specific trans-membrane proteins which, overall, increase membrane material and membrane fluidity, such as *COLD-REGULATED413* (Levitt 1980, Breton et al. 2003). Because photosynthesis and membranes are sensitive to temperature, they are likely involved in the perception of cold (Lamers et al. 2020).

In laboratory settings, cold acclimation capacity can be measured by comparing plants submitted to a cold acclimation treatment (typically chilling) to control non-acclimated plants. After cold acclimating for varying lengths of time, plants are exposed to freezing and their tolerance is often measured by electrolyte leakage or by performing whole-plant survival tests. Electrolyte leakage gives an indirect measurement of tissue damage by estimating the amount of electrolyte that has diffused from plant tissues. Hence, by exposing plant tissues to different sub-zero temperatures, the damage that these temperatures induce, for instance through punctured membranes following the formation of ice crystals, can be monitored by incubating plant tissues into water (Thalhammer et al. 2014). In plants capable of cold acclimation, the damage induced

by freezing decreases over the progression of cold acclimation. Whole-plant freeze tests provide a more direct measurement of damage by measuring the survival of plants exposed to sub-zero temperatures, usually plants are gradually removed from increasingly colder temperatures to obtain a survival curve as a function of temperature (Mayer et al. 2014). Survival is usually determined at re-growth, hence for up to a week after plants are stressed. Other values are used to infer stress levels or as an index of cold tolerance during cold acclimation, including chlorophyll content which correlates with photosynthesis (photosynthesis tends to be generally lower in stressful situations), proline content or levels of soluble carbohydrates which also increase during cold acclimation (Colton-Gagnon et al. 2014, Thalhammer et al. 2014).

2.2.4 The expression of cold acclimation

The mechanisms behind the perception of cold in plants have not been completely elucidated. However, studies have determined a likely sequence of events that immediately follow an exposure to low temperatures. Cold causes membranes to become rigid and the cytoskeleton to reorganize itself (Orvar et al. 2000). These lead to the opening of Ca2+ channels and an influx of Ca2+ into cells, which activates cold-specific MAP kinases and downstream signalling cascades that lead to the activation of cold-responsive transcription factors (Orvar et al. 2000). Signalling molecules like cytokinins, ABA, reactive-oxygen species and ethylene are also involved in the response to cold (Lamers et al. 2020). Because of the central position of photosynthesis in cell processes, the photosynthetic apparatus likely plays a role in the perception of cold and signalling, notably through levels of photosynthates and reactive-oxygen species (Ensminger et al. 2006). There is also evidence that chromatin may directly perceive changes in temperature (Kumar et al. 2010, Park et al. 2018). Overall, the perception and signalling of cold is complex and multifactorial.

In plants that can cold acclimate, cold initiates responses that lead to extensive changes in gene expression that typically affect 10-30% of their genome (Lee et al. 2005, Ouellet et al. 2013, Park et al. 2015). These include transcription factors and structural genes that produce the cold-hardy phenotype, including the structural proteins heat-shock proteins which act like chaperones during temperature stress, dehydrins and anti-freeze proteins as described in the previous section (Thomashow et al. 1990, Barrett 2001, Welling et al. 2002). A well-known pathway that regulates the expression of structural genes involves transcription factors known as *C-REPEAT BINDING*

FACTORS/DROUGHT-RESPONSIVE ELEMENTS BINDING (*CBF*s/*DREB*s) (Barrett 2001). Independently discovered in cold and drought response (Yamaguchi-Shinozaki et al. 1994, Thomashow et al. 1997), overexpression of *CBF* genes improves cold hardiness in plants (Jaglo-Ottensen et al. 1998, Ryu et al. 2014). These transcription factors are members of the *APETALLA AP2* class and are rapidly induced in cold exposure (Zhang et al. 2010). It was reported that *CBF* expression correlates with temperature fluctuations in the field, along with Ca^{2+} levels which are induced by temperature decreases as well (Hiraki et al. 2019). When expressed, *CBF*s subsequently bind to a core element of consensus sequence (A/G)CCGAC (termed CRT, DRE or LTRE) that is located in the promoter region of cold-regulated genes (Barrett 2001, Welling et al. 2002). Their constitutive overexpression confers high freezing tolerance in many plant species but can severely limit plant growth (Achard et al. 2008, Jeknic et al. 2014, Wisniewski et al. 2015). As their expression typically peaks within the first few hours of cold exposure, the timing and level of expression are likely important for cold acclimation (Medina et al. 2011).

Cold acclimation allows temperate plants to change their phenotype in order to continue performing under cold and better survive freezing. This example of phenotypic plasticity induces important physiological change that are crucial for the persistence of temperate plants in cold environments. Its evolutionary and mechanistic connection to vernalization, which control flowering time, are not well understood. Understanding this link could shed light on the mechanisms that control phenotypic plasticity in plants.

2.3 – Plasticity and temperate-climate adaptation in grasses

2.3.1 The temperate grass system to study phenotypic plasticity

Temperate grasses (Poaceae, subfamily Pooideae), are widely distributed in temperate regions. They regroup important cereals like wheat, barley and rye and the closely-related model plant *Brachypodium distachyon*. Pooid grasses have radiated into temperate climates from the temperature drop and increased seasonality during the Oligocene (34My), notably through traits controlling cold tolerance and phenology (Sandve et al. 2010, McKeown et al. 2016, Zhong et al. 2018, Schubert et al. 2019). Cold acclimation and vernalization seem to be connected in temperate grasses, making them valuable to study the mechanisms controlling phenotypic plasticity in cold climates (Galiba et al. 2009, Dhillon et al. 2010, Deng et al. 2015).

Most of the research on cold acclimation and vernalization in temperate grasses was performed on economically important cereals crops. Variety development in these have led to clear-cut differences in growth habits. In wheat, cultivars are generally either classified as spring genotypes, which are sown in the spring and which flower at the end of the summer, or as winter genotypes, which are sown in the fall, overwintering as seedlings and which flower earlier than the spring genotypes in the summer. Winter genotypes require vernalization to flower and have higher winter hardiness than their spring counterparts (Fowler et al. 1996, Laudencia-Chingcuanco et al. 2011). Studies that investigated the differences between these groups of cultivars, notably by crossing these to produce recombinant isogenic lines, have led to a better understanding of the traits that control winter hardiness and vernalization, which was also performed in barley (Danyluk et al. 2003, Trevaskis et al. 2007, Ganeshan et al. 2008, Dhillon et al. 2010, Deng et al. 2015).

2.3.2 Vernalization and cold acclimation in temperate grasses

Vernalization in the temperate grasses studied so far is characterized by the activation of a transcription factor from the MADS-box family called *VERNALIZATION1* or *VRN1* that functions like a flowering inducer (Danyluk et al. 2003, Trevaskis et al. 2003). *VRN1* is primarily involved in flower morphogenesis but appears to be involved in other processes, such as root architecture in wheat and barley (Preston et al. 2008, Voss-Fels et al. 2018). In vernalization-requiring plants like winter genotypes of wheat, *VRN1* expression is activated by cold exposure. Once *VRN1* expression crosses a given threshold, *VRN1* remains active until the end of the life cycle in annual plants, conferring flowering competence. Hence, when other flowering signals are also present, e.g. higher temperatures and an inductive photoperiod, vernalized plants can transition to the reproductive stage.

VRN1 is controlled by an activating transcriptional memory encoded in chromatin. Cold exposure induces changes in the chromatin state of *VRN1*, notably through a depletion of the silencing epigenetic mark tri-methylated of histone 3 lysine 27 (H3K27me3) and the activating tri-methylated histone 3 lysine 4 (H3K4me3) (Oliver et al. 2009, Oliver et al. 2013, Woods et al. 2017, Huan et al. 2018). This epigenetic change occurs gradually and quantitatively in response to cold exposure, leading to higher expression of *VRN1* which remains high even when the cold signals are removed. *VRN1* expression is notably regulated by the chromatin modifying complexes PRC2 and Trithorax-group proteins (TrxG) that have a conserved function in regulating

temperature-controlled flowering (Alvarez-Venegas 2010, Diallo et al. 2012, Bratzel et al. 2015). Interestingly, PRC2 and TrxG are also involved in regulating phenotypic plasticity, in stress and in developmental response in plants, indicating that the mechanisms controlling *VRN1* may also be involved in other responses, like cold acclimation (Kleinmanns et al. 2014, Marasca et al. 2018). Cold stress memories were recently described in *Arabidopsis* but whether these mechanisms exist in temperate grasses in cold stress response or during cold acclimation is unknown.

Cold acclimation genes in temperate grasses have likely evolved from stress-responsive ancestors, and include the conserved gene families CBFs, cold-regulated genes COR and dehydrins DHN although there are also important species-specific differences (Zhong et al. 2018, Schubert et al. 2019). Hence, similarly to vernalization, cold acclimation is at least a partly conserved response in temperate grasses. It was previously proposed that cold acclimation and vernalization are linked in wheat and barley (Fowler et al. 1996, Galiba et al. 2009, Dhillon et al. 2010). The vernalized state and freezing tolerance are negatively correlated in some cases where vernalized plants show reduced cold acclimation capacity (Fowler et al. 1996). Moreover, both vernalization requirement and cold acclimation capacity are dependent on alleles of *VRN1* in wheat, hence indicating that *VRN1* is a probable connective node between cold acclimation and vernalization (Ganeshan et al. 2008, Dhillon et al. 2010, Laudencia-Chingcuanco et al. 2011). The temperate grass *Brachypodium distachyon* has recently emerged as a useful model to study cold tolerance in plants.

Although cold acclimation and vernalization are induced by similar signals, few studies have investigated in detail the mechanistic connection between these two responses. This could be due to the complexity of the wheat and barley genomes, to their inconvenient use in laboratory settings, and possibly because cold acclimation and vernalization are independent in *Arabidopsis* (Bond et al. 2011). Studying these responses in an undomesticated plant, with higher genetic and potentially higher phenotypic diversity, could reveal to a larger extent the mechanisms controlling them. Furthermore, understanding the connection between environmental cues and phenotypic plasticity would likely be more informative in a wild plant. The plant *Brachypodium distachyon*, an undomesticated Pooid grass relatively recently described as a model organism, is a useful species for the study of phenotypic plasticity in temperate-climate adaptation.

2.3.3 Brachypodium distachyon

Brachypodium distachyon is a wild grass native to the Mediterranean region that grows as a spring or winter annual (Colton-Gagnon et al. 2014, Des Marais et al. 2016). Seeds were collected from many locations in the plant's native range and gave rise to hundreds of lines, including T-DNA insertion lines developed for functional genomics studies (BrachyTAG , DOE-JGI). The genome of a main inbred line Bd21 was first sequenced, followed by the genomes of 54 lines used to build a pan-genome for population genetics studies (Vogel 2010, Gordon et al. 2017). Interestingly, inbred lines developed from natural populations display a range of vernalization requirements, some lines do not require any vernalization treatment while others require up to 8 weeks of cold exposure to flower (Colton-Gagnon et al. 2014, Ream et al. 2014). Studies of their population structure revealed that vernalization requirement, whose degree correspond to specific *VRN1* alleles as observed in other temperate cereals, is an important discriminant in the species (Ganeshan et al. 2009, Dhillon et al. 2010, Gordon et al. 2017). The induction of *VRN1*'s expression through cold exposure, its epigenetically-encoded transcriptional memory and its effects on flowering were confirmed in *B. distachyon* (Colton-Gagnon et al. 2014, Ream et al. 2014, Woods et al. 2017).

B. distachyon is also capable of increasing its tolerance to freezing during cold acclimation. The plant possesses the major actors of cold acclimation, such as the transcription factors *C-REPEAT BINDING FACTOR1, 2 and 3* (*CBF1, CBF2, CBF3*) which are homologous to the CBFs previously identified in Arabidopsis and other species, and cold-stress response structural genes such as the membrane-protein *COLD-REGULATED 413* likely involved in increasing membrane fluidity, the anti-freeze protein *ICE RECRYSTALLIZATION INHIBITOR* (*IRI*), and the dehydrin *COLD-REGULATED 410* (Colton-Gagnon et al. 2014, Ryu et al. 2014, Bredow et al. 2016, Mayer et al. 2020). However, the freezing tolerance reached after cold acclimation is modest compared to other temperate grasses. After cold acclimation, spring and winter wheat varieties can, for example, decrease the temperature at which 50% of the plants survive by 6ºC and 18ºC respectively, while in *B. distachyon* this increase is only of 2ºC (Ganeshan et al. 2009, Colton-Gagnon et al. 2014). These observations suggest that either *B. distachyon* has a limited capacity to cold acclimate, or that the cold acclimation treatments performed in laboratory settings do not induce the full extent of the cold acclimation capacity of the species. Moreover, all studied lines of *B. distachyon* showed so far the same capacity to cold acclimate regardless of their vernalization

requirement, therefore regardless of their geographical origin and the *VRN1* allele they possess (Colton-Gagnon et al. 2014, Gordon et al. 2017). Although it is possible that alleles of *VRN1* do not influence cold acclimation, unlike what was previously observed in wheat and barley, it is unlikely as this species' genome has a high synteny with the wheat genome, and a study suggested that vernalization limits freezing tolerance in *B. distachyon* (Dhillon et al. 2010, Deng et al. 2015, Feng et al. 2017, Scholthof et al. 2018). This observation also suggests that the conditions of the laboratory cold acclimation treatments do not elicit a response that could differentiate between different lines of *B. distachyon*.

Studying the mechanisms of cold adaptive responses in *B. distachyon* should be performed by replicating natural cues. The lack of progress in understanding the mechanisms of cold adaptation was at least partly attributed to discrepancies between the conditions of laboratory studies and the natural environment (Gusta et al. 2013). As phenotypic plasticity in cold-adapted plants follows natural cues, replicating these in laboratory settings can reveal the connection between cold acclimation and vernalization. Memory mechanisms, as observed in vernalization, may also be involved in cold acclimation in *B. distachyon*. Recently, studies have demonstrated the implication of stress memories and chromatin changes during cold acclimation and deacclimation in *Arabidopsis* (Zuther et al. 2019, Vyse et al. 2020). The existence of such mechanisms in temperate grasses, however, remains to be determined. As cold acclimation and vernalization are independent in *Arabidopsis*, the implication of memory mechanisms in cold acclimation may further connect these two processes in temperate grasses, and hence highlight possible mechanisms behind phenotypic plasticity and adaptation. Hence, the connection between cold acclimation and vernalization, and the implication of memory mechanisms in *B. distachyon* should be studied in responses triggered by natural signals.

3. TREATMENT ANALOGOUS TO SEASONAL CHANGE DEMONSTRATES THE INTEGRATION OF COLD RESPONSES IN BRACHYPODIUM DISTACHYON

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3.1 Abstract

Anthropogenic climate change precipitates the need to understand plant adaptation. Crucial in temperate climates, adaptation to winter is characterized by cold acclimation and vernalization, which respectively lead to freezing tolerance and flowering competence. However, the progression of these responses during fall and their interaction with plant development are not completely understood. By identifying key seasonal cues found in the native range of the cereal model *Brachypodium distachyon*, we designed a diurnal-freezing treatment (DF) that emulates summer-to-winter change. DF induced unique cold acclimation and vernalization responses characterized by low *VERNALIZATION1* (*VRN1*) expression. Flowering under DF is characterized by an upregulation of *FLOWERING LOCUS T* (*FT*) post-vernalization independent of *VRN1* expression. DF, while conferring flowering competence, favors a high tolerance to freezing and the development of a winter-hardy plant structure. The findings of this study highlight the contribution of phenotypic plasticity to freezing tolerance and demonstrate the integration of key morphological, physiological, and molecular responses in cold adaptation. The results suggest a fundamental role for *VRN1* in regulating cold acclimation, vernalization, and morphological development in *B. distachyon*. This study also establishes the usefulness of reproducing natural cues in laboratory settings.

3.2 Introduction

The unpredictable effects of climate change have imposed challenges to natural ecosystems and agriculture. The detrimental effects of environmental stresses on food production will become more problematic in the future (USGCRP, 2017). Unfortunately, the limited understanding of plants' adaptive mechanisms to changing environments restrains our ability to predict and prepare for these consequences. Plant adaptation is a complex concept that transcends stress responses, plant development, behavior, and evolution. Undertaking research on this topic requires a global perspective on how plants respond to change. Temperate plants have evolved to persist under seasonal climates and their adaptation to cold and freezing is a useful system for adaptation studies. However, there are still gaps in the integrative understanding of cold adaptation, possibly due to the disparity between controlled and natural environments (Gusta & Wisniewski, 2013). Indeed, cold is a major stressor in temperate regions and climatic events, such as late frost, will be

increasingly problematic in the future. Hence, understanding the mechanisms behind plant adaptation to cold is crucial for the development of hardier plants.

Freezing tolerance is an important adaptive trait in temperate plants (Chouard, 1960). Winter hardy plants innately possess or can acquire the structure and physiology to grow under cold and survive freezing (Körner, 2016; Thomashow, 1999). In fact, it was proposed that plant cold-adaptive characteristics can be divided into three groups: (i) genotypic traits (irreversible within one plant's lifetime), (ii) modification of plant structure (in response to the environment) and (iii) acclimation or physiological adjustments (that are reversible; Körner, 2016). The latter group, also called cold acclimation, has been the subject of substantial research in plants. Cold acclimation is often accompanied by the production of osmolytes, cryoprotective molecules, ice formation inhibitors, and metabolic shifts that increase tolerance to freezing and the plant's performance under cold (Thomashow, 1999). Cold acclimation is orchestrated by the expression of cold-regulated genes, notably through the C-repeat binding factor pathway (Thomashow, 1999). Despite the recognized importance of plant morphology in freezing tolerance, the interaction between cold acclimation and morphological development has not been thoroughly studied (Körner, 2016). Cold acclimation is the main mechanism by which plants increase their freezing tolerance. Studies define cold acclimation as early events of cold response (Bond et al., 2011). However, under cold conditions, both early and longer-term responses likely contribute to the establishment of a freezing tolerant phenotype. Providing temperatures are not so cold as to completely inhibit growth, plant development and the morphology acquired under cold conditions may hence play a role in freezing tolerance (Equiza et al., 2001; Patel & Franklin, 2009).

While cold hardiness is important for surviving cold stress, plants also maximize their persistence in temperate climates by adjusting their phenology to seasonality (Chouard, 1960). The cold-mediated regulation of flowering time, often coupled to a longer photoperiod, ensures that flowering occurs when winter is over. Indeed, temperate plants usually require a relatively long exposure to cold temperatures to acquire the capacity to flower through a process known as vernalization (Chouard, 1960). In temperate cereals, vernalization is characterized by the activation of the MADS-box transcription factor *VERNALIZATION1* (*VRN1*) and the quantitative accumulation of its transcripts in response to cold (Danyluk et al., 2003). The activation of *VRN1* occurs in tandem with epigenetic changes on the *VRN1* gene, such as the depletion of histone 3 lysine 27 trimethylation (H3K27me3; Oliver et al., 2013; Oliver et al., 2009; Woods et al., 2017).

Because the activation of *VRN1* is maintained after exposure to cold, vernalization has been referred to as the "memory of winter".

Plants generally respond to colder temperatures and lower photoperiod during fall. These are thought to be important signals for cold acclimation and vernalization and could possibly induce structural change. Although these processes are triggered by similar signals, the connection between their regulations is not well known. Probably because cold acclimation and vernalization appear to occur independently in *Arabidopsis* (Bond et al., 2011), most research efforts that investigated their interaction focused on temperate cereals. Indeed, cold acclimation capacity and responsiveness to vernalization treatments appear to be linked in wheat and barley (Dhillon et al., 2010; Fowler et al., 1996). A negative correlation between the vernalized state and freezing tolerance has been reported as plants reaching vernalization saturation started to lose their freezing tolerance (Fowler et al., 1996). Furthermore, it was shown that vernalization requirement and cold acclimation capacity appear to be linked to alleles of *VRN1* in wheat (Ganeshan et al., 2008; Laudencia-Chingcuanco et al., 2011). *VRN1* has been proposed as a connective node between cold acclimation and vernalization (Dhillon et al., 2010). Studies have also highlighted the role of *VRN1* in regulating elements of plant phenotypic development (Preston & Kellogg, 2008; Voss-Fels et al., 2018). *VRN1* may hence play a fundamental role in cold adaptation in temperate cereals. Temperate cereal crops are complex systems to study the interaction between growth, cold acclimation, and vernalization because of the complex relationship between these traits and their inconvenient use in laboratory settings. Moreover, knowledge gained from studying these domesticated crops may not reflect the natural variation and the adaptive mechanisms potentially found in wild organisms. The undomesticated cereal model *Brachypodium distachyon* can thus be viewed as a useful candidate species to study cold adaptation and its regulation in a natural context.

The temperate grass *B. distachyon* is native to the Mediterranean region, where it grows as a spring or winter annual (Colton-Gagnon et al., 2014; Des Marais & Juenger, 2016). The species displays a range of vernalization requirements and has the capacity to cold acclimate (Colton-Gagnon et al., 2014; Ream et al., 2014; Ryu et al., 2014). Compared to wheat, however, *B. distachyon* has so far displayed a limited capacity to increase its tolerance to freezing upon cold acclimation. Unlike spring and winter wheat that can, for example, increase their tolerance to freezing by 6ºC and 18ºC respectively (decrease in lethal temperature for 50% of the plants, LT_{50}; Ganeshan et al., 2008), *B. distachyon* accessions have shown a modest gain in freezing tolerance

of 2ºC regardless of their vernalization requirement (Colton-Gagnon et al., 2014). The limited capacity for acclimation of *B. distachyon* is particularly intriguing because this species has been shown to have an extensive natural variation in vernalization requirements. While it is possible that the species possesses a limited cold acclimation capacity, we hypothesized that the low temperature treatments commonly used under controlled conditions are unsuccessful in eliciting the extent of the species' freezing tolerance. By developing a method to simulate seasonal change, we have attempted to further characterize the species' freezing tolerant phenotype and highlighted a regulatory function for *VRN1* in cold acclimation, and plant morphology in *B. distachyon*.

3.3 Results

3.3.1 Diurnal freezing models the transition from summer to winter in *B. distachyon*'s natural range

It was previously shown that when cold-acclimated for 28 days under a typical constant-chilling treatment (4ºC), the freezing tolerance of *B. distachyon* is estimated at a lethal-temperature 50% (LT_{50}) of -10ºC (Colton-Gagnon et al., 2014). This LT_{50} appears to be the maximal tolerance of this species when acclimated under constant-chilling, as up to 49 days of cold acclimation under either short or long-day photoperiod does not further increase its freezing tolerance (Appendix 1 Fig. 1). However, substantially lower freezing temperatures were measured in *B. distachyon*'s natural range. These observations may indicate that in addition to inducing visible chilling stress, constant-chilling might not reproduce the cues responsible for complete cold acclimation in *B. distachyon* (Appendix 1 Fig. 1). Therefore, we attempted to find a more appropriate experimental protocol to induce sturdier cold acclimation in the species and investigated the seasonal cues at geographically distinct locations in the species' natural range (represented by habitats H1 to H4). These locations correspond to the seed collection sites of four accessions of *B. distachyon*, from lowest to highest latitude: H1 in Iraq (Bd21-3), H2 in Spain (Bd30-1), H3 in Turkey (Bd18-1), and H4 in Ukraine (Bd29-1). The climatic conditions at these natural habitats H1 to H4 are respectively warm semi-arid (Bsh), hot-summer Mediterranean (Csa), warm-summer Mediterranean/cold semi-arid (Csb/Bsk), and humid subtropical/oceanic (Cfa/Cfb) according to the Koppen-Geiger classification system and may represent the extent of *B. distachyon*'s geographical range (Fig. 3.1, Appendix 1 Fig. 1).

Meteorological data reporting monthly averages of temperature (tmp), diurnal temperature range (dtr), frequency of frost days (frs), and daily hours of light (pp) that span 1901-2017 was used to study seasonal change in the four locations. Principal component analysis (PCA) was performed to highlight the difference in atmospheric conditions between seasons across the four habitats and between H1-4 (Fig. 3.1B). This analysis shows that the principal component 1 (PC1) appears to capture the seasonality shared among the habitats H1-4 while PC2 describes differences between the conditions in habitats H1-4. It appears that seasons are clearly defined across the four habitats and that atmospheric conditions are more markedly different between seasons than between the selected habitats (Fig. 3.1B). Moreover, we plotted the monthly diurnal temperature range (dtr) over the monthly mean temperature (tmp) at habitats H1 to H4 (Fig. 3.1C). These representations depict the temperature variations experienced in a typical day at each month in each habitat, based on 1901-2017 monthly average values. According to this data, *B. distachyon* experiences relatively high diurnal temperature variations, that are highest during the summer (>20°C in H1) and lowest in winter (<6°C in H4) with a yearly average of 11.25°C across habitats H1-4 (Fig. 3.1C, Appendix 1 Table 1).

To visualize the change in atmospheric conditions during the progression of seasons, we plotted the atmospheric variables monthly tmp, dtr, frequency of frost days (frs), and daily hours of light (pp) in a circular diagram (Fig. 3.1D). This diagram illustrates how seasons are characterized by gradual change in the atmospheric variables tmp, dtr, frs, and pp. The lowest monthly values are towards the center of the circle and the highest on the edge (Fig. 3.1D). Unsurprisingly, summers have highest tmp, dtr, and pp while on the opposite, winters show highest frs and lowest tmp, dtr, and pp. Indeed, the transition from summer to winter sees gradual decreases first in photoperiod (pp), second in mean temperature (tmp), and third in diurnal temperature range (dtr), while the frequency of frost days (frs) increases during fall. In other words, photoperiod leads the change, followed by mean temperature, dtr, and frs.

In an attempt to unite the cues that signal summer-to-winter change, we have selected specific values of the seasonal atmospheric variables that are representative of (i) summer, (ii) fall, and (iii) winter. We combined the lowest photoperiod (end of fall) to a mean temperature typical of fall, a high dtr typical of summer, and a high frs typical of winter into a single treatment (called diurnal freezing; DF; circled in Fig. 3.1D; Fig. 3.1E). The DF treatment is characterized by cycles of 24 hours that simulate winter-like nighttime frost with a minimum of -1°C, and a maximum of

22°C during the day. This temperature range models a summer-like diurnal temperature range and a fall-like mean temperature of 8.7°C. The DF treatment associates this temperature regime to a late fall-like photoperiod of 8 hours of daily light (Fig. 3.1E, Appendix 1 Table 1).

3.3.2 Constant-chilling and diurnal freezing emulate distinct cold conditions and induce divergent responses in *B. distachyon*

To further characterize the progressively colder temperatures of the fall, we compared the naturally occurring chilling and freezing at *B. distachyon* habitats H1-4 and DF to constant-chilling (CC), a typical laboratory cold treatment. Hours and rates of chilling (between 0°C and 8°C) and freezing (temperature below 0°C) were determined using 3-hourly meteorological data from nearby meteorological stations (Appendix 1 Table 2). Both chilling and freezing events were observed at habitats H1, H2, H3, and H4 between September and March. In all habitats, the occurrence of freezing increases as the hours of chilling increase (Fig. 3.2). Also, both chilling and freezing rates increase with the progression of fall and peak at wintertime (Fig. 3.2C). Unsurprisingly, DF reproduces the relation between the occurrence of chilling and freezing, along with chilling and freezing rates that approximate the conditions in habitats H1-4 (Fig. 3.2B-C). Conversely, the absence of freezing in CC, coupled to a chilling rate twice as high as the maximum natural chilling rate, clearly show that the CC treatment does not reproduce the natural occurrence of cold in these habitats (Fig. 3.2B-C).

To measure the growth response of *B. distachyon* to CC and DF, we measured the number tillers and leaf chlorophyll content in plants exposed to either treatment for 7-56 days. *B. distachyon* developed fewer tillers under CC than under DF. After 56 days of exposure to either treatment, DF plants tended to be more similar to plants growing under control conditions than plants growing under CC conditions (Fig. 3.2D). Moreover, all accessions lose more chlorophyll when exposed to CC than DF (Fig. 3.2E). Notably, DF did not induce visible chilling stress injuries as observed under CC (Appendix 1 Fig. 1C). Hence, CC reproduces maximum chilling conditions that limit growth and reduce plant chlorophyll content. Conversely, DF simulates conditions that are closer to natural events and leads to less growth reduction that CC.

3.3.3 DF leads to higher freezing tolerance

To compare the cold acclimation response under CC and DF, we measured the freezing tolerance, the transcript accumulation of cold-regulated (*COR*) genes and the levels of non-structural carbohydrates and proline in plants exposed to either treatment. We measured survival to freezing temperatures of Bd21-3, Bd30-1, Bd18-1, and Bd29-1 subjected to CC or DF for 7 days by performing whole-plant freeze tests during which plants were exposed to gradually lower freezing temperatures (Fig. 3.3). DF-treated plants showed measurably higher survival to freezing in all accessions. Moreover, we measured the survival of Bd21-3 CC28 and DF28 plants that were subjected to either treatment for 28 days. The results show that at -12°C, more than 60% of DF28 survive compared to almost 0% of CC28 (Fig. 3.3B). Therefore, we estimated that the LT50 of DF28 plants (which we were not able to measure) is probably below -12°C.

Transcript accumulation of cold-regulated (*COR*) genes at the first 16 and 24 hours of exposure to CC or DF suggests that cold acclimation occurs under both treatments. However, *COR* gene profiles are different between the two treatments as illustrated by the early high levels of *ICE-RECRYSTALLIZATION INHIBITOR* (*IRI*) observed under DF (Fig. 3.3C). Interestingly, all accessions seem to respond similarly to either CC or DF. We further deepened our analysis by measuring the contents of proline and non-structural carbohydrates. Both treatments induced to similar levels the accumulation of raffinose, glucose, fructose, and high-density polymerization fructans (Fig. 3.3D). Nevertheless, the accumulation of sucrose, whole-soluble sugars, starch, and total non-structural carbohydrates were higher in CC-treated plants. Similarly, the accumulation of proline was higher in CC-treated than in DF-treated plants (Fig. 3.3E). Altogether, CC and DF induce distinct cold acclimation in *B. distachyon*. DF-treated plants gain a higher freezing tolerance but accumulate lower levels of total non-structural carbohydrates and proline compared to CC-treated plants.

3.3.4 CC and DF induce contrasting vernalization and flowering responses

To determine the effects of DF on flowering time, we measured the number of days to heading in Bd21-3 (facultative accession with a low vernalization requirement) and Bd18-1 (winter accession with high vernalization requirement) that were vernalized under CC, a typical vernalization treatment, or DF. Plants were vernalized under either treatment for 7-56 days prior to being transferred to a flowering-inducing treatment (long-day conditions). Compared to non-

vernalized controls, both cold treatments decreased time to flowering in Bd21-3 and Bd18-1. Although both treatments induced flowering, when vernalized for up to 21 days, CC-treated Bd21-3 flowered earlier than DF-treated Bd21-3. Similarly, CC-treated Bd18-1 vernalized for 7 and 14 days also flowered earlier than DF-treated Bd18-1. However, the flowering time of CC-treated and DF-treated plants in all later time points were equivalent (Fig. 3.4). Hence, vernalization under DF could effectively induce a flowering response in both *B. distachyon* accessions but did so slightly slower than the CC treatment.

We further measured the transcript levels of the cold-responsive vernalization gene *VERNALIZATION1* (*VRN1*) whose expression is known to provide flowering competence in *B. distachyon*. *VRN1* transcripts accumulate to higher levels in CC than in DF in all four accessions tested (Fig. 3.4B). Linear regression of form $y=mx+b$ showed that, according to the m coefficient in CC and DF fitted models, *VRN1* transcript levels accumulate ~5.8 times faster under CC than under DF (Fig. 3.4B). Plotting the levels of *VRN1* transcripts against the corresponding days to heading in the vernalization-requiring accession Bd18-1 shows that DF-treated plants reach minimum flowering time with lower *VRN1* transcript levels than in CC-treated plants (Appendix 1 Fig. 2). Again, as CC and DF induce similar flowering responses, linear regression shows that the accumulation of *VRN1* transcripts under DF induces vernalization with ~5.6 lower *VRN1* transcript levels than under CC (Appendix 1 Fig. 2). Hence, plants exposed to DF show a vernalization response that leads to flowering competence with significantly lower *VRN1* transcript levels, which indicates that lower expression of VRN1 than previously observed under CC are necessary to reach flowering competence in *B. distachyon*. Moreover, the vernalization response under DF also suggests the influence of DF-responsive factors on vernalization and flowering.

The vernalization response is characterized by the activation of *VRN1* that sees its chromatin transition from a closed to an open state under cold exposure. Thus, we measured the levels of histone H3, repressive histone mark H3K27me3, and polymerase-II-bound DNA at the *VRN1* locus on non-vernalized (NV) control (56-week-old plants grown under short-day 22°C), and vernalized CC56 (CC) and DF56 (DF) Bd21-3 plants. NV show the highest levels of H3 and H3K27me3, and no binding of polymerase II at the *VRN1* locus (Fig. 3.4C). CC leads to significantly lower H3 and H3K27me3 levels, and significantly higher signals of polymerase II binding to *VRN1* compared to both NV and DF. Compared to NV, DF shows lower nucleosome density levels around the first exon of *VRN1* (*CaRG* and *R1*) and lower H3K27me3 levels towards

the end of the first intron (*R6*), indicating a vernalization response. However, the overall chromatin state of *VRN1* observed in DF-vernalized plants appears to be similar to NV rather than CC-vernalized. Therefore, the chromatin state of *VRN1* measured under DF suggests a moderate vernalization response compared to the highly relaxed state and the highly active transcription measured under CC.

Because CC and DF induced a similar flowering response and contrasting epigenetic and transcriptional state of *VRN1*, we measured the expression of *FLOWERING LOCUS T (FT),* whose expression promotes flowering as previously identified in *B. distachyon* (Ream et al., 2014). Prior to the transfer to flower-inducing conditions, transcript levels of *FT* are higher in plants vernalized under CC compared to plants vernalized under DF (Fig. 3.4D). However, when transferred to flowering conditions, plants vernalized under DF accumulate *FT* transcripts to significantly higher levels than CC vernalized plants despite lower VRN1 levels (Fig. 3.4E). Hence, change in *FT* may occur independently of *VRN1* expression under DF. To determine the effects of *VRN1* expression and the acquisition of flowering competence under CC and DF, we measured the transcript levels of *VRN1* and *FT* in previously described *VRN1* overexpressor (UBI:*VRN1*) and knock-down (ami*VRN1*) lines that respectively display rapid flowering without vernalization and strong flowering delay when vernalized in response to CC and DF (Ream et al., 2014; Woods et al., 2016). As expected, UBI:*VRN1* shows higher transcript levels of *VRN1* under both cold treatments (Appendix 1 Fig. 3). As observed with Bd21-3 (Fig. 3.4E), both *VRN1* transgenic lines that were vernalized under DF show higher transcript levels of *FT* once transferred to flowering conditions, compared to CC-vernalized plants (Appendix 1 Fig. 3). Therefore, these results suggest that CC and DF induce different vernalization and flowering responses and that vernalization under DF appears to lead to a higher expression of *FT* independently of the expression of *VRN1*.

3.3.5 High *VRN1* transcript levels limit cold acclimation and freezing tolerance

Plants grown under CC accumulate high levels of *VRN1* transcripts and display a moderate tolerance to freezing. Conversely, plants grown under DF develop a high tolerance to freezing with lower levels of *VRN1*. Hence, we investigated the link between *VRN1* expression, cold acclimation, and freezing tolerance in *VRN1* overexpressor (UBI:*VRN1*) and knock-down (ami*VRN1*) transgenic lines. With a similar non-acclimated freezing tolerance and a lower cold-acclimated freezing tolerance, UBI:*VRN1* showed a lower capacity to cold-acclimate under both CC and DF

compared to 10A and ami:*VRN1* plants in whole-plant freeze test (Fig. 3.5A). Within the first 16 hours of exposure to DF, the profiles of *COR* gene transcript accumulation in the *VRN1* transgenic lines suggest that VRN1 influences *COR* gene transcription (Fig. 3.5B). Although *VRN1* transgenics show complex differences in the transcription profiles of *COR* genes, the transcript levels of the cold-responsive transcription factors *C-REPEAT BINDING FACTORs 1-3* (*CBF1-3*) were significantly different between all lines at 4 and 16 hours for CBF1, at 16 hours for CBF2, and at 12 hours for CBF3; UBI:*VRN1* showed the lowest while ami*VRN1* showed the highest transcript levels (Fig. 5B). In addition to being significantly different from one another, both transgenic lines also showed lower transcript levels compared to the control line 10A for the structural *COR* gene *COR410* at 12 and 16 hours.

To determine whether the VRN1 protein was directly interfering with the transcriptional regulation of *CBF* genes, we performed a ChIP-qPCR assay on the ACV5-tagged VRN1 fusion protein in the UBI:*VRN1* background. The results suggest that VRN1 binds to the promoters of *CBF1* and *CBF3,* and hence that high VRN1 levels affect cold acclimation by interacting with *CBF*s promoters.

3.3.6 *VRN1* influences plant morphology and winter hardiness

As the DF treatment is closer to natural conditions, studying how the growth, cold acclimation, and vernalization responses are integrated under this treatment may better explain winter hardiness in *B. distachyon*. Indeed, DF-treated plants developed a distinctive plant structure. We recorded final height, final leaf number, number of tillers, number of spikes, dry weight, and weight of seeds in control, CC- and DF-treated Bd21-3 and Bd18-1. CC56 and DF56 were both shorter than control CTR56 and tend to produce more spikes and heavier seeds (Fig. 3.6A). However, CC and DF led to two distinct plant morphologies with large differences in the number of final leaves and tillers. Indeed, DF-treated plants developed compact plant structure with a high number of leaves and tillers and consequently tended to produce more biomass (Fig. 3.6A). In addition to producing more leaves and tillers than CC-treated plants, DF plants acquired a compact structure compared to control plants with similar number of tillers. Indeed, the length between each node (where tillers emerge) is significantly smaller in DF plants (Appendix 1 Fig. 4). This structure appears to better insulate the crown tissues of the plant, which are believed to be an important structure for surviving freezing (Appendix 1 Fig. 4).

Phenotypic measurements along with the corresponding *VRN1* transcript levels (Fig. 3.4A) and days to heading (Fig. 3.4B) in CC7-56 and DF7-56 of both Bd21-3 and Bd18-1 were summarized in a heatmap. Associated dendrograms show that CC35-56 followed by CC21-28 form distinct phenotypic groups, highlighting the effects of long-term constant chilling on plant phenotype. In contrast, DF-treated plants cluster together along with CTR0, CTR56, and CC7-14. Generally, the number of final leaves decreases over time under both CC and DF until vernalization saturation. However, only DF plants showed a subsequent increase in final leaf number (Fig. 3.6B, Appendix 1 Fig. 5). In addition, the heatmap shows that *VRN1* is an important discriminating factor between CC and DF-treated plants. As CC and DF lead to disparate plant morphologies and drastically different transcription of *VRN1*, we investigated the effects of high *VRN1* expression on plant morphology.

B. distachyon is a long-day plant that does not flower under 8h light/day (e.g. control SD22°C, CC, and DF). Hence, when grown for 56 days under non-inductive photoperiod, UBI:*VRN1* plants adopted a distinct plant stature compared to control and ami*VRN1* plants (Fig. 3.6C). UBI:*VRN1* were taller and displayed fewer tillers under all treatments. Moreover, UBI:*VRN1* DF56 plants that have flowered had around twice the height and half the number of tillers and leaves compared to 10A (empty-vector control) and ami:*VRN1* plants (Appendix 1 Fig. 6). When grown under DF, *ami:VRN1* plants adopt a shorter stature than control plants (Fig. 3.6C). These results show that *VRN1* expression influences plant morphology. Possibly, the phenotypic difference between CC-treated and DF-treated plants can be at least partly attributed to the levels of *VRN1* transcripts, as also suggested by the dendrogram (Fig. 3.4B): high levels prevent the development of short-statured plants with high numbers of tillers and leaves. Importantly, UBI:*VRN1* failed to produce a compact plant structure under DF. These results allowed us to generate a model of the effects of *VRN1*'s expression on vernalization, plant phenotype, and winter hardiness (Fig. 3.6D).

3.4 Discussion

3.4.1 DF induces high freezing tolerance in *B. distachyon*

The DF treatment was designed to maximize the signals of seasonal change towards winter by combining specific values of seasonal atmospheric variables that are typical of summer, fall, and winter in four habitats inside the natural range of *B. distachyon*. Compared to constant-chilling

(CC), the DF treatment induced higher freezing tolerance in all tested accessions. The transcript accumulation profiles of *COR* genes, as well as the levels of proline and non-structural carbohydrates indicate that DF induces a different cold acclimation response compared to the one elicited by CC. Cold acclimation induced by freezing temperatures has been described in temperate cereals, *Brassica napus* and Arabidopsis (Herman et al., 2006; Kacperska & Kulesza, 1987; Takahashi et al., 2019). This response, termed sub-zero acclimation or described as a secondary stage of cold acclimation, appears to regroup acclimation mechanisms different from those of chilling-induced acclimation. Plants exposed to transient non-damaging frost and diurnally freezing temperatures were shown to undergo changes in photosynthesis, organelle structure, phospholipid content and composition, cell wall composition, and water potential that contribute to increasing freezing tolerance (Andrews et al., 1974; Kacperska & Kulesza, 1987; Le et al., 2008; Sikorska & Kacperska-Palacz, 1979; Takahashi et al., 2019). Hence, by exposing plants to negative temperatures, DF likely induces cold acclimation mechanisms different from those induced by chilling (CC).

Under DF, *B. distachyon* developed similarly to control conditions, but acquired more leaves and a more compact plant stature (Fig. 3.6A-B). This compact plant stature contributes to insulating the crown tissues (Appendix 1 Fig. 4) and was described as a morphological adaptation to cold climates in alpine plants (Körner, 2016). This more extensive growth response compared to CC can in part be attributed to the promotion of leaf initiation by warmer temperatures (Li et al., 2019). Moreover, it was also shown that sub-zero acclimation can affect plant growth capability and that variations in the excitation state of photosystem II caused by day/night temperature changes can affect growth and plant structure (Gray et al., 1997; Kacperska & Kulesza, 1987). As such, the compact plant stature displayed by DF-treated plants could hypothetically be the result of individual or a combination of environmental factors, such as freezing, warm temperatures, and diurnal temperature variation and likely contributed to the significantly higher freezing tolerance measured in DF28 compared to CC28.

Because the daily temperatures cycles are relatively wide in *B. distachyon*'s natural range, the occurrence of freezing and chilling appear to be closer to the freeze-thaw cycles of DF than to stable chilling temperatures (Fig. 3.1C, Fig. 3.2A-C). This particularity simultaneously allows the occurrence of cold and sub-zero acclimation, coupled to a morphological response. Hence, the response induced by DF may elicit mechanisms of cold acclimation and freezing tolerance closer

to what the species undergoes in a natural context. Although a range of vernalization responsiveness was found in *B. distachyon*, studies so far show that all accessions underwent vernalization when exposed to cold (Ream et al., 2014) and because they all showed the capacity to increase their freezing tolerance, no "true" spring accession was found in the species (Colton-Gagnon et al., 2014). Because *B. distachyon* can grow under cold conditions, acquiring an adaptive morphology to mitigate the negative effects of freezing during cold exposure may hence be part of its freezing tolerance strategy.

3.4.2 High VRN1 expression inhibits freezing tolerance

The results show that high expression of *VRN1* limits cold acclimation and the acquisition of a compact plant stature. Our results show that *VRN1* overexpressors have a limited ability to cold acclimate and tolerate freezing and show lower freezing tolerance and lower *COR* gene expression (Fig. 3.5A-B). This observation can be at least partly explained by the direct binding of the *VRN1* protein to the *CBF1* and *CBF3* promoters. As *B. distachyon* is an obligate long-day plant, light conditions under control, CC, and DF treatments (8 hours light per day) did not induce flowering. Under non-inductive treatments (SD, CC, and DF), *VRN1* overexpressors grew into taller plants with fewer tillers and leaves and *VRN1* knock-down plants had a shorter stature under DF but showed a similar number of tillers and leaves. As the *VRN1* overexpressors failed to develop a freezing tolerant plant structure normally induced by DF (Fig. 3.6C), *VRN1* seems to be involved in cold acclimation and the regulation of plant morphology, with high *VRN1* expression inhibiting freezing tolerance in *B. distachyon*. Interestingly, previous work on barley demonstrated that VRN1 binds and regulates the expression of diverse target genes, such as genes involved in hormone metabolism and *CBF* (Deng et al., 2015). In addition to regulating vernalization in cereals, *VRN1* is active in wheat meristems during flower morphogenesis (Preston & Kellogg, 2008). Furthermore, recent work demonstrated that *VRN1* influences root architecture in barley and wheat (Voss-Fels et al., 2018). Our results suggest that Bd*VRN1* plays a basic role in cold adaptation by regulating vernalization, cold acclimation, and plant morphology. This finding is in accordance with putative functions of *VRN1* reported in this species and other temperate cereals (Dhillon et al., 2010; Feng et al., 2017). Recent work suggested that vernalization, in that case associated with high *VRN1* expression, limits freezing tolerance in *B. distachyon* (Feng et al., 2017). Our results indicate that vernalization can occur with relatively low expression of *VRN1*,

and hence can coincide with high freezing tolerance, as illustrated by DF-treated plants. Our findings support that *VRN1* is involved in cold acclimation and plant development, and as such, its expression levels are determinant in acquiring a freezing tolerant phenotype.

3.4.3 DF induces a distinct vernalization and flowering response

CC and DF induced distinct responses characterized by markedly different *VRN1* levels. CC-treated plants showed a lower tolerance to freezing and higher *VRN1* expression levels compared to DF-treated plants. According to the function of *VRN1* in regulating the freezing tolerant phenotype, the higher expression levels measured under CC are likely involved in limiting freezing tolerance but unsurprisingly have induced a strong vernalization response. Under both treatments, vernalization is characterized by the activation of *VRN1* and the associated epigenetic changes (lower nucleosome density and depletion in H3K27me3) that were previously observed in *B. distachyon* and in barley (Oliver et al., 2013; Woods et al., 2017). However, CC- induced vernalization showed higher *VRN1* expression that extended long after vernalization saturation (when plants had fully transitioned to flowering competence). This highly active transcriptional state was linked to the presence of RNA polymerase II and few nucleosomes on the *VRN1* locus, indicating an extensively relaxed chromatin state. Conversely, the lower transcript levels of *VRN1* measured under DF coincided with higher nucleosome and H3K27me3 levels compared to CC and was overall closer to the non-vernalized chromatin state of *VRN1*. Interestingly, CC-vernalized and DF-vernalized plants flowered at relatively the same time but had contrasting levels of both *VRN1* and the flowering gene *FT* under cold-treatment, but also under flowering conditions post-vernalization (Fig. 3.4D-E). Vernalization under DF induced lower *FT* expression but led to higher *FT* expression once transitioned to a flowering treatment. This change in *FT* expression occurred independently of *VRN1* expression levels, as also shown in *VRN1* transgenic lines (Appendix 1 Fig. 3). It was previously shown that *FT* overexpression *B. distachyon* lines also flower rapidly without vernalization (Ream et al., 2014). Hence, strictly speaking, the higher expression of *FT* in DF-vernalized plants once transitioned to flowering could compensate for the lower *VRN1* levels induced by DF. Studies have shown that there is a regulatory loop between *VRN1* and *FT*, as high transcript levels of *FT* were measured in UBI:*VRN1* and high levels of *VRN1* were measured in *FT* overexpressing mutants (Ream et al., 2014). The DF treatment seems to induce the expression of *FT* post-vernalization independently of this regulatory loop, which suggests the existence of

different flowering mechanisms than previously described. Overall, these results show that vernalization and the acquisition of flowering competence can occur with a relatively weaker activation of *VRN1* expression than previously described (Colton-Gagnon et al., 2014).

3.4.4 DF is an artificial treatment that elicits seemingly more balanced cold responses

Cold induces acclimation during early exposure and leads to both physiological and morphological changes that can contribute to maximizing survival to freezing. Vernalization becomes relevant when flowering can occur, thus in later stages (probably at springtime, when photoperiod increases in the case of *B. distachyon*). Therefore, it seems logical that cold-induced reproductive growth would occur subsequently and without inhibiting the development of freezing tolerance. CC and DF treatment lead to two different outcomes regarding the unfolding of cold acclimation, plant growth, vernalization, and flowering in *B. distachyon*. On the one hand, DF treatment resulted in cold-acclimated, short-statured, and flowering-competent plants. On the other hand, CC treatment resulted in highly vernalized plants that displayed lower cold acclimation and signs of chilling stress. Therefore, the response induced by DF appears to be more effective in inducing freezing tolerance and flowering. Because DF more closely models the natural onset of cold, this manifestation of cold acclimation and vernalization may better reflect the plants' cold-adaptive traits. Nevertheless, the DF treatment is artificial and the responses it elicits may diverge from the plant's natural cold responses. The DF treatment is a representational approach to reproduce and combine extreme natural cues of a given geographical range. The combination of extreme signals like the co-occurrence of low photoperiod with repetitive freezing and high diurnal temperature range are not typically experienced by plants. However, this unique combination may have exacerbated some of the responses associated with seasonal change, such as the existence of morphological mechanisms of freezing tolerance and an alternative induction of vernalization and flowering. Hence the DF treatment appears to have experimental value. Other crucial factors such as water availability, light quality, and light intensity were maintained constant in our study and would deserve more attention in such treatments, especially as drought appears to have applied an important selective pressure on the species (Des Marais & Juenger, 2016). It is of course impossible to reproduce the complexity of nature indoors, however, our study shows that attempting to simulate natural conditions of plant's native range can lead to new and informative observations. Bridging the gap between basic experimental research and field studies is a crucial

step in making relevant conclusions about the relationship between natural phenomena and biology, especially when investigating the consequences of anthropogenic climate change. Altogether, the approach presented in this study can contribute to the understanding of the effect of natural environmental conditions and could be applied to other plant species with different climatic specificities. Importantly, the DF treatment contributed to revealing a basic function for *VRN1* in cold adaptation. The regulation of its expression levels appears to be central to an adaptive unfolding of cold acclimation and morphological change that increase freezing tolerance. As studies have shown, the implication of *VRN1* in regulating cold adaptation makes this gene a prime subject to understand the regulation and the evolution of cold adaptation in plants, as previously suggested (McKeown et al., 2016).

3.4.5 CC induces chilling stress and sub-optimal cold acclimation

Although CC has been a useful treatment in the discovery and study of cold responses in many species, it does not seem to reproduce the natural patterns of cold acclimation conditions present in the native range of *B. distachyon* and appears to induce chilling damages along with limited cold acclimation in the species. It has already been proposed that the lack of progress in effectively improving winter hardiness in plants is partly due to a failure to reproduce in laboratory settings the complexity of natural conditions (Gusta & Wisniewski, 2013). We observed larger accumulation of total non-structural carbohydrates and proline in response to CC than to DF (Fig. 3.3). The lower levels of non-structural carbohydrates measured in DF plants may be linked to a different allocation, for example towards biomass (Fig. 3.6). Even though the accumulation of proline is considered as a marker of cold acclimation in many species, it does not correlate with freezing tolerance in *B. distachyon* (Colton-Gagnon et al., 2014). In fact, proline is known to accumulate in plants during stress (Hayat et al., 2012), and the higher proline concentrations measured under CC may hence be a sign of stress (Appendix 1 Fig. 1). Overall, it seems that the levels of proline and non-structural carbohydrates reflect the different responses induced by CC and DF rather than indicating cold acclimation and the levels of freezing tolerance.

3.4.6 Constant-chilling vernalization leads to over-vernalization

This study suggests that previous observations of negative correlations between cold acclimation and the vernalized state in temperate cereals may have been biased by high expression

of *VRN1* induced by the traditional use of CC to study cold acclimation and vernalization under controlled conditions. For many years, the number of leaves at senescence (final leaf number) has been used as an indicator of the number of days to heading or of the transition between the vegetative to the reproductive stage (Wang et al., 1995). Here, the relationship between days to heading and final leaf number is visible in early time points in both CC and DF-vernalized plants (Appendix 1 Fig. 5). However, plants exposed to DF begin to re-accumulate leaves after vernalization saturation (*i.e.* 28 days in Fig. 3.6A, Appendix 1 Fig. 5). Thus, as also observed in *VRN1* overexpressors, the number of leaves appears to be mostly indicative of high expression of *VRN1*, rather than the vernalized state (Fig. 3.6C, Appendix 1 Fig. 6). From these observations, we can suggest two stages during DF-induced vernalization: (i) acquisition of flowering competence and (ii) development after vernalization saturation. Probably because the expression of *VRN1* is relatively low throughout vernalization under DF, its effects on the number of leaves and tillers is attenuated once vernalization saturation is reached (Appendix 1 Fig. 5). Therefore, the acquisition of flowering competence would be a "checkpoint" event during the development of *B. distachyon*, rather than a developmental determinant. As vernalization saturation is reached under DF, *B. distachyon* can resume its vegetative growth and, subsequently, once flowering signals are present (e.g. higher temperatures and long-days) can transition to reproductive growth by upregulating *FT* (Fig. 3.4E, Appendix 1 Fig. 3). As such, the unfolding of development and the acquisition of flowering competence could be linked to the expression of *VRN1* during vernalization and flowering to the expression of *FT* post-vernalization. A tentative model summarizes the influence of *VRN1* on winter hardiness and flowering in Fig. 3.6D.

CC-induced vernalization is characterized by high expression of *VRN1* and *FT* (Fig. 3.4) and prompt flowering with no change in *VRN1* or *FT* expression, as seen in wild-type and *VRN1* overexpressing and knock-down transgenic plants. Therefore, CC appears to prime plants differently to flowering than DF. It was previously described that overly long exposure to cold induces growth-inhibitory effects in monocots. This phenomenon, termed over-vernalization, is attributed to delayed development and reduced numbers of buds, leaves, and spikes (Derera & Ellison, 1974; Weiler & Langhans, 1968). Notably, the number of leaves and tillers are lower in *VRN1* overexpressors (Fig. 3.6C, Appendix 1 Fig. 6). CC-induced vernalization also leads to fewer tillers, leaves, and spikes which may be linked to the growth inhibition imposed by the treatment and to the high expression of *VRN1*. If we consider plant stature, growth, and tillering as factors

that influence winter hardiness in *B. distachyon*, the growth inhibition and *VRN1* overexpression induced by constant-chilling inhibit winter survival. As the overly high *VRN1* expression may not be required to achieve flowering and negatively affects freezing tolerance, it appears that the over-vernalization induced by CC is therefore ultimately deleterious to winter hardiness. Because CC is far from modelling the atmospheric conditions measured in *B. distachyon*'s natural range, this state of over-vernalization may hence deviate substantially from what can be observed in natural populations of *B. distachyon*.

3.4.7 Conclusions

This study reports an innovative approach to model atmospheric cues of seasonal change. By combining cues specific of summer, fall, and winter, the DF treatment induced cold acclimation, morphological change towards a freezing tolerant plant structure and led to flowering competence through an expression of *VRN1* significantly lower than previously described. The results show that high expression of *VRN1* inhibits freezing tolerance and that constant-chilling treatments induce artificial responses that limit freezing tolerance in *B. distachyon*. The work presented in this study also suggests that *VRN1* plays a fundamental role in cold adaptation by regulating flowering, cold acclimation, and morphological development. By providing a glimpse of how cold adaptation responses are integrated in *B. distachyon*, this study shows that modeling elements of the natural context in laboratory experiments can provide new perspectives to scientific knowledge.

3.5 Methods

3.5.1 Climatic and meteorological datasets

Monthly average data for temperature, diurnal temperature range, and frost frequency were retrieved from the *Climatic Research Unit TS4.01 dataset* (Harris et al., 2014) covering data from 1901-2017 at stated or estimated collection sites of the parent accessions of *Brachypodium distachyon* inbred lines Bd21-3, Bd30-1, Bd18-1, and Bd29-1 corresponding to four habitats named H1, H2, H3, and H4, respectively. The data was retrieved by GPS coordinates from datasets archived by the Centre for Environmental Data Analysis (University of East Anglia Climatic Research Unit et al., 2017). Climates at the four habitats, as well as the data used to generate the map displayed in Fig. 3.1A, were obtained from a Köppen-Geiger climate world map that was re-

analyzed in 2017 and produced with data from 1986 to 2010 (Kottek et al., 2006). Hourly temperature data used to generate Fig. 3.1C, Fig. 3.2A-C, and Appendix 1 Fig. 1B were retrieved from the *HadISD: Global sub-daily, surface meteorological station data, 1931-2017, v2.0.2.2017f* (J. H. Dunn et al., 2015; Met Office Hadley Centre & National Centers for Environmental Information - NOAA, 2018) for specific stations as summarized in Appendix 1 Table 2. The data and raster file of the climate map were analyzed in R to produce Fig. 3.1A (R Core Team, 2013).

3.5.2 Plant growth and treatments

B. distachyon inbred lines Bd21-3, Bd30-1, Bd18-1, and Bd29-1 seeds were soaked for two hours and stratified at 4°C in the dark for 7 days. Stratified seeds were planted 3X3 in 3-inch 0.5 L pots filled with 160g of G2 Agromix (Fafard et Frères Ltd., Saint-Remi, QC, Canada) which were placed in an environmental growth chamber (Conviron, Winnipeg, MB, Canada) at 22°C under photosynthetically active radiation (PAR) intensity of 150 µmol m^{-2} s^{-1} for 8 hours of light per day. When plants reach the three-leaf stage (~14 days under control conditions), they were either transferred to the constant cold treatment (CC) at 4°C in an environmental growth chamber or to the diurnal freezing treatment (DF - see details in Appendix 1 Table 1) programmed into a LT-36VL growth chamber (Percival Scientific, Perry, IA, USA). CC and DF light conditions were identical to control (8 hours of light per day at 150 µmol m^{-2} s^{-1}) and all plants were kept equally watered throughout the treatments. To induce flowering, plants were transferred to 16 hours of light per day also at a PAR intensity of 150 µmol m^{-2} s^{-1} on a growth bench at 25°C and maintained watered until senescence.

3.5.3 Phenotypic measurements

Days to heading were determined from the date plants were moved to flower inducing conditions to the date when plants showed first visible emergence of heads (flowers). The number of tillers and plant height were determined prior to being transferred to flowering conditions except when mentioned otherwise. Total chlorophyll was extracted using methanol from fresh and ground pooled leaf tissue of three plants and observed by spectrophotometry as previously described (Ritchie, 2006). Final leaf number, number of spikes, dry weight, and seed weight were determined after senescence. Dry weight measurements were performed on total aerial tissue (without seeds) after thorough drying of plant tissues.

3.5.4 Survival to freezing

Plant survival to freezing was measured in whole-plant freeze tests (WPFT) in a LT-36VL growth chamber (Percival Scientific, Perry, IA, USA). The freezing program decreases the chamber temperature from -1°C to -12°C at the rate of 1°C per hour. Prior to the freeze test, the pots were watered to soil saturation and randomly placed in the growth chamber. Three randomly selected pots containing 9 plants each were removed after each hourly plateau from -7°C to -12°C. The plants were then left to thaw at 4°C in the dark for 24 hours, then switched to 22°C with no light for an additional 24 hours before being moved back to control conditions. Percent survival was determined after a week of recovery under control conditions. Prior to planting, all pots contained insulating pads to prevent drastic soil freezing and emulate natural soil cooling conditions. Plants exposed to WPFT were at the three-leaf stage in all experiments except for CC28 and DF28 plants that had a higher number of tillers.

3.5.5 Proline and sugar quantification

Tissue used for proline and sugar quantification were pooled aerial tissue from 27 plants per replicate and dehydrated, extracted, and quantified as previously described (Colton-Gagnon et al., 2014).

3.5.6 RNA extraction and RT-qPCR

Plant tissue was sampled from whole aerial tissue of plants at the three-leaf stage for cold acclimation samples (Fig. 3.2D) and 1 g of leaf tissue for vernalization samples (Fig. 3.3B-C). Sampled tissue was flash-frozen in liquid nitrogen before storage at-80°C. Each sampling was performed by pooling plant tissue from three plants. Samples were then extracted using EZ-10 spin column plant RNA miniprep kit (CAT#: BS82314, Bio Basic, New York, NY, USA) following the manufacturer's protocol. Reverse-transcriptase cDNA was synthesized using iScript™ advanced cDNA synthesis kit for RT-qPCR (CAT# 1725037, Bio-Rad, Hercules, CA, USA) as stated in the manufacturer's protocol. Relative transcript levels were determined by RT-qPCR reactions with Green-2-Go qPCR mastermix (CAT# QPCR004, Bio Basic) using a CFX Connect Real Time system (BioRad) and relative transcript levels were analyzed following the ΔΔCT method using *UBC18* gene as reference on biologically independent replicates (Hong et al.,

2008; Ream et al., 2014; Woods et al., 2017). The genes studied were previously identified as important cold-responsive genes in *B. distachyon* and include *CBF1*, *CBF2*, *CBF3* (Colton-Gagnon et al., 2014; Ryu et al., 2014), *IRI* (Bredow et al., 2016; Colton-Gagnon et al., 2014), and *VRN1* (Colton-Gagnon et al., 2014; Ream et al., 2014). Primer sequences can be found in Appendix 1 Table 3.

3.5.7 *VRN1* transgenic lines

VRN1 mutant lines UBI:*VRN1* and ami:*VRN1* were previously described and published (Ream et al., 2014; Woods et al., 2016).

3.5.8 Chromatin immunoprecipitation and qPCR

Chromatin immunoprecipitation were performed from a pool of three plants' cross-linked aerial tissue. ChIP was performed with anti-Histone H3 antibody (CAT#ab1791, Abcam, Cambridge, UK), anti-Histone H3 antibody (tri methyl K27) (CAT#ab6002, Abcam), and anti-RNA polymerase II antibody (Clone CTD4H8, Sigma-Aldrich, St. Louis, MO, USA) performed on the VRN1 locus. For the VRN1 protein binding analysis, ChIP was performed using anti-ACV5 antibody (CAT# A2980, Sigma-Aldrich) targeted to the VRN1-ACV5 fusion protein expressed by UBI:*VRN1* plants and a mock no-antibody control. Immunoprecipitated samples were analyzed by qPCR using the reagents described above for RT-qPCR and expressed by percent input (H3) or percent H3 as previously described (Mayer et al., 2015) without removing the mock signal from IP signals. Primer sequences can be found in Appendix 1 Table 3.

3.5.9 Statistical Analysis

One-way ANOVA tests followed by Tukey's test were performed in JMP® (1989-2019). Statistical significance was determined with $P<0.05$ on at least three independent biological replicates, including fold values for qPCR data. Error bars represent standard deviations between biological replicates. Linear model fits were performed in R using *lm()* for Fig. 3.4B-C.

3.5.10 Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *UBC18* (Bradi4g00660), *VRN1* (Bradi1g08340), *CBF1*

(Bradi3g51630), *CBF2* (Bradi1g49560), *CBF3* (Bradi4g35650), *IRI* (Bradi5g27350), *COR410* (Bradi3g51200) and *FT* (Bradi1g48830).

3.6 Supplemental data

Appendix 1 Figure 1. Freezing tolerance in CC-treated *B. distachyon* and associated chilling stress.

Appendix 1 Figure 2. *VRN1* transcript levels in relation to days to heading in CC and DF treated vernalization-requiring Bd18-1.

Appendix 1 Figure 3. Expression of VRN1 and FT in non-vernalized, vernalized in CC and DF, and flowering post-vernalization VRN1 transgenic lines.

Appendix 1 Figure 4. The compact plant structure produced by DF may better insulate crown tissues.

Appendix 1 Figure 5. Phenotype of Bd21-3 and Bd18-1 in response to CC and DF at 7-56 days of exposure.

Appendix 1 Figure 6. Phenotype of DF56 *VRN1* transgenic plants at senescence.

Appendix 1 Table 1. Summary of the dataset on habitats H1-4 and the diurnal-freezing treatment (DF), and the detailed temperature and light cycles of DF.

Appendix 1 Table 2. Accessions selected for this study, the corresponding geographic location of their parental seed collection site and associated climate.

Appendix 1 Table 3. Primers used in this study.

3.7 Acknowledgments

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3.8. Author contributions

B.F.M. and J.B.C. designed the research; B.F.M. and A.B. performed the experiments and analyses; B.F.M., A.B., and J.B.C. wrote the manuscript.

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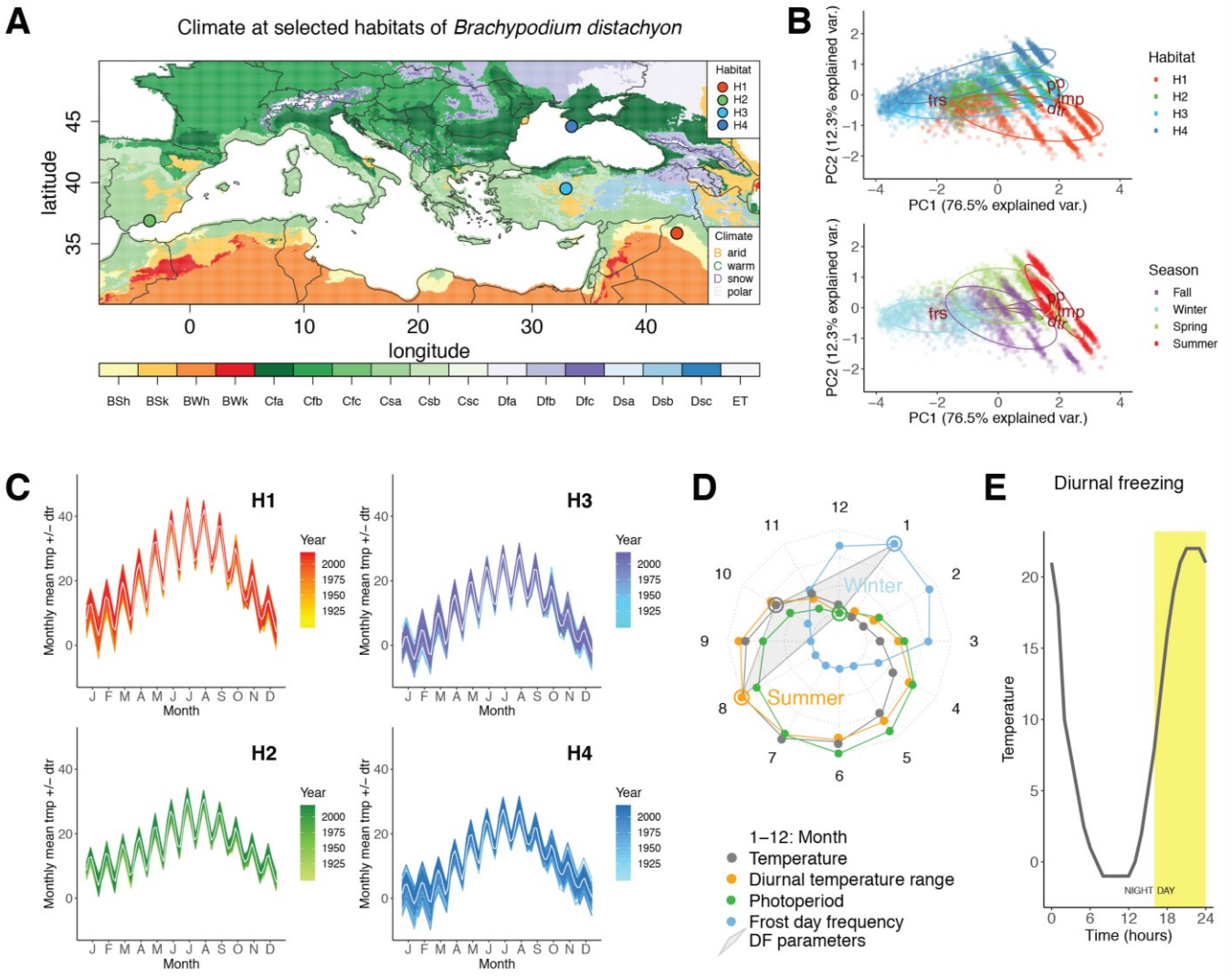

Figure 3.1: Specific seasonal cues observed at four habitats of *B. distachyon* can be combined into a diurnal freezing treatment to mimic seasonal change.

(**A**) Climate at selected geographical locations (habitats) that correspond to the parental seed collection sites of accessions Bd21-3 (H1), Bd30-1 (H2), Bd18-1 (H3), and Bd29-1 (H4). The colors correspond to the following climate: Group B: Dry (arid) climates - BSh: Hot semi-arid, BSk: Cold semi-arid, BWh: Hot desert, BWk: Cold desert; Group C: Temperate/mesothermal climates - Csa: Mediterranean hot summer, Csb: Mediterranean warm/cool summer, Csc: Mediterranean cold summer, Cfa: Humid subtropical, Cfb: Oceanic, Cfc: Subpolar oceanic; Group D: Continental/microthermal climates, Dfa: Hot-summer humid continental, Dfb: Warm-summer humid continental, Dfc: Subarctic, Dsa: Mediterranean-influenced hot-summer humid continental,

Dsb: Mediterranean-influenced warm-summer humid continental, Dsc: Mediterranean-influenced subarctic; Group E: Polar climates - ET: Tundra. (**B**) Principal component analyses illustrating clusters of the climatic data by habitat H1-4 (top panel) or by season across the four habitats (lower panel) over the following variables: average temperature (tmp), diurnal temperature range (dtr), photoperiod or daily hours of light (pp), and frequency of frost days (frs). (**C**) Diagram depicting a typical daily temperature variation for each month at each habitat. Values represent the monthly average diurnal temperature range centered around the monthly average temperature from data spanning 1901-2017 in H1-4. (**D**) Radar plot summarizing the gradual monthly change of tmp, dtr, pp, and frs that characterize seasonal change and representative values (circled) selected as parameters of a diurnal freezing treatment (DF): frs observed in winter, dtr observed in summer and values of tmp and pp observed in the fall. (**E**) Representation of a 24-hour-cycle of DF

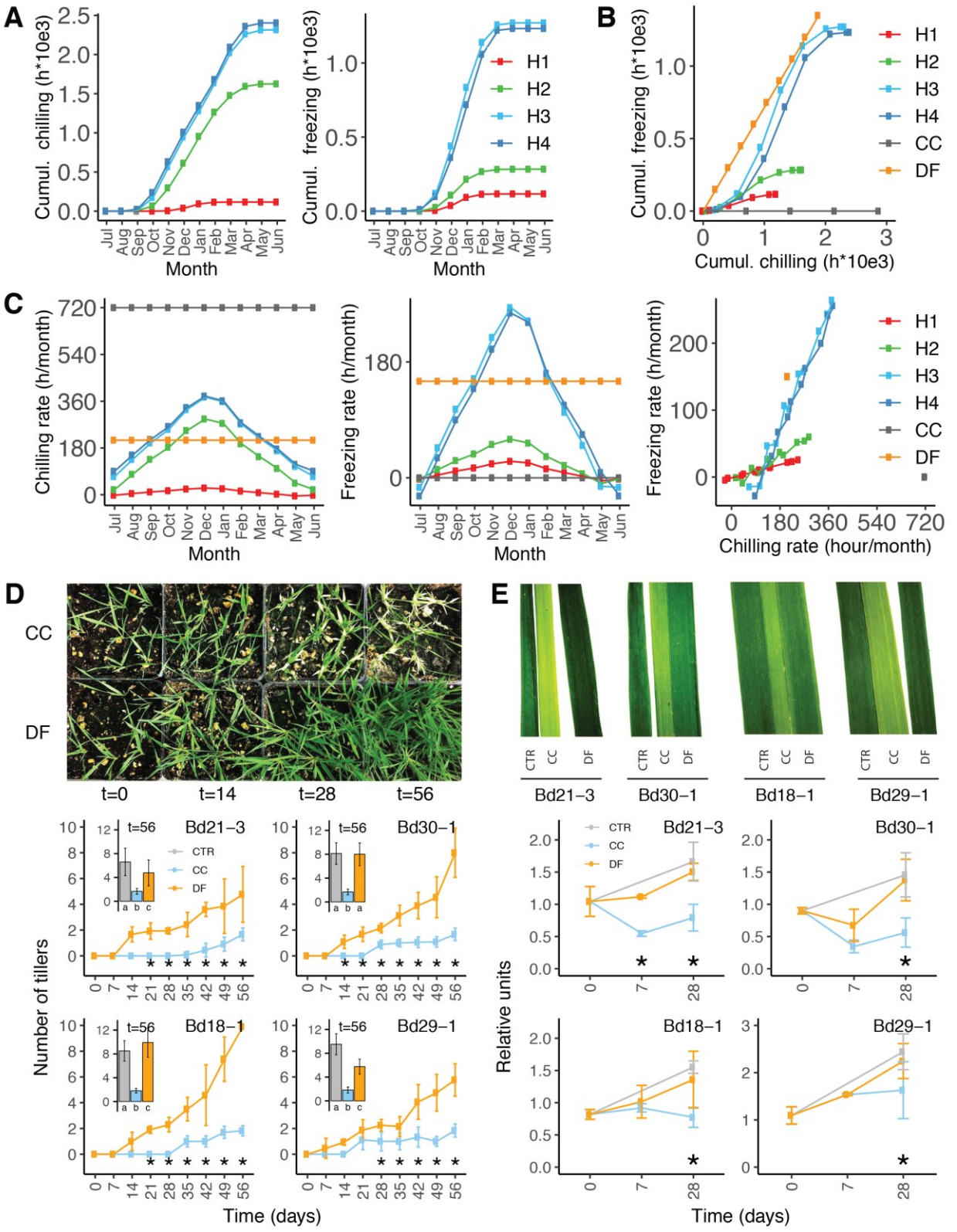

Figure 3.2: Constant-chilling and diurnal freezing simulate distinct chilling and freezing conditions and induce divergent growth responses.

(A) Cumulative hours of chilling (0 ºC <<8ºC) and freezing (<0 ºC) in four habitats of B. distachyon H1-4 compared to constant-chilling (CC) and diurnal-freezing (DF) treatments. (**B**) Cumulative freezing in relation to cumulative chilling in H1-H4, CC, and DF. (**C**) Actual rate of chilling and freezing in the four habitats based on meteorological stations from 1973-2017 (Appendix 1 Table 2) compared to CC and DF. (**D**) Number of tillers in control (CTR), CC, and DF-treated B. distachyon accessions Bd21-3, Bd30-1, Bd18-1, and Bd29-1 for 7 to 56 days. * *indicate statistical differences between CC and DF; p<0.05; error bars represent standard deviations between three biological replicates.* (**E**) Relative total chlorophyll contents in CTR, CC, and DF measured in Bd21-3, Bd30-1, Bd18-1, and Bd29-1 at 0, 7, and 28 days. * *indicate statistical differences between CC and DF; p<0.05; error bars represent standard deviations between three biological replicates.*

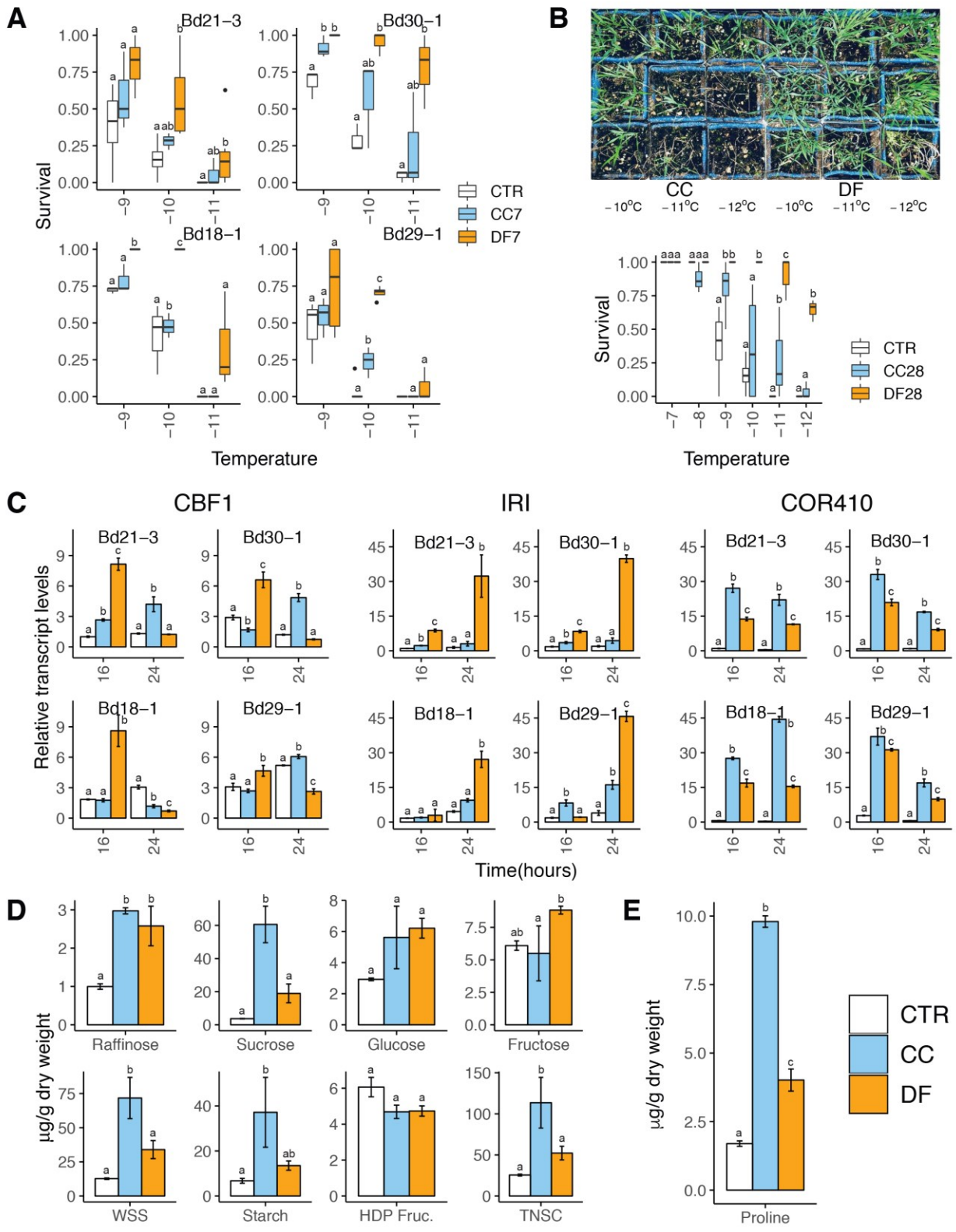

Figure 3.3: Constant-chilling and diurnal freezing induce contrasting cold acclimation and freezing tolerance.

(**A**) Survival to freezing in Bd21-3, Bd30-1, Bd18-1, and Bd29-1 after 7 days in either control conditions (CTR; short-day 22ºC), constant-chilling (CC), or diurnal freezing (DF) measured in whole-plant freeze tests in which temperature hourly decreases by 1°C down to -12°C. (**B**) Survival to freezing in Bd21-3 after 28 days in either control (CTR), constant-chilling (CC), or diurnal freezing (DF). (**C**) Relative transcript accumulation of C-REPEAT BINDING FACTOR 1 (CBF1), ICE-RECRISTALLIZATION INHIBITOR (IRI), and COLD-REGULATED 410 (COR410) at 16 or 24 hours after exposure to constant-chilling (CC) or diurnal freezing (DF). (**D**) Non-structural carbohydrate contents in CTR, CC, and DF-treated plants after 7 days in either treatment. (**E**) Proline contents in CTR, CC, and DF-treated plants after 7 days in either treatment. *In all panels, error bars represent standard deviations between three biological replicates; different letters represent statistically significant differences; $p<0.05$.*

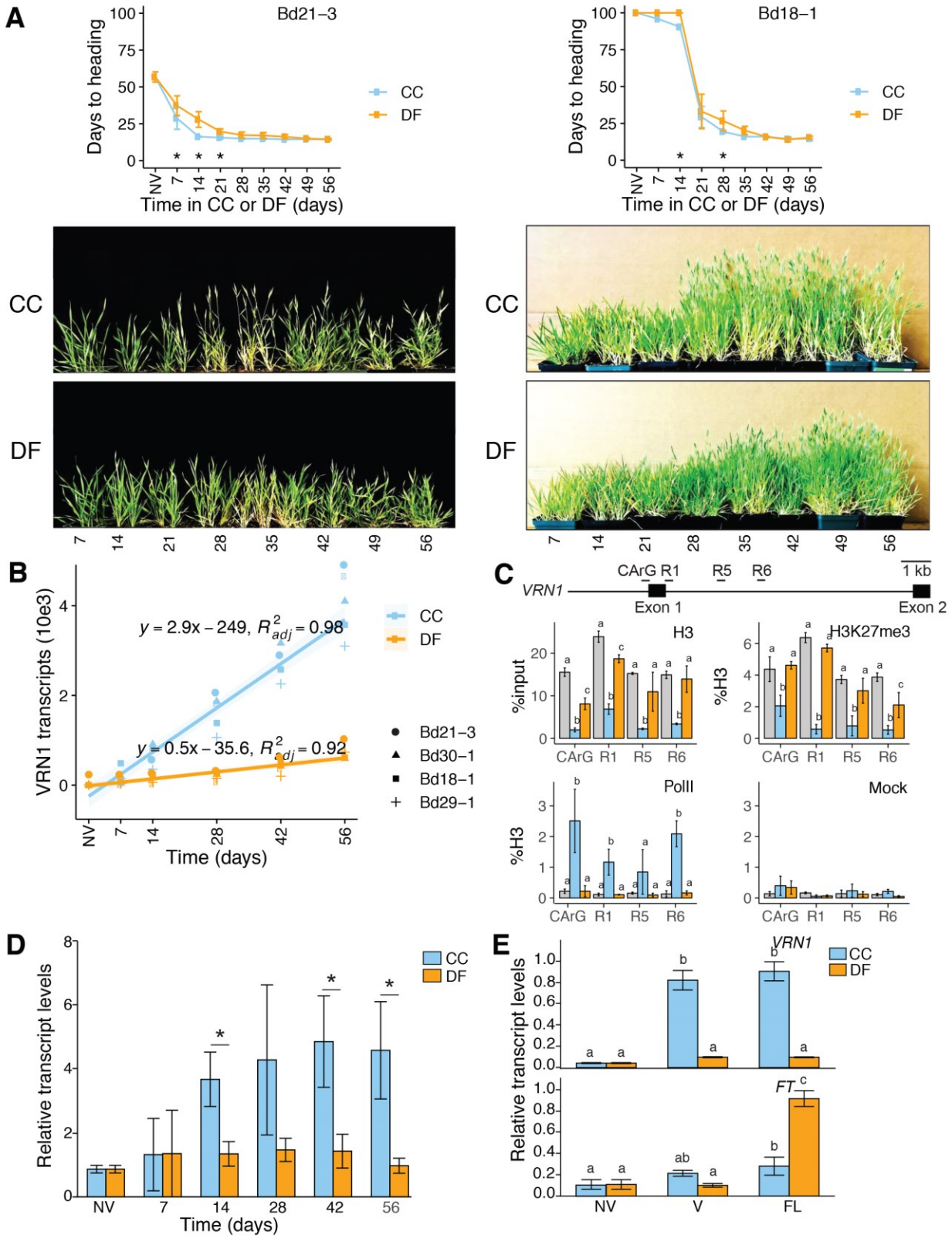

Figure 3.4: Constant-chilling and diurnal freezing lead to flowering competence but induce contrasting vernalization and flowering responses.

(**A**) Days to heading in weak vernalization-requiring Bd21-3 and strong vernalization-requiring Bd18-1. Prior to being transferred to flower inducing conditions, plants were either non-vernalized (NV; grown under non-inductive control conditions) or vernalized for 7 to 56 days (7-56) under constant-chilling (CC) or diurnal freezing (DF). (**B**) Relative levels of *VRN1* transcripts in Bd21-3, Bd30-1, Bd18-1, and Bd29-1 in a non-vernalizing or vernalization treatment CC or DF for 7-56 days. Linear regression of the form $y=mx+b$ was fitted on both CC and DF-induced *VRN1* transcript levels (y) over time under either treatment (x). (**C**) Chromatin state of the *VRN1* gene in Bd21-3 at 56 days of exposure to CC or DF; levels of H3, H3K27me3, levels of polymerase II (Pol II) binding and mock control at four regions of VRN1 (regions adapted from (Woods, Ream et al. 2017)). (**D**) Relative levels of *FT* transcripts in Bd21-3 non-vernalized (NV) or vernalized for 7 to 56 days (7-56) under constant-chilling (CC) or diurnal freezing (DF). (**E**) Relative transcript levels of *VRN1* in Bd21-3 and of the flowering gene FT in non-vernalized control (NV), CC56, or DF56 (vernalized; V), or a week after being transferred to flower inducing conditions (flowering; FL) after vernalization. *In all panels, error bars represent standard deviations between three biological replicates; different letters represent statistically significant differences;* $p<0.05$.

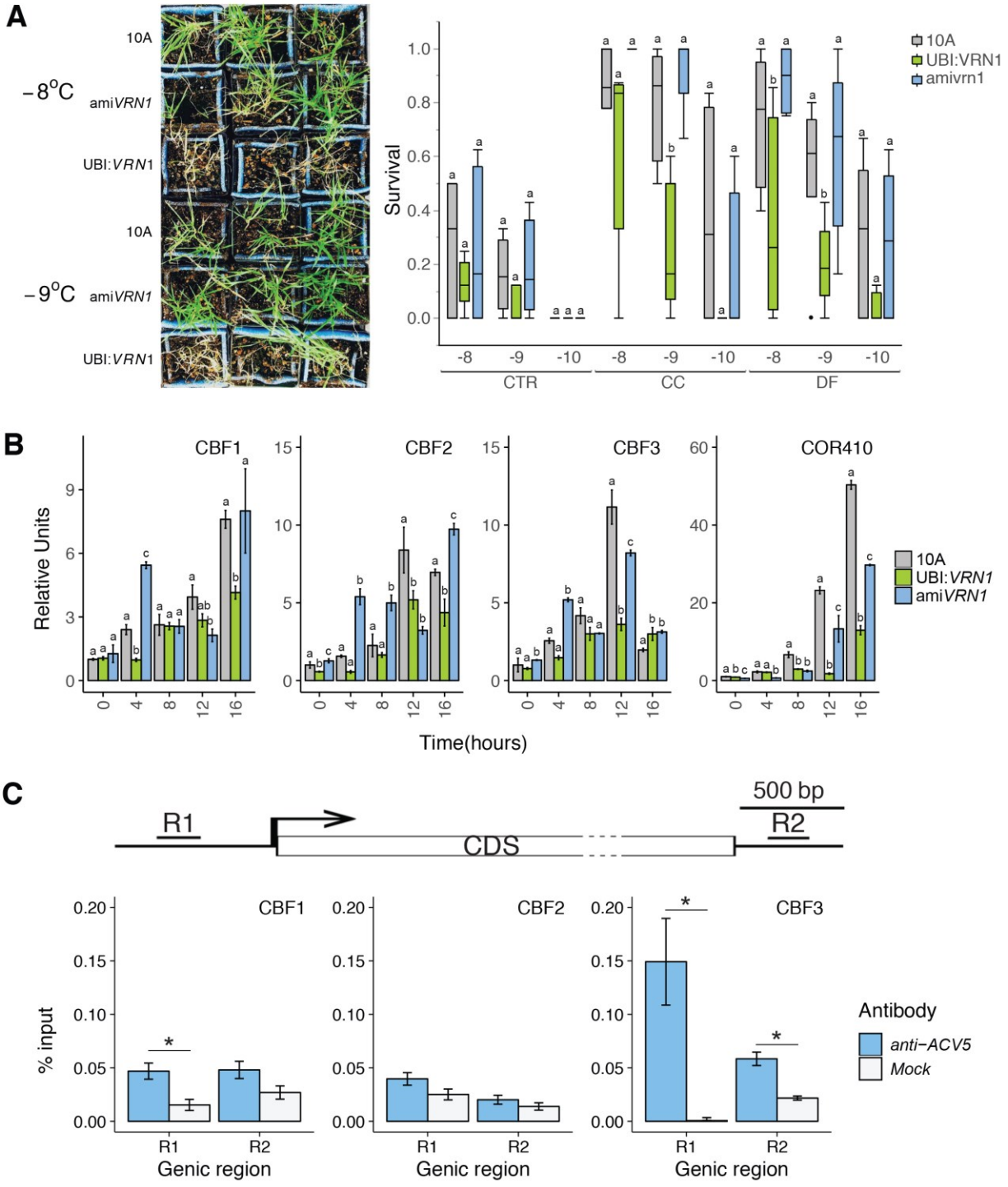

Figure 3.5: High *VRN1* expression limits cold acclimation and freezing tolerance.

(**A**) Survival to freezing measured of empty-vector pANIC 10A control (10A), *VRN1* overexpressor (UBI:*VRN1*), and *VRN1* knock-down (ami*VRN1*) lines grown under control (CTR), and cold-acclimated under constant-chilling (CC) or diurnal freezing (DF). (**B**) Relative levels of

cold acclimation gene transcripts at 0, 4, 8, 12, and 16 hours under DF. (**C**) Chromatin immunoprecipitation showing binding of the ACV5-tagged VRN1 protein in UBI:*VRN1* background on cold-responsive transcription factors *C-REPEAT BINDING FACTOR CBF1*, *CBF2*, and *CBF3*. *In all panels, error bars represent standard deviations between three biological replicates; different letters represent statistically significant differences; $p<0.05$.*

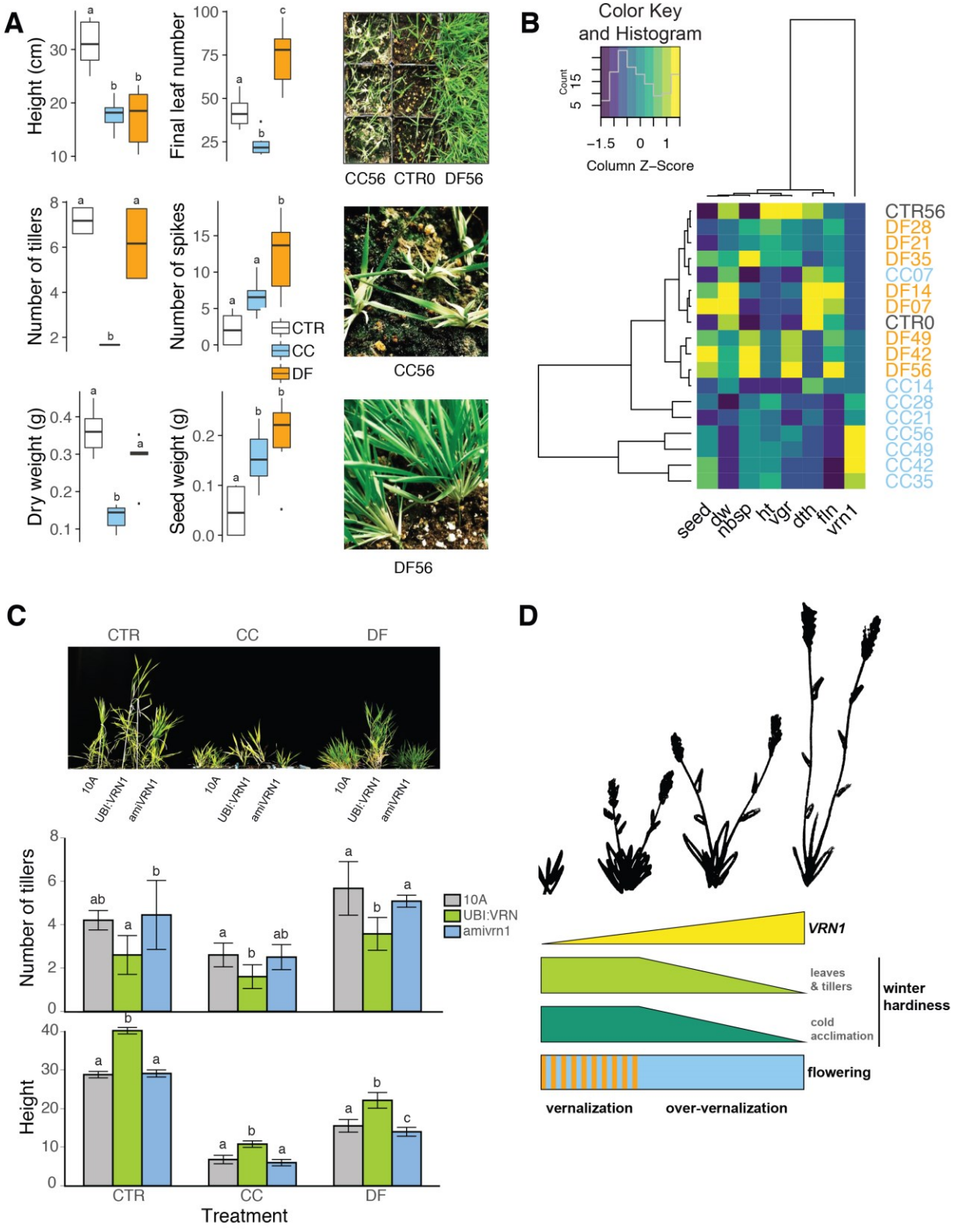

Figure 3.6: The development of a winter hardy phenotype is mediated by the expression levels of *VRN1*.

(**A**) Phenotypic data on control (CTR56), CC, and DF plants exposed for 56 days to either treatment (CC56 and DF56) in Bd21-3 and Bd18-1. Pictures illustrating the contrast between CTR0 (equivalent to CC0 and DF0), CC56, and DF56 plant phenotypes (*B. distachyon* Bd30-1). *Error bars represent standard deviations between six biological replicates; different letters represent statistically significant differences; $p<0.05$*. (**B**) Heatmap and dendrogram summarizing these differences in phenotype in CC or DF-treated Bd21-3 and Bd18-1 (CC7-56, DF7-56, CTR0, and CTR56). Abbreviations: vrn1: *VRN1* transcript levels, vgr: number of tillers, ht: height, dth: days to heading, fln: final leaf number, nbsp: number of spikes, dw: dry weight, seed: total seed weight. (**C**) Morphology of empty-vector pANIC 10A control (10A), *VRN1* overexpressor (UBI:*VRN1*), and *VRN1* knock-down (ami*VRN1*) lines grown in control (CTR56; short-day 22ºC), constant-chilling (CC56), and diurnal freezing (DF56) (no flowering occurs under these treatments). *Error bars represent standard deviations between biological replicates; different letters represent statistically significant differences; $p<0.05$*. (**D**) Model summarizing the relationship between *VRN1* expression levels, flowering, and winter hardiness.

3.10 Connecting text

Chapter 3 revealed that the morphology of *B. distachyon* is likely influenced by the signals of seasonal change. Hence, in addition to cold acclimation, the species can gain a morphology that contributes to higher freezing tolerance. This study described the occurrence of a morphological stress response in the context of freezing tolerance. Along with cold acclimation and vernalization which are respectively physiological and phenological plasticity responses, the morphological response of *B. distachyon* to diurnal-freezing can hence be considered part of temperate-climate adaptive phenotypic plasticity of *B. distachyon*, completing the picture of physiological, morphological and phenological phenotypic plasticity to be studied.

Cold acclimation is typically described as the result of early responses to cold and which lead to a more cold-tolerant physiology. Moreover, morphological responses are observed over longer times and are not typically described as a cold acclimation response. However, as shown in Chapter 3, morphology likely contributes with physiological responses to freezing tolerance. Therefore, in Chapter 4, we consider this response as a "morphological cold acclimation".

Although Chapter 3 showed that *VRN1* regulates cold acclimation, vernalization and morphology, the mechanisms that regulate the transition between early responses that affect physiology and longer-term responses that affect morphology are still unknown. Hence, Chapter 4 focuses on deciphering the mechanisms that regulate physiological and morphological cold acclimation. Because vernalization is regulated by mechanisms of transcriptional memory and these are involved in regulating phenotypic plasticity (namely learning and the evolution of responses over time), it is likely that these are also involved in regulating other temperate-climate adaptive phenotypic responses like cold acclimation.

4. TRANSCRIPTIONAL MEMORIES MEDIATE THE PLASTICITY OF COLD STRESS RESPONSES TO ENABLE MORPHOLOGICAL ACCLIMATION IN B. DISTACHYON

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4.1 Abstract

Plants that successfully acclimate to stress can resume growth under stressful conditions. The grass *Brachypodium distachyon* can grow a cold-adaptive morphology during cold acclimation. Studies on transcriptional memory (TM) revealed that plants can be primed for stress by adjusting their transcriptional responses, but the function of TM in stress accclimation is not well understood. We investigated the function of TM during cold acclimation in *B. distachyon*. qPCR, RNA-seq and ChIP-qPCR analyses were performed on plants exposed to repeated episodes of cold to characterize the presence and stability of TM during the stress and growth responses of cold acclimation. TM mainly dampened stress responses as growth resumed and as *B. distachyon* became habituated to cold stress. Although permanent on vernalization gene *VRN1*, TM were short-term and reversible on cold-stress genes. Growing under cold also coincided with the acquisition of new and targeted cold-induced transcriptional responses. Overall, TM provided plasticity to cold stress responses during cold acclimation in *B. distachyon*, leading to stress habituation, acquired stress responses, and resumed growth. Our study shows that chromatin-associated TM are involved in tuning plant responses to environmental change and, as such, regulate both stress and developmental components that characterize cold-climate adaptation in *B. distachyon*.

4.2 Introduction

Understanding the mechanisms of phenotypic plasticity in plants is crucial to building a sustainable agriculture, especially under the current worldwide climate and environmental crises. Human activities have caused abrupt environmental changes that will affect the global climate well beyond this century, imposing stress of increasing intensity to plants and crops (Gray & Brady, 2016; USGCRP, 2017; Bathiany *et al.*, 2018). Environmental disruptions can be widely problematic; for example, cold spells and late frosts can menace crop production in different environments such as temperate Canada or sub-tropical India (Aggarwal, 2008; Kutcher *et al.*, 2010). Finding ways to employ or enhance the acclimation mechanisms of plants can hence contribute to building more resilient food production systems.

Stressful conditions generally trigger responses that, once expressed, alleviate the negative effects of stress on plant growth and development. Depending on the severity of the stress and their adaptability, plants either die, enter a state of dormancy until conditions improve, or

acclimate and continue growing (Ciarmiello *et al.*, 2011). In the literature, acclimation is usually confined to reversible physiological changes, although stress conditions can irreversibly influence plant structure (Liu & Su, 2016; Klem *et al.*, 2019; Mayer *et al.*, 2020). Recently, stress-responses were found to be particularly plastic, improving over multiple exposures (Ding *et al.*, 2012; Li *et al.*, 2019; Zuther *et al.*, 2019; Mayer *et al.*, 2020). These modified responses are influenced by the stress exposures themselves during which plants build "experience" through stress memories (Crisp *et al.*, 2016; Yeung *et al.*, 2018).

Stress responses are often linked to extensive changes in gene expression (Ingram & Bartels, 1996; Thomashow, 1999; Zhang *et al.*, 2006). Studies have hence reported cases of stress memories linked to transcription, called transcriptional memories (TM). Genes that show TM typically display different transcriptional responses when the same stimulus is applied repeatedly (Avramova, 2015; Lämke & Bäurle, 2017). Events of TM were associated to specific epigenetic remodelers and to changes in chromatin marks left by stress exposure, which can, at least in part, explain the encoding of stress memories (Ding *et al.*, 2012; Lamke *et al.*, 2016). The di- and tri-methylation of histone 3 lysine 4 (H3K4me2/3) were identified as markers of TM in various plant abiotic stress contexts, including heat, cold, drought and salt stress (Liu *et al.*, 2014; Shen *et al.*, 2014; Feng *et al.*, 2016; Lamke *et al.*, 2016; Liu *et al.*, 2018; Zeng *et al.*, 2019), while DNA methylation is involved in stress-responses, TM and adaptation to environmental stress (Verhoeven *et al.*, 2010; Jiang *et al.*, 2014; Mayer *et al.*, 2015; Sanchez & Paszkowski, 2014; Wibowo *et al.*, 2016). By changing the regulation of gene expression, mechanisms of TM contribute the plasticity of stress responses. As plants can progress from "shock-like" stress responses to resuming growth in stressful conditions, the plasticity of stress response and associated TM mechanisms may hence mediate the plasticity of responses, leading to successful acclimation and morphology. Although plant stress-response mechanisms are generally well studied, the function of transcriptional memories in this stress-to-growth context needs further investigation.

The traits that characterize cold adaptation in the cereal model *Brachypodium distachyon*, namely cold acclimation and vernalization, provide a useful system to study the interaction between stress and growth, and the function of TM in acclimating to environmental change. Recently established as a model for cold-induced responses in temperate cereals, this undomesticated grass responds to cold by (i) physiologically acclimating, (ii) gaining flowering

competence through vernalization, and (iii) growing a cold-hardy morphology (Li *et al.*, 2012; Colton-Gagnon *et al.*, 2014; Ream *et al.*, 2014; Ryu *et al.*, 2014; Mayer *et al.*, 2020). The acquisition of freezing tolerance, aimed at maximizing survival to winter, encompasses the physiological changes associated with cold acclimation and the morphological plasticity that leads to freezing tolerant plant structures (Chouard, 1960; Thomashow, 1999; Körner, 2016). In addition, timely flowering is crucial in seasonal climates, and in temperate plants, this is ensured by a process known as vernalization. In temperate grasses, overwintering provides flowering competence by activating the transcription factor *VERNALIZATION1* (*VRN1*), precursor to flowering under favorable conditions (Danyluk *et al.*, 2003; Oliver *et al.*, 2009; Woods *et al.*, 2017; Mayer *et al.*, 2020). Under cold, the chromatin state of *VRN1* becomes gradually depleted of the silencing epigenetic mark histone 3 lysine 27 trimethylation (H3K27me3) and enriched in the activating H3K4me3, which remain after cold exposure (Oliver *et al.*, 2009; Chen & Dubcovsky, 2012; Oliver *et al.*, 2013; Woods *et al.*, 2017; Huan *et al.*, 2018). Hence, vernalization ensures timely flowering over long-term cold exposure through an epigenetically-regulated permanent TM. Although well studied in vernalization, little is known about the function of TM in cold acclimation and morphological responses to cold. Recent work in *Arabidopsis* has demonstrated the existence of cold-stress memories affecting freezing tolerance, transcriptomic and metabolomic responses to chilling and has highlighted the involvement of chromatin modifications in cold memory (Zuther *et al.*, 2019; Vyse *et al.*, 2020), although other studies have also indicated the existence of cold memory or changed chromatin states associated with cold exposure other than vernalization (Zhu *et al.*, 2008; Kwon *et al.*, 2009; Mayer *et al.*, 2015; Bittner *et al.*, 2020). Unlike in *Arabidopsis*, however, vernalization, and the expression of freezing tolerance that encompass cold acclimation and morphology are interconnected in *B. distachyon*, notably through *VRN1* which, other than regulating vernalization, influences cold acclimation and the acquisition of a winter-hardy morphology (Bond *et al.*, 2011; Mayer *et al.*, 2020). Hence, studying the function of TM when growth is resumed in cold conditions in *B. distachyon* can provide valuable insights on the mechanisms that regulate phenotypic plasticity in cold-climate responses and during stress acclimation in plants.

4.3 Materials and methods

4.3.1 Plant growth and cold treatments

B. distachyon Bd21-3 seeds were planted in 3X3 inch 0.5 L pots containing 160g of G2 Agromix (Fafard et Frères Ltd., Saint-Remi, QC, Canada), grown an environmental growth chamber (Conviron, Winnipeg, MB, Canada) under non-stress control conditions (8/16 hours dark/light at 22°C; PAR intensity of 150 µmol m−2 s−1) for 14 days. Plants were subsequently transferred to 4°C or under diurnal-freezing in an LT-36VL growth chamber (Percival Scientific, Perry, IA, USA). The diurnal-freezing treatment is characterized by air temperature cycles reaching -1ºC at night and 22ºC during the day as described in Fig. 4.3a and as explained in more details in Mayer *et al.* (2020). Plant were kept equally watered throughout.

4.3.2 Measures of freezing tolerance

Survival to freezing was measured in whole-plant freeze tests (WPFT). Plants were subjected to gradually decreasing temperatures from −1°C to −12°C at the rate of 1°C per h in a LT-36VL growth chamber (Percival Scientific). Pots each were watered to soil saturation and placed randomly in the chamber, and three randomly selected pots containing nine plants each were removed after selected hourly plateau from −8°C to −12°C. Pots were left to thaw at 4°C in the dark for 24 h, then switched to 22°C with no light for an additional 24 h before being transferred to control conditions. Percent survival was determined after 1 week of recovery under control conditions. This experiment was repeated three times. Electrolyte leakage of leaf tissue was measured as described in Lee & Zhu (2010) on five leaf replicates, which was performed three times.

4.3.3 RNA extraction and RT-qPCR

For each biological triplicate, pool of aerial tissue from at least three plants was collected, flash-frozen in liquid nitrogen, and extracted using EZ-10 RNA kit (CAT#: BS82314, Bio Basic, New York, NY, USA) following the manufacturer's protocol. Reverse-transcriptase cDNA was obtained using iScript™ (CAT# 1725037, Bio-Rad, Hercules, CA, USA) and RT-qPCR performed using Green-2-Go (CAT# QPCR004, Bio Basic) using CFX Connect Real Time (BioRad) both following manufacturer's protocol. Relative transcript levels were analyzed by the ΔΔCT method using *UBC18* gene as reference on three biologically independent replicates (Hong *et al.*, 2008;

Mayer *et al.*, 2020). The cold-regulated genes selected for this study were previously studied and exhibit different functions during cold acclimation in *B. distachyon*. These include the transcription factors *C-REPEAT BINDING FACTOR1,2 and 3* (*CBF1, CBF2, CBF3*) homologous to the CBFs previously identified in Arabidopsis and other species (Colton-Gagnon *et al.*, 2014; Ryu *et al.*, 2014), which likely induce the expression of following structural genes: the membrane-protein *COLD-REGULATED413*, the dehydrin *COLD-REGULATED410* (Colton-Gagnon *et al.*, 2014; Mayer *et al.*, 2020), and possibly the anti-freeze protein *ICE RECRYSTALLIZATION INHIBITOR* (*IRI*) (Bredow *et al.*, 2016; Mayer *et al.*, 2020). The transcription factor *VERNALIZATION1* (*VRN1*) is involved in vernalization but was recently shown to play an important function in cold acclimation and freezing tolerance (Ream *et al.*, 2014; Mayer *et al.*, 2020). Primer sequences can be found in Appendix 2 Table 5.

4.3.4 Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed on three biological replicates as previously described (Mayer *et al.*, 2015) using anti-Histone H3 antibody (CAT#ab1791, Abcam, Cambridge, UK), anti-Histone H3 tri-methyl-K27 (CAT#ab6002, Abcam), anti-Histone H3 di-methyl-K4 (CAT#ab11946, Abcam) and anti-Histone H3 tri-methyl-K4 (CAT#ab8580, Abcam). Immunoprecipitated samples were analyzed by qPCR and expressed by percent input or percent H3.

4.3.5 Global DNA methylation assay

Global DNA methylation was performed using Imprint Methylated-DNA Quantification Kit (Sigma-Aldrich Corp., St. Louis, MO, USA) following the manufacturer's protocol. Genomic DNA was extracted by standard phenol-chloroform extraction from two independent biological replicates, each a pool of aerial tissue from at least three plants. Each replicate was measured in technical quadruplicate using a Microplate reader (BioRad).

4.3.6 Sample preparation for RNA-sequencing

For the diurnal-freezing priming experiment, three plants for each treatment were collected 10 minutes before light. For the primed response to chilling experiment, three plants for each treatment were collected 3 hours after plants were moved to chilling. After collection, plant tissue

was flash-frozen and stored at -80°C until extraction. This experiment was performed twice. RNA was extracted using the RNeasy Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Two libraries, composed of three biological replicates each, were built using NEBNext® Multiplex Oligos for Illumina® (CAT#E7600S, New England Biolabs, Ipswich, Massachusetts, USA) and sequenced using Illumina HiSeq 4000 (DF-priming experiment) and NovaSeq 6000 (primed response to chilling) (Illumina, San Diego, California, USA) by Le Centre d'Expertise et de Services Genome Québec.

4.3.7 Transcriptome analysis

Using Galaxy, FASTQ reads (pre-released project available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA629906?reviewer=g0dlh6m54716vpjpa333uf1tpr), were analysed with FastQC and processed with Trimmomatic to remove adapters (Andrews, 2010; Bolger *et al.*, 2014; Afgan *et al.*, 2018). Using RNA Star, trimmed reads were mapped to Bd21v.3.1 obtained on Phytozome (**Brachypodium distachyon** Bd21v.3.1 DOE-JGI, http://phytozome.jgi.doe.gov) and reads counted using FeatureCounts (Vogel, 2010; Dobin *et al.*, 2013; Liao *et al.*, 2014). Using DEseq2 with cold/priming and replicates as factors, fold-change was calculated for S1/S0, S4/S0 and S4/S1 and for naïve and primed responses to chilling by comparing cold-treated with their respective non-stress controls (Anders & Huber, 2010). Genes displaying significant differential expression (p-adj < 0.05) and an absolute fold change > 2 (FC > 2) in both replicates were selected for further analysis.

4.3.8 Gene ontology enrichment analysis

GO analyses were performed using agriGO 2.0 (Tian *et al.*, 2017) with FDR adjusted p-value of 0.05 on genes FC>2, GOseq on genes FC>4 (Young *et al.*, 2010), ReViGO with associated over-represented p-adj, *Oryza sativa* as GO terms size database and SimRel for the semantic similarity measure (Supek *et al.*, 2011), and annotated *B. distachyon* gene list from phytozome. GO terms showed in Fig. 4e were obtained by summarizing agriGO terms with ReViGO. Treemaps were visualized using the package *treemap* in R (R Core Team, 2013).

4.3.9 Transcription factors and transcriptional regulators analysis

Transcription factors were identified using data from iTAK (Zheng *et al.*, 2016) and visualized using *heatmap.2* in R (R Core Team, 2013).

4.3.10 Statistical analyses

All experimental data, except for RNA-seq results, were analyzed by one-way ANOVA followed by Tukey HSD using JMP (SAS Institute, Cary, NC, USA). Statistical significance was determined with $p<0.05$ on at least three independent biological replicates, including fold values for qPCR data.

4.4 Results

4.4.1 Intermittent exposure to chilling resulted in higher survival than a continuous treatment

To determine whether *B. distachyon* can acquire cold-stress memories, we exposed plants to a cold acclimation treatment interrupted by a recovery period in control conditions. By performing whole-plant freeze tests, we measured the survival to freezing of plants that were exposed to chilling continuously for 14 or 21 days, or intermittently for 21 days (first for 14 days, then for 7 days separated by a 3-day recovery in control conditions; Fig. 4.1a). Plants exposed to chilling for a total of 21 days were the most tolerant to freezing. However, those that were submitted to an intermittent treatment survived better than those exposed continuously to chilling (Fig. 4.1b). Interestingly, the second 7-day exposure induced an increase in LT_{50} from ~25% to ~90% at -9ºC (re-acclimated compared to recovery plants), compared to an increase from ~15% to ~40% after the first 14-day exposure (acclimated compared to non-acclimated plants). Therefore, these results indicate that *B. distachyon* cold acclimated faster and more efficiently during the second exposure to chilling.

4.4.2 Repeated chilling led to global and gene specific chromatin changes

As chilling induces both cold acclimation and vernalization in *B. distachyon*, we investigated the chromatin response during both continuous and intermittent chilling treatments. We first measured the levels of global DNA methylation. Levels of global DNA methylation decreased significantly after 7 days in chilling but gradually increased back to control levels after

14 and 21 days of continuous exposure (Fig. 4.1c). Moving plants to recovery after 14 days in chilling did not alter the global levels of DNA methylation, while a subsequent re-exposure induced a hypermethylation significantly higher than the continuous 21-day treatment (Fig. 4.1c). Therefore, continuous exposure to chilling led to a transient hypomethylation, while a second exposure to chilling led to global DNA hypermethylation.

As a depletion of the silencing mark H3K27me3 occurs during vernalization in *B. distachyon*, which is a long-term epigenetic response, we investigated H3K27me3 levels on the gene *VERNALIZATION1* (*VRN1*), in acclimated, recovered and re-acclimated plants. To determine whether this mark is also involved in cold acclimation, we measured H3K27me3 levels on the transcription factor *C-REPEAT BINDING FACTOR1* (*CBF1*), the dehydrin *COR410*, the anti-freeze protein *ICE RECRYSTALLIZATION INHIBITOR* (*IRI*), which are involved in cold acclimation in *B. distachyon* (Colton-Gagnon *et al.*, 2014; Ryu *et al.*, 2014; Bredow *et al.*, 2016; Mayer *et al.*, 2020). After 14 days of chilling, nucleosome levels significantly decreased on *VRN1*, *CBF1*, *COR410* and *IRI*, while levels of H3K27me3 generally decreased, suggesting that these genes were activated upon cold exposure (Fig. 4.1d; Appendix 2 Table 1). Interestingly, 7 days of re-acclimation led to even lower levels of H3K27me3, while nucleosome levels increased on the four genes (Fig. 4.1d; Appendix 2 Table 1). Therefore, cold acclimation led to fewer nucleosomes and lower levels of H3K27me3, while reacclimation led to denser chromatin, suggesting that *B. distachyon* had a different chromatin response upon re-exposure to chilling.

4.4.3 Cold acclimation induces the formation of transcriptional memories

The transcriptional response of cold acclimation typically occurs, unlike vernalization, during the first few hours of cold exposure. Hence, to determine if cold acclimation led to the formation of transcriptional memories, we measured the transcript levels of *CBF1*, *COR410, IRI* and *VRN1* at 1, 3 6 and 24 hours of exposure and re-exposure to chilling, and under control conditions after 14 days of cold acclimation. The transcript levels of all four genes were measurably higher at least at the first hour of recovery compared to non-acclimated controls (Fig. 4.2a). Interestingly, transcript levels of *IRI* increased within the first 6 hours of recovery, while those of *VRN1*, known to be regulated a permanent transcriptional memory, remained elevated through recovery. Re-exposure to chilling led to a lower transcriptional response at all time points in *CBF1* but only at the later ones in *COR410* and *IRI*, while *VRN1* showed higher activation at all

time points (Fig. 4.2a). Therefore, cold exposure induced the formation of transcriptional memories that affected the transcript levels of *IRI* during recovery, of *CBF1* upon re-exposure and of *VRN1* throughout.

As chilling led to the formation of transcriptional memories affecting *CBF1*, *COR410*, *IRI* and *VRN1*, we investigated whether these were connected, at their gene loci, to nucleosome occupancy, to levels of H3K27me3 and to the levels of chromatin marks involved in stress-induced transcriptional memories H3K4me2 and H3K4me3. These were measured during the early transcriptional response of cold acclimation at 3 hours into the first and second exposures, and into recovery. The nucleosome and H3K27me3 levels measured under chilling were lower in plants exposed for a second time, indicating that these adopted a looser chromatin structure (Fig. 4.2b; Appendix 2 Table 1). Moreover, the levels of H3K4me2 and H3K4me3 peaked at recovery, suggesting that these were deposited after the first episode of cold, and dropped to lower levels upon re-exposure, suggesting that they are involved in the acquired transcriptional responses of *CBF1*, *VRN1 IRI* and *COR410* (Fig 4.2b). Therefore, the transcriptional memories observed for *CBF1*, *COR410*, *IRI* and *VRN1* occur along with different epigenetic signatures at their gene loci.

4.4.4 Longer exposures to diurnal-freezing translate into higher freezing tolerance

We previously showed that chilling induces artificial responses and limits cold acclimation in *B. distachyon* partly through a high expression of *VRN1* that inhibits the transcription of *CBFs* (Mayer *et al.*, 2020). Hence, the high *VRN1* expression induced during the first exposure to chilling has likely interfered with the transcriptional responses measured during the second one. To address this limitation, we investigated cold acclimation under diurnal-freezing, which models more closely the signals inducing cold acclimation and vernalization in the native range of *B. distachyon* (Mayer *et al.*, 2020). Diurnal-freezing is characterized by repeated 24-hour temperature cycles that fluctuate between day (22ºC) and sub-zero night temperatures (-1ºC) in a low photoperiod (8/16 hours light/dark), and is hence paced by daily cycles of low-temperature stress and recovery (Fig. 4.3a). Whole-plant freeze tests performed on plants exposed to 7 and 28 cycles of diurnal-freezing indicate that exposure to diurnal-freezing induces a gradually higher tolerance to freezing (Fig. 4.3b). In addition, while whole-plant freeze-test also indicate this trend in plants exposed to 1 and 4 cycles of diurnal-freezing (Fig. 4.S1), electrolyte leakage assays show that plants exposed to 4 cycles were less damaged by freezing than plants exposed to only 1 cycle of diurnal-freezing,

which were both less damaged than non-treated control plants (Fig. 4.3b). Therefore, the freezing tolerance of *B. distachyon* increased over repeated cycles of diurnal-freezing, indicating that these gradually prime *B. distachyon* to survive freezing (Fig. 4.3b-c).

4.4.5 Transcriptional responses evolve over cycles of diurnal-freezing

To determine whether cold-induced transcriptional responses change over time in diurnal-freezing, we measured the transcript levels of *CBF1*, *COR410*, *IRI*, and *VRN1*. In addition, to deepen our analysis, the study of additional genes involved in cold acclimation in the species, namely the transcription factors *CBF2* and *CBF3* and the structural gene *COR413* were also included. The transcript levels of the seven genes were measured at 0, 5, 8, 12 and 16 hours of exposure to low-temperature during a first and a fourth cycle of diurnal-freezing (Fig. 4.3a, d). Except for *IRI*, all transcripts accumulated within 16 hours of exposure to the first cycle. When exposed to the fourth cycle, transcript levels were higher for *IRI* and *VRN1*, and lower for *CBFs* compared to the first, indicating that repeated cycles of diurnal-freezing led to the establishment of transcriptional memories on these genes.

To follow the evolution of transcript levels from the first to the fourth cycle of diurnal-freezing, we selected two time-points: one during low-temperature and which showed the highest difference in transcript levels from cycle 1 to cycle 4 (time-point=16 in Fig. 4.3d), and another time-point during diurnal-freezing when temperatures were high (time-point=0 or 24 in Fig. 4.3d). Both time-points respectively represent low-temperature stress and recovery within each cycle of diurnal-freezing. Transcript levels of *CBFs* decreased sharply after cycle 1 at stress time-points, but slowly increased from cycle 1 to 4 at recovery (Fig. 4.3d). The transcript levels of *IRI* attained gradually higher levels from cycle 1 to 4, especially at recovery, while the transcriptional response of *COR410* and *COR413* remained relatively similar at stress time-points but decreased at recovery (Fig. 4.3d). *VRN1* transcripts gradually increased from cycle 1 to 4 through both stress and recovery time-points (Fig. 4.3d). Therefore, all genes showed altered transcriptional responses to cycles of diurnal-freezing. *CBFs* and *COR410-413* showed clear signs of transcriptional memory that decreased their expression at stress and recovery respectively, while *IRI* showed a transcriptional memory that increased its transcriptional response at recovery.

4.4.6 Six profiles describe the evolution of transcriptional responses in diurnal-freezing

To characterize the transcriptional change induced by diurnal-freezing, we compared the transcriptomes of plants exposed to the stress time-point of cycle 1 (S1) or of cycle 4 (S4) of diurnal-freezing, along with non-treated controls (S0; Fig. 4.3a). A total of 6725 genes showed a fold-change larger than 2 (FC>2) when comparing at least one condition to another: cycle 1 of diurnal-freezing compared to control (S1 compared to S0; S1/S0), cycle 4 of diurnal-freezing compared to control (S4 compared to S0; S4/S0), or cycle 4 of diurnal-freezing compared to cycle 1 (S4 compared to S1; S4/S1), which were summarized in a Venn diagram (Fig. 4.4a). We further classified these genes depending on whether these were upregulated, downregulated or non-responsive between the response to cycle 1 and the response to cycle 4 (S1/S0 and S4/S0; Fig. 4.4b). Combining the results from the Venn diagram and the change in expression between the cycles 1 and 4, we obtained a total of 17 categories, which describe in detail the transcript level outcomes in plants responding to diurnal-freezing (Table 4.1; Fig. 4.4c). These could be further regrouped into six expression profiles (Fig. 4.4c-d). *Stable* genes showed no change in response to diurnal-freezing (S4/S1). *Transient* genes responded only in S1/S0, while *late-responsive* genes responded only in S4/S0. *Complex-convergent* and *complex-divergent* genes responded in both S1/S0 and S4/S0, but *complex-convergent* had lower expression in S4/S0 compared to S1/S0 converging towards initial non-stress levels, and *complex-divergent* had increased expression between S4/S0 and S1/S0 diverging further from initial non-stress levels. *Offset/oscillating* moved from up to downregulated (offset with FC>2, oscillating with FC<2) or from down to upregulated (oscillating FC<2) between S1/S0 and S4/S0. Hence, these six expression profiles describe the main outcomes of transcriptional change between the first and the fourth cycle of diurnal-freezing (Table 4.1; Fig 4.4c-d).

4.4.7 The progression from S1 to S4 responses suggest a transition from stress to growth in diurnal-freezing

The response to cycle 1 of diurnal-freezing (S1 response) and cycle 4 of diurnal-freezing (S4 response) shared respectively 61% and 65% of their transcriptomes (the same transcripts in the same amount, corresponding to *stable* genes). In addition, 15% and 16% of their transcriptomes contained the same transcripts, but in different amounts (corresponding to *complex-convergent*, *complex-divergent* and *offset/oscillating* genes), while unique transcripts corresponded to 24% and

19% of their transcriptomes respectively (*transient* or *late-responsive* genes; Table 4.2). To characterize the progression from S1 to S4, enriched gene ontology terms shared between, and unique to, S1 and S4 were visualized along with the average S4/S1 fold-change of associated genes (Fig. 4.4e; Appendix 2 Fig. 2-3). Photosynthesis and ribosome biogenesis genes were mostly upregulated, while oxidation/reduction and metabolism genes were mostly downregulated between S1 and S4 (Fig 4.4e). Further analysis revealed that transcription factor activity, responses related to oxygen and heme binding were significantly enriched in S1 and S4 responses (Table 4.3). Interestingly, *iron ion binding*, *response to auxin*, *photosynthesis* and *sequence-specific DNA binding* were enriched in S1, but not in S4, indicating that S1 partly included transient responses. Specifically, the absence of the term *photosynthesis* in S4, and the upregulation of associated genes between S1 and S4 suggest that the normal expression of photosynthesis was restored in S4 (Fig. 4.4e; Table 4.3). Hence, the response to diurnal-freezing was plastic, and clearly changed between S1 and S4. Priest *et al.* (2014) described 22 distinct gene modules that characterize the plasticity of abiotic stress response in *B. distachyon*. Comparing the diurnal-freezing responsive genes to these modules support that S1 was associated with transiently expressed transcription factors and S4 with growth-related responses and restored photosynthesis (Appendix 2 Fig. 4). Therefore, repeated cycles of diurnal-freezing led to extensive reorganization of cold-induced responses from S1 to S4, indicating that *B. distachyon* exhibited plastic responses during cold acclimation.

4.4.8 Transcriptional memories regulate transient stress responses

To characterize the transcriptional regulation that accompanied the response plasticity in diurnal-freezing, we investigated the distribution of transcription factors within the six expression profiles identified in Fig. 4.4c-d. *Transient* and *complex-convergent* expression profiles were especially enriched in transcription factors, while the *stable* profile regrouped the most (Table 4.4). Interestingly, there were twice as many transcription factors in the *transient* than in the *late-responsive* expression profile. Hence, as *transient* genes are expressed in S1 and *late-responsive* genes in S4, S1 regrouped more transcription factors both in proportion and absolute number compared to S4. Moreover, *transient* transcription factors relate mostly to stress responses, *late-responsive* to growth and chromatin remodeling, while *complex-convergent* to both stress and development-related factors (Table 4.4). Hence, as suggested by the previous gene ontology

analyses, the response to diurnal-freezing generally progressed from the expression of stress-related to growth-related transcriptional responses.

Genes responsive in S1 and which showed significant change in expression between S4 and S1 (i.e. part of the *transient* T3 and T4 and *complex* genes), were regulated by transcriptional memories (Fig. 4.4a, d, Table 4.1). Hence, 19% of the diurnal-freezing responsive genes were memory genes and responded differently from the first to the fourth cycle of diurnal-freezing, as also illustrated by RT-qPCR analysis (Appendix 2 Fig. 5). Memory genes were enriched in transcription factors generally involved in stress response. For example, most members of the family AP2/ERF were regulated by transcriptional memories (Appendix 2 Fig. 6). This analysis indicated that *CBF1* and *CBF2* were *complex-convergent*, *IRI* and *CBF3 complex-divergent* and *COR410* and *COR413 stable* genes. *VRN1* behaved like *complex-divergent* genes (but with lower fold-change). Hence, RNA-seq data support our previous results that *CBFs, IRI* and *VRN1* displayed transcriptional memory.

4.4.9 The transcriptional memories of genes involved in cold acclimation are mostly reversible

Plants exposed to diurnal-freezing became increasingly tolerant to freezing and gained transcriptional memories. Hence, diurnal-freezing primed *B. distachyon* to respond to freezing, with the response to cycle 1 of diurnal-freezing is considered naïve, and the response to cycle 4 is considered primed. To determine the stability of transcriptional memories on genes involved in cold acclimation, primed plants (exposed to 4 cycles of diurnal-freezing) were exposed to an extended recovery (lag phase) for 1, 3, 6 or 9 days at 22ºC, after which their response to a "trigger" cycle of diurnal-freezing was measured (Fig. 4.5a). At the stress time-point of diurnal-freezing, the response of *COR410* and *COR413* was naïve throughout, while the *VRN1* response remained primed after all lag times (Fig. 4.5b). Moreover, the transcriptional responses of *CBF1*, *CBF2*, *CBF3* and *IRI* gradually returned to the naïve state after 3-6 days of lag time. Therefore, the transcriptional memory was stable for *VRN1*, but reversible for *CBF1*, *CBF2*, *CBF3* and *IRI*. Interestingly, all seven genes responded differently to priming at the recovery time-point compared to the stress time-point of diurnal-freezing (Fig. 4.5b). For instance, the transcriptional response of *CBF1*, *CBF2*, *CBF3* and *IRI* were naïve at all stages at recovery, hence showing no signs of memory, while *VRN1*, *COR410* and *COR413* showed reversible memories. Overall, diurnal-

freezing induced transcriptional memories that were reversible for *CBF1*, *CBF2*, *CBF3* and *IRI* at the stress time-point of diurnal-freezing, *COR410/413* and *VRN1* at the recovery time-point, but that remained stable for *VRN1* at stress time-point.

4.4.10 Primed plants respond less intensely to chilling and retained the responses acquired in diurnal-freezing

Genes involved in cold acclimation demonstrated different types of transcriptional memories at the stress and recovery time-points of diurnal-freezing, which could indicate that temperature cycles influenced their expression, *e.g.* inducing a change in circadian regulation when moved to diurnal-freezing. To address this possibility and confirm the presence of transcriptional memory, we applied cold at an unexpected time (middle of the day as opposed to dusk/night as in diurnal-freezing) and measured the transcriptional response of primed plants exposed to chilling after 1 lag-cycle (when memories had not reverted to the naïve state) compared to naïve plants exposed to chilling, and to the non-stressed primed and naïve control plants (Fig. 4.5c). In primed plants, *CBF1* showed a more sustained response at 6 hours, while *CBF2* and *CBF3* responded to lower levels at 3 hours compared to naïve plants. Moreover, *IRI* levels were higher in primed plants both in chilling and control conditions, confirming that *IRI* acquired a strong transcriptional memory. Conversely, *COR410* and *COR413* showed no difference of expression between primed and naïve plants. As expected, the expression of *VRN1* accumulated to higher levels in primed plants, which have likely undergone early vernalization (Fig. 4.5b). Therefore, primed plants exposed to chilling showed higher expression of *CBF1*, *IRI* and *VRN1*, and lower expression of *CBF2* and *CBF3*, confirming that these genes were regulated by transcriptional memories formed under diurnal-freezing.

Furthermore, we compared the transcriptome of naïve and primed plants at 3 hours of exposure to chilling compared to their non-stressed controls. The primed response contained approximately half the number of responsive genes than the naïve response, with 775 genes (of which 313 were primed-specific), compared to 1404 in the naïve response (of which 942 were naïve-specific; Fig. 4.6a, Table 4.5). 412 genes showed no memory, while 942 were altered by transcriptional memories, as also illustrated by RT-qPCR analysis (Table 4.5; Appendix 2 Fig. 7). All six expression profiles identified in diurnal-freezing were represented in the naïve and primed responses to chilling, and primed plants had a 3-fold depletion in *complex-convergent* genes and a

2.3-fold enrichment in *late-responsive* genes compared to the naïve response (Fig. 4.6a, Table 4.5). Because *complex-convergent* genes were regulated by transcriptional memories that dampened their expression antagonistically to *late-responsive* genes, the cold responses acquired in diurnal-freezing were hence retained in primed plants. Moreover, the ratio of memory to no-memory genes identified in diurnal-freezing was especially high in genes common to the naïve and primed response and especially low in genes unique to the primed response (~1-1.5 and 0.3 respectively; Table 4.5). Diurnal-freezing also affected the response of all naïve-specific genes and of those that responded differently to chilling in primed plants. Altogether, over 70% of the naïve response were memory genes.

4.4.11 The primed response to chilling is depleted in transcription factors and enriched in structural genes

Genes unique to the naïve response to chilling (naïve-specific) were enriched in *nucleic acid binding* and *sequence-specific DNA binding transcription factor activity* whereas genes unique to the primed response to chilling (primed-specific) were enriched in *metabolic*, *oxido-reduction*, *oxoacid*, *organic acid* and *cellular* processes (Appendix 2 Table 2). Although the primed response contained 3 times fewer specific genes than the naïve response, these were associated to 4 times the number of enriched gene ontology terms of naïve-specific genes. Primed-specific genes were hence connected to more diverse functions, related to plant metabolism and cellular processes, unlike naïve-specific genes which mainly function in the regulation of transcription. Among the genes that were differently expressed when comparing the naïve to the primed response, 66% were dampened by diurnal-freezing (Appendix 2 Table 3). Of these genes, which became hyposensitive to cold, many were stress-response transcription factors, unlike the genes that became hypersensitive (Appendix 2 Tables 3-4). Indeed, the primed response contained fewer stress-response transcription factors of the families AP2/ERF, bHLH, WRKY and C2C2 (Fig. 4.6c-d; Appendix 2 Fig. 8). Overall, the transcriptional response of primed plants to chilling was mostly characterized by the differential expression of structural genes.

4.4.12 Repeated priming in diurnal-freezing led to the onset of similar transcriptional memories but a different chromatin response

To investigate whether a second episode of priming could reinforce transcriptional memories and induced chromatin changes, we compared the transcriptional response of genes involved in cold acclimation and their associated levels of H3, H3K27me3, H3K4me2, H3K4me3 and the levels of global DNA methylation to two episodes of priming in diurnal-freezing separated by 3-day recovery. The transcriptional response of all genes evolved similarly in diurnal-freezing during both priming episodes (Fig. 4.7a). However, the first and second episodes of priming showed distinct chromatin signatures. Overall, genes became generally depleted in H3 and H3K27me3 after the first priming episode, which remained at recovery along with increased levels of H3K4me3. During the second episode of priming, these also became generally depleted in H3K4me3, but enriched in H3K4me2 (Appendix 2 Fig. 9; Fig. 4.7b). Global DNA methylation increased at the first priming episode, but decreased over recovery and during the second episode of priming, indicating contrasting chromatin responses to diurnal-freezing (Fig. 4.7c). These results suggest that depletions of H3 and H3K27me3 were linked to the transcriptional memories established during the first episode of priming, that H3K4me3 participated in the maintenance of transcriptional memories during recovery and its demethylation into H3K4me2 occurred with the transcriptional response to a second priming episode (Fig. 4.7d). Overall, the chromatin composition of genes involved in cold acclimation evolved in response to repeated episodes of priming in diurnal-freezing without affecting the onset of transcriptional memories, suggesting that chromatin composition can evolve separately from transcriptional responses.

4.5 Discussion

4.5.1 Cold acclimation in *B. distachyon* is characterized by a transition from stress to growth responses

B. distachyon gradually acquired higher freezing-tolerance in diurnal-freezing (Fig. 4.3b-c). This treatment first induced transcriptional responses typical of cold acclimation, including the activation of the transcription factors *CBFs*, and structural genes *IRI*, *COR410* and *COR413* previously identified in cold acclimation studies in *B. distachyon* (Fig. 4.3d; Colton-Gagnon *et al.*, 2014; Ryu *et al.*, 2014; Bredow *et al.*, 2016; Mayer *et al.*, 2020). Over repetitions of diurnal-freezing cycles, the transcriptional responses associated with cold acclimation were dampened

while the initial downregulation of photosynthesis, which is a typical sign of stress, returned to control levels (Fig. 4.3d-e, 4.4e, 4.6; Table 4.3-4). As plants grown in diurnal-freezing eventually produce biomass equivalent to non-stressed control plants, *B. distachyon* could hence successfully acclimate to the treatment (Crisp *et al.*, 2016; Mayer *et al.*, 2020). Interestingly, almost 40% of the cold-response transcriptome changed at the fourth cycle of diurnal-freezing, where initial stress response genes became generally replaced by genes linked to primary metabolic processes, cellular growth and to regulators implicated in growth and development (Fig. 4.4, Table 4.4, Appendix 2 Fig. 2-3). Furthermore, responsive genes were enriched in iron binding, heme binding and oxygen signaling genes. Plant hemoglobins are important for normal plant growth (Hebelstrup *et al.*, 2006), and by regulating the interplay between oxygen and nitric oxide, mediate stress and growth responses to environmental changes (Gupta *et al.*, 2011; Simontacchi *et al.*, 2015). Therefore, these results indicate that *B. distachyon* readjusted stress responses, growth and cellular homeostasis over time in diurnal-freezing (Table 4.3). As this treatment was designed to reproduce the natural signals of cold acclimation in *B. distachyon* (Mayer *et al.*, 2020), the stress-to-growth transition indicate that morphology may be an extension of cold acclimation in the species.

The transiently high expression of *CBF*s, important regulators of cold acclimation in several plant species, is likely important for morphological cold acclimation in *B. distachyon*. Well studied in cold acclimation, *C-repeat binding factors* (*CBF*) are known regulators of structural cold-regulated genes' expression (Jaglo-Ottensen *et al.*, 1998). In response to repeated chilling and diurnal-freezing, the initial transcriptional responses of *CBF*s are dampened (Fig. 4.2a, 4.3d, 4.5b). Moreover, the transcript levels of *CBFs* positively correlated with those of *COR410* and *COR413* in diurnal-freezing (Fig. 4.5b-c, Appendix 2 Fig. 10). *CBF*s induce the expression of *COR* genes by binding to the C-repeat motif in their promoter (Thomashow, 1999), but this has yet to be determined experimentally in *B. distachyon*. Here, results indicate that the maintenance of *COR410/413* expression post-cold is correlated to the transcriptional memory status of *CBFs*, suggesting the existence of a *CBF/COR* regulatory link in *B. distachyon* (Fig. 4.5b-c, Appendix 2 Fig. 10). Furthermore, *CBF1*, *CBF2* and *CBF3* have redundant and essential cold-acclimating functions in *Arabidopsis*, confer freezing tolerance in many plant species, and are involved in other processes, such as seedling and chloroplast development (Gilmour *et al.*, 2004; Savitch *et al.*, 2005; Benedict *et al.*, 2006; Jia *et al.*, 2016; Zhao *et al.*, 2016). Their regulation receives inputs from the circadian clock, light, and temperature for an appropriate response (Nakamichi *et al.*,

2009; Jiang *et al.*, 2017; Liu *et al.*, 2017). The constitutive overexpression of *CBF*s usually provide high freezing tolerance but severely limit plant growth and delay development in many plant species, notably through the accumulation of DELLA proteins (Achard *et al.*, 2008; Jeknic *et al.*, 2014; Wisniewski *et al.*, 2015). Therefore, the high expression of *CBFs* in diurnal-freezing may be transient to allow *B. distachyon* to grow.

4.5.2 Cold acclimation in *B. distachyon* relies on a habituation response mediated by transcriptional memories

Cold acclimation and the increase of freezing tolerance occurred with the establishment of transcriptional memories. These mainly dampened the initial cold-induced transcriptional responses, including those of the typical genes involved in cold acclimation (Fig. 4.2a, 4.3d-e). Approximately 20% of diurnal-freezing responsive genes showed strong transcriptional memories with 75% of these leading to downregulation (Table 4.1). Moreover, over 67% of naïve-response genes to chilling became non-responsive in primed plants, revealing that most transcriptional memories were downregulating memories (Table 4.5). Generally, studies report memory genes that show stronger and faster responses (i.e. showing hyperactivation) under repeated exposure to a priming stimulus (Ding *et al.*, 2012; Lamke *et al.*, 2016; D'Urso & Brickner, 2017; Liu *et al.*, 2018), although different types of memory were also described (Ding *et al.*, 2013). A recent review coined that plant memory genes can fit into two classes, either by showing a sustained expression during stress recovery or a hyperactivation upon re-exposure (Bäurle & Trindade, 2020). According to the definition that memory genes show an altered response to a given repeated stimulus (Avramova, 2015; Lämke & Bäurle, 2017), the present study reports transcriptional memories events that mainly lead, instead, to hypoactivation.

The decrease of cold-stress responses suggests that *B. distachyon* habituated to cold stress in diurnal-freezing (Fig. 4.2a, 4.3d-e, 4.6). By definition, habituation is a reversible response that becomes weaker over-time (Rankin *et al.*, 2009; Gagliano *et al.*, 2018). This term was previously used to describe plant responses to mechanical stimuli (Gagliano *et al.*, 2014), cell cultures growing autonomously from hormones and growth factors (Christou, 1988; Pischke *et al.*, 2006), or from toxins and growth inhibitors (Brochu *et al.*, 2010; Mélida *et al.*, 2010), and more recently, along with the term *sensitization*, to describe transcriptional memories in abiotic stress response (Liu *et al.*, 2014; Liu & Avramova, 2016; Csermely *et al.*, 2020). In general, the justifications of

habituation responses relate to energy balance, suggesting that organisms mitigate the energy cost of constantly responding to a given stimulus (Duijn, 2017). In our system, *B. distachyon* shows reversible hypoactivation on cold-regulated genes and can fully acclimate by resuming growth in diurnal-freezing (Fig. 4.3d-e, 4.5b; Mayer *et al.*, 2020). Hence, habituation likely coincided, along with the return of growth-associated gene expression, with a diversion of the energy spent on initial stress responses towards the acquisition of a freezing-tolerant plant morphology.

4.5.3 The regulation of phenotypic plasticity

The phenotype acquired in diurnal-freezing is the result of dynamic signals that, over time, led to converging stress and growth responses. Recovery from stress is a critical period during which stress memories can be either discarded, encoded for future responses, or reinforced to maintain the expression stress-activated genes (e.g. *VRN1*; Crisp *et al.*, 2016). In *Arabidopsis*, recovery from cold exposure, or deacclimation, induces rapid hormonal, structural, metabolic and transcriptomic changes that relate to growth and development likely to kickstart growth in spring (Zuther *et al.*, 2015; Pagter *et al.*, 2017). The higher temperatures of diurnal-freezing cycles allow *B. distachyon* to grow and gain high freezing tolerance, unlike constant low temperature which can also induce chilling stress in the species (Fig. 4.3b; Mayer *et al.*, 2020). Hence, the alternance of low/high temperature signals in diurnal-freezing, or stress/recovery, appears to favour an equilibrium between stress-response and growth, which could explain the "carried-over" expression of growth-associated genes during cold exposure, and the dampening of acute cold-stress responses over time (Fig. 4.4e, Appendix 2 Fig. 2-4, 6, Table 4.3). Similarly, transcripts of *IRI*, *CBF*s and *VRN1* gradually accumulate at recovery time-points in diurnal-freezing, hence gaining higher basal expression outside of cold exposure (Fig. 4.3e). The gradual loss of transcriptional memory of *VRN1* or the rapid loss associated with *IRI* also indicate that the extended expression of cold-activated genes into the recovery time-point of diurnal-freezing is an acquired response to the treatment (Fig. 4.5a). Therefore, the phenotype displayed by *B. distachyon* in diurnal-freezing suggests a gradual convergence of cold-stress and growth responses. It is important to consider that, although this treatment models seasonal cues, the phenotypic plasticity displayed by *B. distachyon* in diurnal-freezing can be the result of both cold-adaptive plasticity traits and responses to the treatment itself (Fig. 4.4, Table 4.1, 4.5). Although the experiments of this study do not allow to differentiate between the two, the diurnal-freezing

phenotype is probably a combination of both. For instance, plants exposed to diurnal-freezing become fully vernalized (Mayer *et al.*, 2020). Moreover, the establishment of similar transcriptional memories were observed in repeated chilling and diurnal-freezing, which induce contrastingly different responses in *B. distachyon* (Fig. 4.2a, 4.3d; Mayer *et al.*, 2020). This supports that the shaping of cold responses *via* transcriptional memories likely regulate cold acclimation in *B. distachyon*.

Cold acclimation and vernalization are adaptive responses that ensure the persistence of plants in temperate climates. The expression of these two phenotypic plasticity responses are linked in temperate grasses, notably through the expression of *VRN1* (Galiba *et al.*, 2009; Dhillon *et al.*, 2010; Deng *et al.*, 2015; Mayer *et al.*, 2020). The results of the present study show that the main regulatory mechanism of vernalization, namely the epigenetically-regulated transcriptional memory of *VRN1*, may also regulate cold acclimation in the temperate grass *B. distachyon* (Fig. 4.2a, 4.3d-e, 4.5b-c). The establishment of stress transcriptional memories have been previously connected to changes in chromatin (Ding *et al.*, 2012; Lamke *et al.*, 2016; Liu *et al.*, 2018). Our results show that repeated cold affected levels of global DNA methylation and of H3K27me3, H3K4me2 and H3K4me3 at the loci of genes involved in cold acclimation in *B. distachyon* (Fig. 4.1c, 4.2b, 4.7b-c). The nucleosome numbers on cold-regulated genes decreased permanently at the first exposure, while H3K4me3 were deposited after extended recovery, and H3K4me2 and H3K27me3 were deposited upon re-exposure to diurnal-freezing (Fig. 4.7b, Appendix 2 Fig. 9). In *Arabidopsis*, H3K4me3 and H3K4me2 mark hyperactive memory genes during heat stress, mediate tolerance to salt and salt-stress priming responses (Shen *et al.*, 2014; Feng *et al.*, 2016; Lamke *et al.*, 2016). While H3K4me2 is usually associated with gene repression (Lämke & Bäurle, 2017; Liu *et al.*, 2019), H3K4me3 is deposited on active cold-regulated genes in potato exposed to cold, along with the repressive mark H3K27me3 (Zeng *et al.*, 2019). However, studies show that levels of H3K27me3 and H3K4me3 tend to be negatively correlated to one another, especially in transcriptional memories linked to vernalization in *Arabidopsis*, barley and *B. distachyon*, which is also supported by our results (Fig. 4.7, Appendix 2 Fig. 11; Finnegan *et al.*, 2005; Oliver *et al.*, 2009; Zhang *et al.*, 2009; Huan *et al.*, 2018). It is intriguing that the establishment of transcriptional memories during a second episode of diurnal-freezing was almost identical to what occurred during the first episode but with contrasting chromatin states (Fig. 4.7). Extended recovery from diurnal-freezing hence induced the onset of different chromatin marks, leading to a slight decrease

in global DNA methylation upon re-exposure rather than a hypermethylation as observed during the first priming episode (Fig. 4.7c). Because cold acclimation extends to morphology in *B. distachyon*, and because vernalization occurs simultaneously, the chromatin responses observed during diurnal-freezing can relate to stress memories, as well as development (e.g. linked to vernalization, and growth stages). These results demonstrate complex links between chromatin marks, stress and development responses in *B. distachyon*.

4.5.4 Conclusion

This study is the first to report that reversible transcriptional memories mediate the progressive return of plant growth following initial stress responses. By regulating transcriptional habituation, transcriptional memories provide plasticity to *B. distachyon*'s stress responses to grow a freezing tolerant morphology during cold acclimation. Hence, in addition to regulating *VRN1*, chromatin-associated transcriptional memories are involved in regulating both stress and developmental responses behind cold-climate adaptation in *B. distachyon*.

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4.7 Author contribution

BFM and JBC designed the research, BFM performed the experiments and analyses, BFM and JBC wrote the manuscript.

4.8 References

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4.9 Supporting information

Appendix 2 Figure 1: Whole-plant freeze tests performed on plants exposed to 1 and 4 cycles of diurnal-freezing compared to non-acclimated plants

Appendix 2 Figure 2: Significantly enriched GO terms in the 17 categories regrouped into 6 expression profiles identified in diurnal-freezing responsive genes

Appendix 2 Figure 3: Transcript levels of genes whose expression change in diurnal-freezing (S4/S1) that are associated with significantly enriched GO terms

Appendix 2 Figure 4: Distribution and differential expression of diurnal-freezing responsive genes in abiotic stress response modules

Appendix 2 Figure 5: RT-qPCR validation of RNA-seq analysis of plants exposed to diurnal-freezing

Appendix 2 Figure 6: Families of transcription factors in the 6 expression profiles identified in diurnal-freezing

Appendix 2 Figure 7: RT-qPCR validation of RNA-seq analysis of primed plants exposed to chilling

Appendix 2 Figure 8: B. distachyon gene modules identified in abiotic stress response and their distribution in in chilling-responsive genes

Appendix 2 Figure 9: Chromatin marks at the loci of genes involved in cold acclimation in response to repeated priming in diurnal-freezing

Appendix 2 Figure 10: Transcript levels of CBF1-3 at stress S and COR410/413 at recovery R are positively correlated

Appendix 2 Figure 11: Correlation R2adj between epigenetic marks at COR gene loci

Appendix 2 Table 1: ChIP-qPCR signals and statistical difference

Appendix 2 Table 2: Gene ontology analysis of chilling-responsive genes

Appendix 2 Table 3: Chilling-responsive genes common to naïve and primed responses that show transcriptional memory and their categorization as diurnal-freezing responsive genes.

Appendix 2 Table 4: Annotated chilling-responsive memory genes common to naïve and primed responses

Appendix 2 Table 5: Primers used in this study

Venn diagram groups							
	A	B	C	D	E	F	G
S1/S0	FC > 2	FC > 2		FC > 2	FC > 2		
S4/S0				FC > 2	FC > 2	FC > 2	FC > 2
S1/S4		FC > 2	FC > 2		FC > 2	FC > 2	

Trend relative to control			Transient		Oscillating	Stable	Complex	Late responsive		Total	%	Group	Transcriptonal profiles	
S0	S4	S4-S0	A	B*	C	D	E*	F	G					
UP 2851 (42%)	UP 2063 (31%)	+					72 (1%)			72	(1%)	U2B	complex-divergent	72
		=				1600 (24%)				1600	(23%)	U1	stable	1600
		-					391 (6%)			391	(5%)	U2A	complex-convergent	391
	=	-	444 (7%)	314 (5%)						758	(11%)	T1-T3	transient	444-314
	DOWN	-					30 (0.4%)			30	(0.4%)	O2	offset	30
= 1092 (16%)	UP	+						69 (1%)	461 (7%)	530	(7%)	L1-L3	late response	69-461
	=	+			16 (0.2%)					16	(0.2%)	O1A	oscillating	16
		-			61 (0.9%)					61	(0.9%)	O1B	oscillating	61
	DOWN	-						174 (2%)	311 (5%)	485	(7%)	L2-L4	late response	174-311
DOWN 2782 (41%)	=	+	462 (7%)	124 (2%)						586	(8%)	T2-T4	transient	462-124
	DOWN (33%)	+					163 (2%)			163	(2%)	D2B	complex-convergent	163
		=				1843 (27%)				1843	(27%)	D1	stable	1843
		-					190 (3%)			190	(2%)	D2A	complex-divergent	190
	Total		906 (13%)	438 (7%)	77 (1%)	3443 (51%)	846 (13%)	243 (4%)	772 (11%)	6725				

* groups that acquire strong transcriptional memories under DF

Table 4.1: Distribution of diurnal-freezing responsive genes according to fold change value at S1 and S4 responses and to the change in expression between S1 and S4.

		S1	S4
Responsive		**5633 (84%)**	**5304 (79%)**
	Up	2851	2756
	Down	2782	2548
	Shared	4289 (76%)	4289 (81%)
	Similar levels	*3443 (61%)*	*(65%)*
	Different levels	*846 (15%)*	*(16%)*
	Unique	1344 (24%)	1015 (19%)
Unresponsive		**1092 (16%)**	**1421 (21%)**
Total		**6725**	

Table 4.2: Distribution of common and unique genes between S1 and S4 responses.

	GO term	ontol.	DFRG				S1				S4			
			DE	Tot	p.adj.or	p.adj.ur	DE	Tot	p.adj.or	p.adj.ur	DE	Tot	p.adj.or	p.adj.ur
S1&S4	transcription factor activity, sequence-specific DNA binding	MF	164	364	8.01E-08	1	81	364	2.99E-11	1	61	364	4.26E-06	1
S1&S4	regulation of transcription, DNA-templated	BP	284	707	8.01E-08	1	109	707	1.17E-06	1	89	707	7.43E-05	1
S1&S4	oxidoreductase activity, acting on paired donors...*	MF	71	150	0.00095	1	31	150	0.00064	1	25	150	0.01005	1
S1&S4	heme binding	MF	108	249	0.00046	1	43	249	0.00419	1	37	249	0.01005	1
S1	iron ion binding	MF	76	180	0.02655	1	33	180	0.0041	1	26	180	0.06271	1
S1	response to auxin	BP	22	34	0.00521	1	12	34	0.03424	1	10	34	0.13828	1
S1	photosynthesis, light harvesting	BP	15	18	0.00095	1	12	18	5.85E-06	1	7	18	0.06271	1
S1	sequence-specific DNA binding	MF	103	215	2.86E-06	1	41	215	0.00127	1	30	215	0.11307	1
DFRG	calmodulin binding	MF	18	26	0.00488	1	3	26	1	1	2	26	1	1
DFRG	response to stress	BP	35	67	0.01228	1	15	67	0.12457	1	13	67	0.17717	1
DFRG	oxidation-reduction process	BP	291	812	0.00654	1	94	812	0.23589	1	81	812	0.18156	1
S1	carboxylic acid metabolic process	BP	13	25	0.72934	1	9	25	0.02881	1	7	25	0.17717	1
S4	trehalose biosynthetic process	BP	12	17	0.05087	1	4	17	1	1	8	17	0.00071	1
S1&S4	structural constituent of ribosome	MF	49	247	1	0.0299	0	247	1	0	1	247	1	0
S1&S4	GTP binding	MF	24	155	1	0.0082	2	155	1	0.0179	0	155	1	0.00177
S1&S4	ribosome	CC	47	239	1	0.0299	0	239	1	0	1	239	1	2.52E-07
S1&S4	translation	BP	51	256	1	0.0299	0	256	1	0	1	256	1	0

*....with incorporation or reduction of molecular oxygen

p.adj.or adjusted p-vlaue for over-representation

p.adj.ur adjusted p-vlaue for under-representation

Table 4.3: Gene ontology enrichment analysis in diurnal-freezing responsive genes (DFRG) and their distribution in S1 and S4 responses using GOseq.

Number of transcription factors and regulators

DFRG profile		%	Category		%	S1	%	S4	%	Representative	General function	Reference
Transient	**128**	10	T1	**41**	9	**128**	26	**0**		C2H2	stress response	Wang et al. 2019 DOI:10.1111/ppl.12728
			T2	**41**	9					E2F-DP	cell cycle, cell growth	Ramirez-Parra et al. 2007 DOI:10.1002/9780470988923.ch6
			T3	**36**	11					GeBP	cytokinin signalling	Chevalier et al. 2008 DOI: 10.1104/pp.107.110270
			T4	**10**	8					HMG	stress response, growth	Kwak et al. 2007 DOI:10.1093/pcp/pcl057
										IWS1	brassinosteroid-responsive	Belkhadir et al. 2015 DOI:10.1111/nph.13269
												Li et al. 2010 DOI:10.1073/pnas.0909198107
										MBF1	stress response, development	Jaimes-Miranda et al. 2020 DOI:10.1093/jxb/erz525
										Tify	stress response, development	Zhang et al. 2015 DOI:10.1007/s10265-015-0755-2
Stable	**278**	8	U1	**126**	8	**361**	73	**361**	83			
			D1	**152**	8							
Complex-convergent	**61**	11	D2B	**16**	10					AP2/ERF	stress response, hormone and development	Xie et al. 2019 DOI:10.3389/fpls.2019.00228
			U2A	**45**	12					LDB	plant growth and development	Mingzhu et al. 2012 DOI:10.1038/cr.2012.63
										WRKY	stress response	
Complex-divergent	**22**	8	D2A	**16**	8							
			U2B	**6**	8							
Late response	**64**	6	L1	**13**	19	**0**		**64**	15	Alfin-like	chromatin modifier	Sanchez and Zhou 2012 DOI:10.1016/j.tibs.2011.03.005
			L2	**13**	7					BES1	plant growth from multiple signals	Li et al. 2018 DOI:10.1016/j.bbagrm.2018.04.003
			L3	**18**	4					PHD	chromatin modifier	Sanchez and Zhou 2012 DOI:10.1016/j.tibs.2011.03.005
			L4	**20**	6					SNF2	chromatin remodelling, may modulate stress-growth	Mlynárová et al. 2007 DOI:10.1111/j.1365-313X.2007.03185.x
Offset/Oscillating	**7**	7	O2	**2**	7	**7**	1	**7**	2			
			O1A	**3**	19							
			O1B	**4**	7							
Total DFRG	**542**	8										

Table 4.4: Distribution of transcription factors 6 expression profiles of diurnal-freezing responsive genes

		N&P same response (FC<2)	N&P different response (FC>2)	Unique to N - non-responsive in P	Response in P only	**Total**	%
DF-responsive profiles	Transient	76	7	127	42	**252**	15
	Complex-convergent	107	16	129	14	**266**	15
	Complex-divergent	9	3	44	11	**67**	4
	Stable	131	17	321	111	**580**	34
	Late response	17	1	39	27	**84**	5
	Offset/Oscillating	7	0	18	6	**32**	2
	Chilling-specific	64	6	264	102	**436**	25
	Total	**412**	**50**	**942**	**313**	**1717**	100
	% Total	24	3	55	18		
	% Naive	29	4	67	-		
	% Primed	53	6	-	40		
DF-responsive	Memory (M)	174 (50%)	27 (59%)	249 (37%)	44 (21%)	**494**	30
	No memory (NM)	173 (50%)	18 (41%)	429 (63%)	167 (79%)	**787**	50
	ratio M/NM	**1**	**1.5**	**0.6**	**0.3**	**0.6**	
	TF/TR	**72**	**6**	**96**	**33**		
In response to CC		**Stable**	**Memory, complex**	**Memory, silenced**	**Acquired response**		
Genes showing memory overall		174	50	942	44	**1210**	70

Table 4.5: Transcriptional behavior of chilling-responsive genes that fit into diurnal-freezing expression profiles and that show transcriptional memory

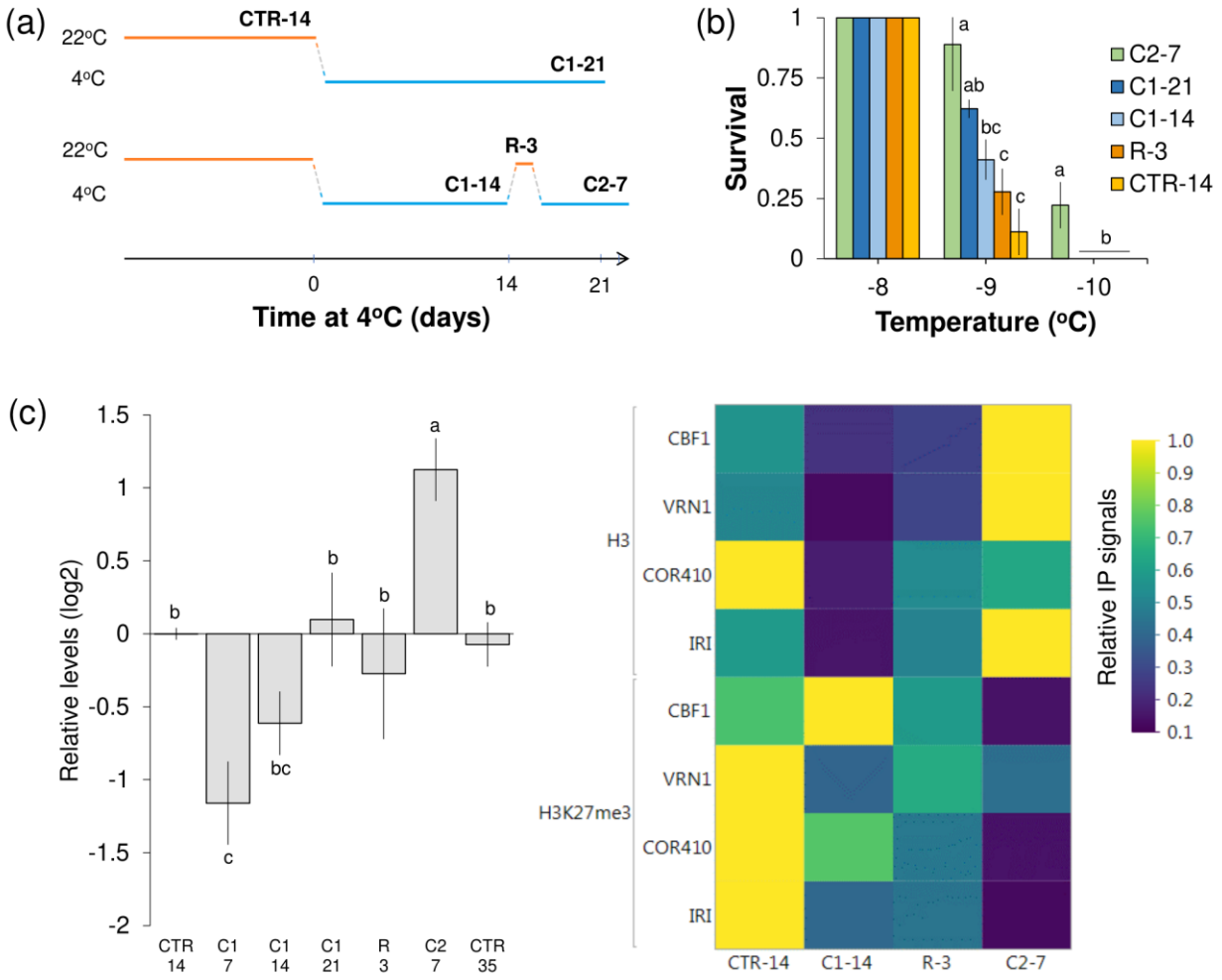

Figure 4.1: Repeated exposure to chilling leads to higher freezing tolerance, and to global and gene-specific epigenetic changes.

(**a**) Experimental design where one period of recovery at 22ºC was inserted during cold acclimation under constant-chilling (CC) at 4ºC; CTR-14: plants grown under control conditions at 22ºC; C1: first exposure to chilling for 14 or 21 days (C1-14 and C1-21); R-3: plants recovered from chilling for 3 days; C2-7: plants re-exposed to chilling for 7 days after recovery R-3. (**b**) Survival to freezing of CTR-14, C1-14, C1-21, R-3, and C2-7 plants measured by whole-plant freeze tests. (**c**) Relative global DNA-methylation; CTR-14, CTR-35: plants grown under control conditions at 22ºC for 14 and 35 days; C1: first exposure to chilling for 7, 14 or 21 days (C1-7, C1-14, C1-21); R-3: recovery from chilling at 22ºC for 3 days; C2-7: second exposure to chilling for 7 days. (**d**) Levels of histone 3 (H3) relative to input levels and of H3K27me3 relative to H3 of cold-regulated genes *C-REPEAT BINDING FACTOR1* (*CBF1*), *VERNALIZATION1* (*VRN1*),

COLD-REGULATED410 (*COR410*) and *ICE RECRYSTALLIZATION INHIBITOR* (*IRI*) at CTR-14, C1-14, R-3 and C2-7. *Different letters indicate statistical difference; $p<0.05$; error bars represent standard deviation between three biological replicates.*

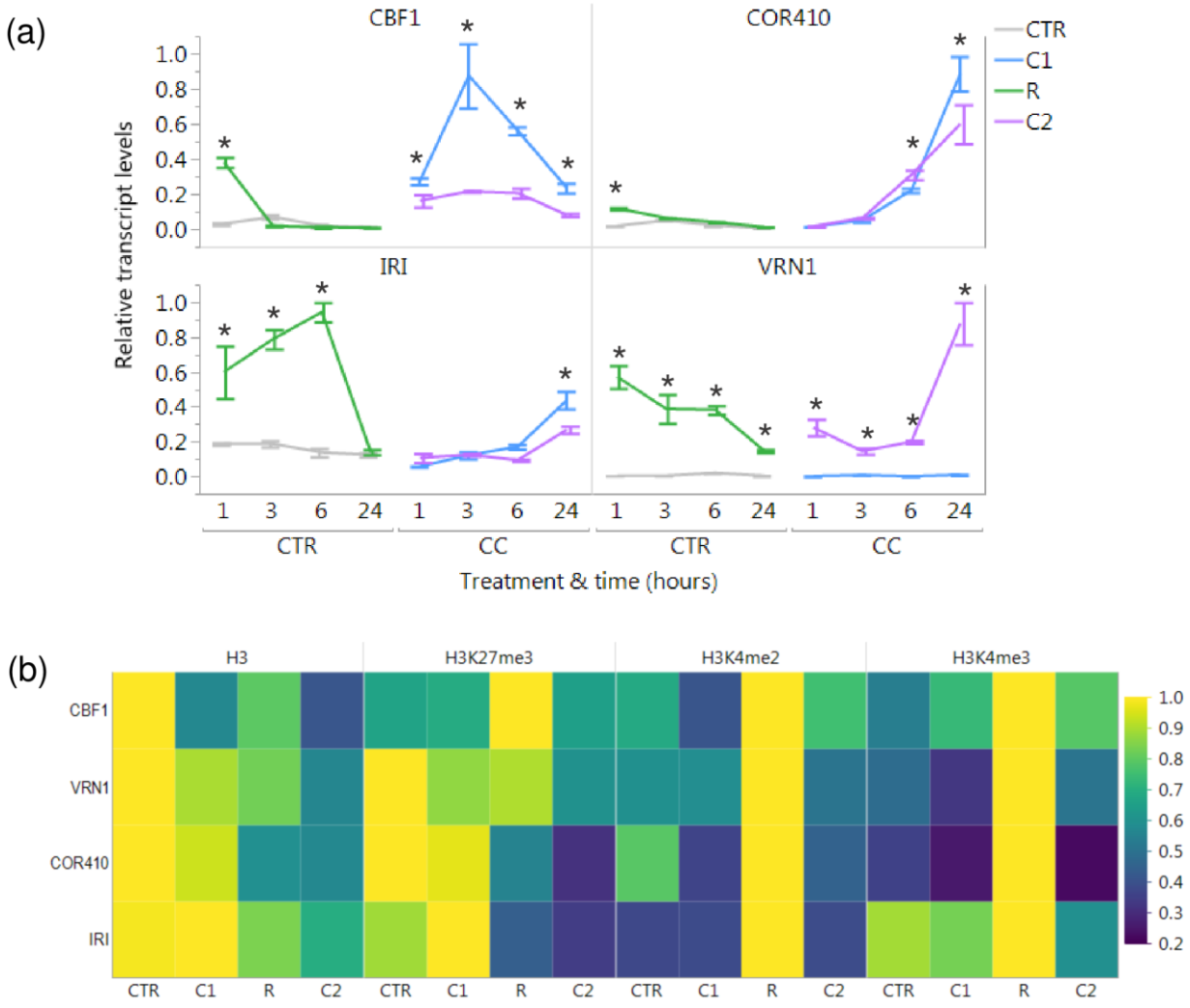

Figure 4.2: Repeated chilling influences transcriptional and chromatin responses to cold.
(**a**) Transcript levels of *COR* genes *CBF1, VRN1*, *COR410* and *IRI* at the first 1, 3, 6 and 24 hours of exposure to a first cold treatment (C1), during recovery from chilling (R) and to a second cold treatment (C2) relative to CTR, C1-14 and R-3. (**b**) Levels of histone H3 relative to input levels and of H3K27me3, H3K4me2 and H3K4me3 relative to H3 on *CBF1, VRN1*, *COR410* and *IRI* at 3 hours into C1, R and C2 compared to control CTR. * *indicate statistical difference; p<0.05; error bars represent standard deviation between three biological replicates.*

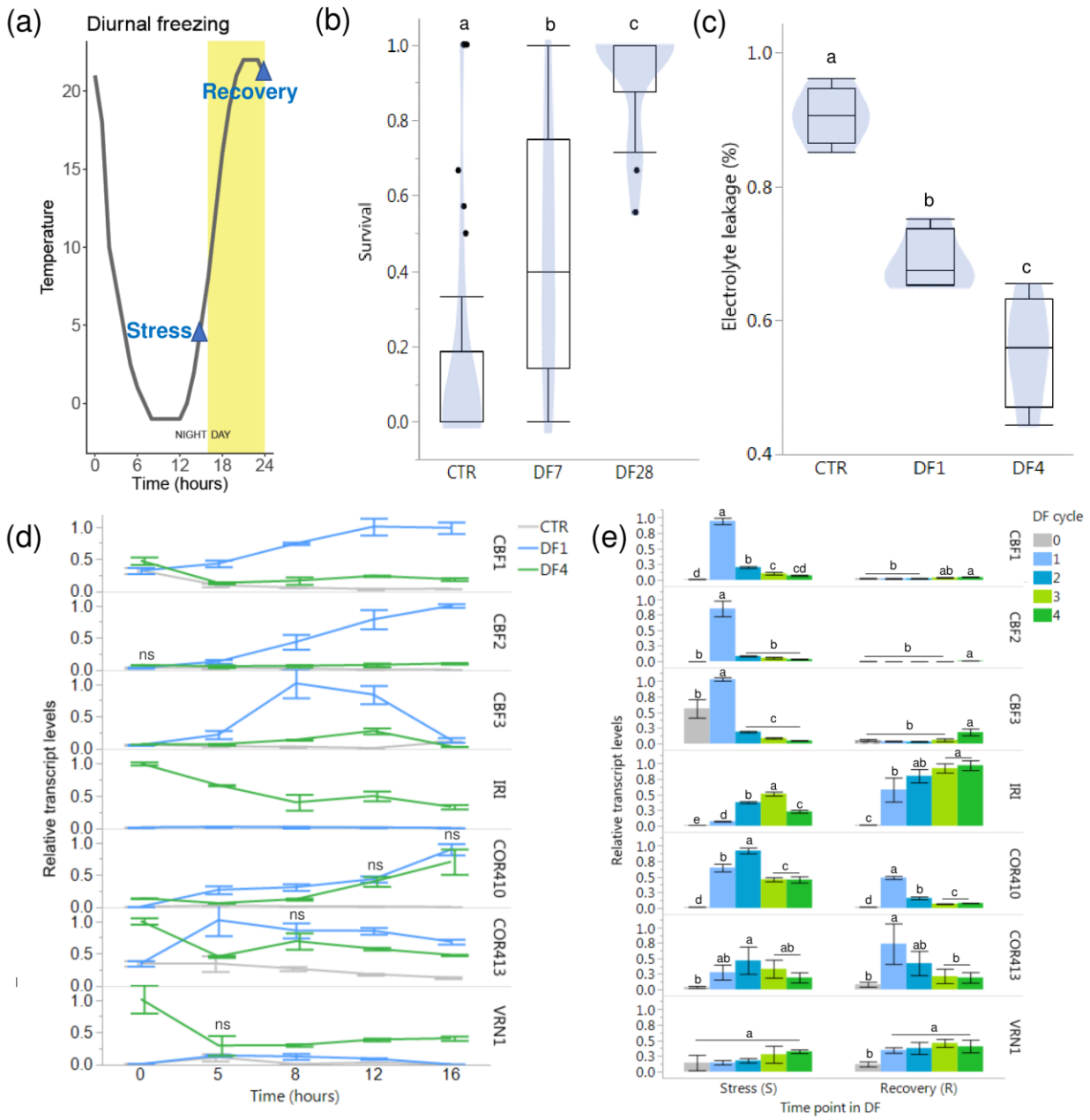

Figure 4.3: Cycles of diurnal freezing gradually increase freezing tolerance and change transcriptional responses to cold.

(**a**) One cycle of diurnal freezing, with time-points S (stress) and R (recovery). (**b**) Freezing tolerance of plants exposed to 7 and 28 cycles of DF (DF7 and DF28) measured in whole-plant freeze tests performed three times independently on 27 plants per temperature plateau between -8°C and -12°C. (**c**) Damage induced by freezing of plants previously exposed to 1 or 4 cycles of

DF (DF1 and DF4) measured by electrolyte leakage. (**d**) Relative transcript levels of *CBFs*, *IRI, COR410*, *COR413* and *VRN1* measured at 0, 5, 8, 12 and 16 of exposure to 1 or 4 cycles of DF (DF1 and DF4) relative to control (CTR). (**e**) Relative transcript level of *COR* genes in response to 1, 2, 3 and 4 cycles of DF (DF1-4) samples at S (S1-4) and R (R1-4) time-points. *Different letters indicate statistical difference; $p<0.05$*; *error bars represent standard deviation between three biological replicates.*

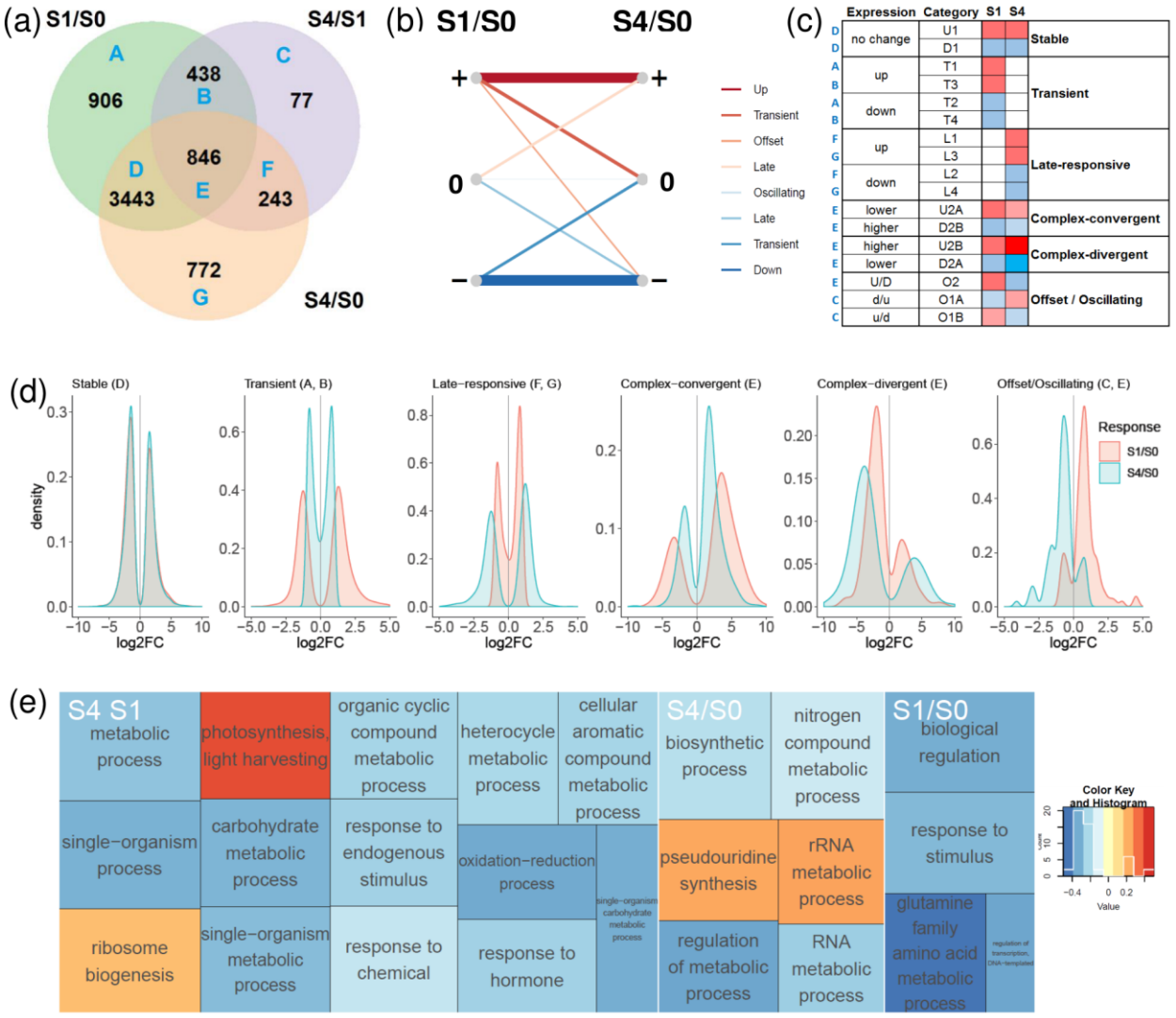

Figure 4.4: Transcriptional responses evolve through repeated cycles of diurnal-freezing following six profiles.

(**a**) Venn diagrams of significantly differentially-expressed genes in S1 (sampled during a first cycle of diurnal-freezing) compared to S0 (control conditions) labelled S1/S0, in S4 (sampled during a fourth cycle of diurnal-freezing) compared to S1 labelled S4/S1,and in S4 compared to S0 labelled S4/S0. (**b**) Sign of differential expression of genes responsive in diurnal-freezing in S1/S0 and S4/S0. + upregulated, 0 non-responsive, - downregulated. Lines are proportional to the number of genes following each trend. (**c**) Genes responsive to diurnal-freezing divided into 17 categories according to their distribution between S1/S0, S4/S1 and S4/S0 as illustrated in (a), and their up or downregulation in S1 and S4 responses (S1/S0 and S4/S0) as illustrated in (b). (**d**) Six

profiles that describe the transcriptional behavior of genes in diurnal-freezing. (**e**) Significantly over-represented GO terms that are unique to the S1 response (S1/S0) to the S4 response (S4/S0) or shared between both (S4 S1). Colours depict the transcriptional change of genes associated with each GO terms between S1 and S4 (S4/S1 - fold change negative to positive, blue to red).

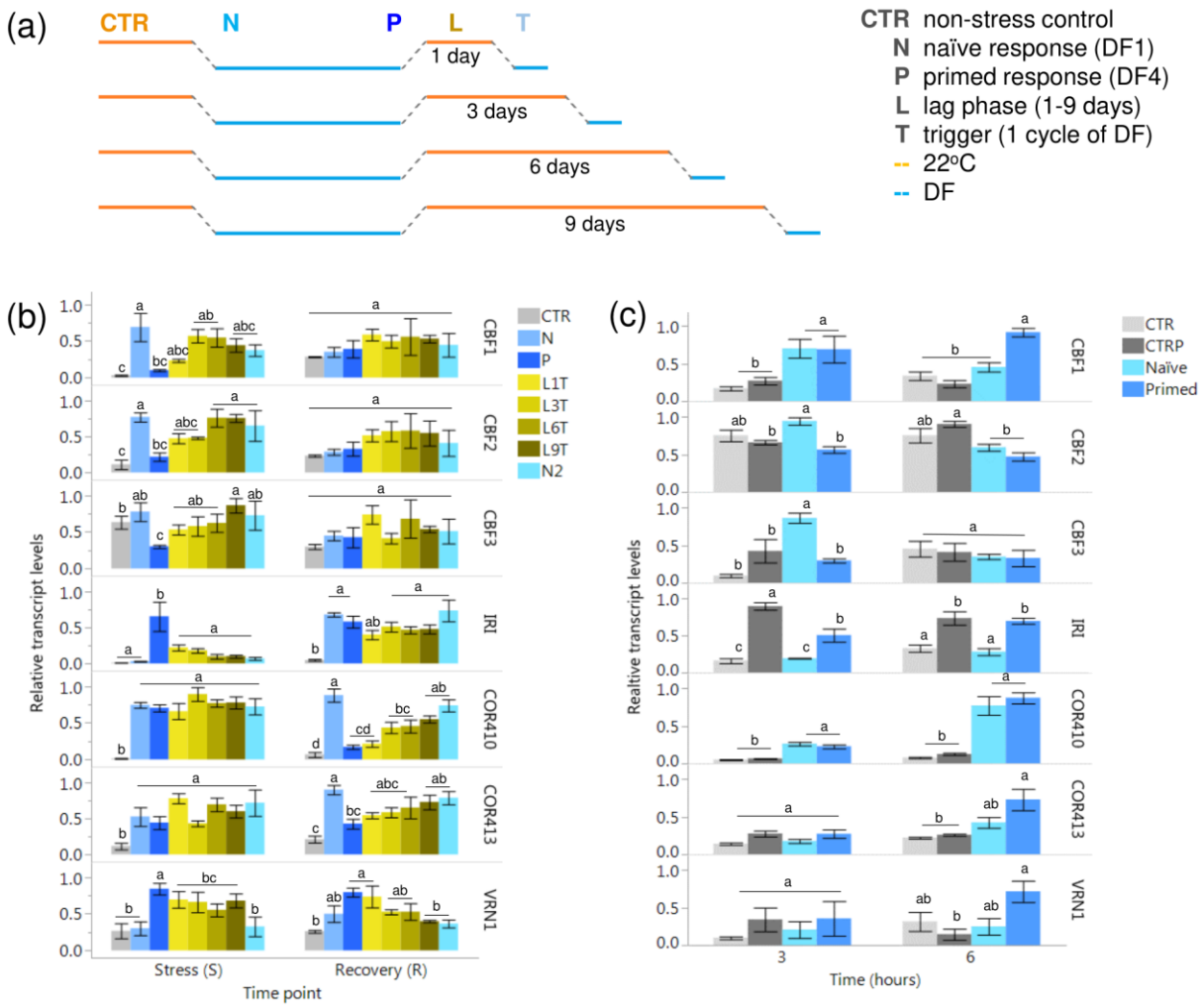

Figure 4.5: Priming induced by diurnal-freezing leads to reversible transcriptional memories on cold-responsive genes.

(a) Diagram of the experimental design, showing the naïve (N) and primed (P) responses, lag phase (L), and a trigger cycle (T). **(b)** Naïve (N), primed (P), trigger responses after 1, 3, 6 and 9 days of lag (L1-9T), and naïve age control (N2). **(c)** Transcriptional levels of cold-responsive genes in response to chilling in primed and naïve plants compared to primed control (CTRP) and naïve control (CTR). *Different letters indicate statistical difference; p<0.05; error bars represent standard deviation between three biological replicates.*

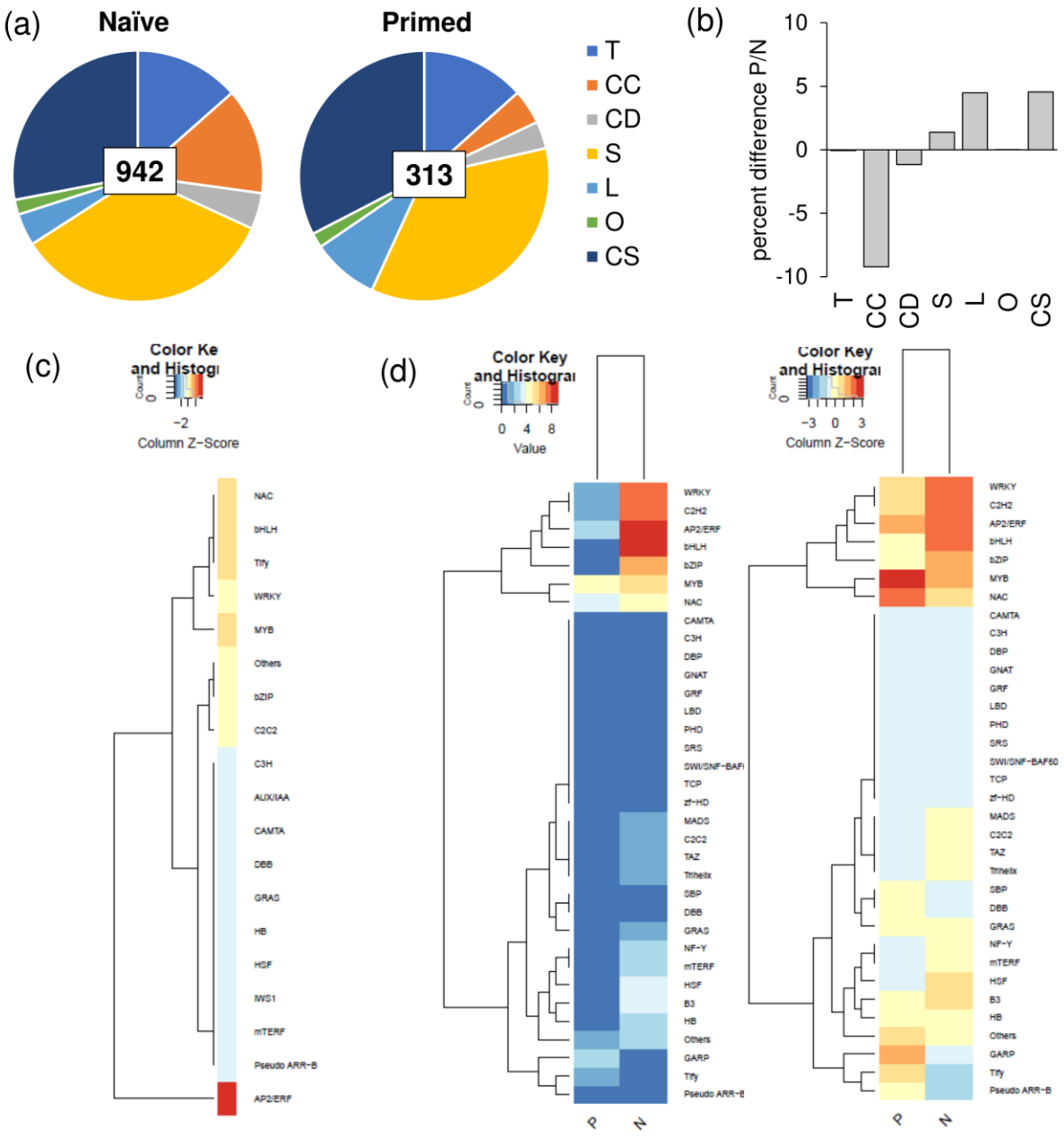

Figure 4.6: Primed plants acquired an attenuated response to chilling.

(a) Proportion of chilling-responsive genes unique to naïve (942 genes) or primed responses (313 genes) that belong to the expression profiles transient (T), complex-convergent (CC), complex-divergent (CD), stable (S), late-response (L) and offset/oscillating (O) or not found to be responsive in diurnal-freezing (chilling-specific; CS). **(b)** Difference between the distribution of genes in diurnal-freezing expression profiles and the chilling-specific category in naïve (N) or in primed response (P). **(c)** Transcription factors and transcriptional regulators that are responsive in

both N and P. **(d)** Number of transcription factors and transcriptional regulators found in genes unique to N and P in absolute value (left) and relative to the total number of genes in P and N (right).

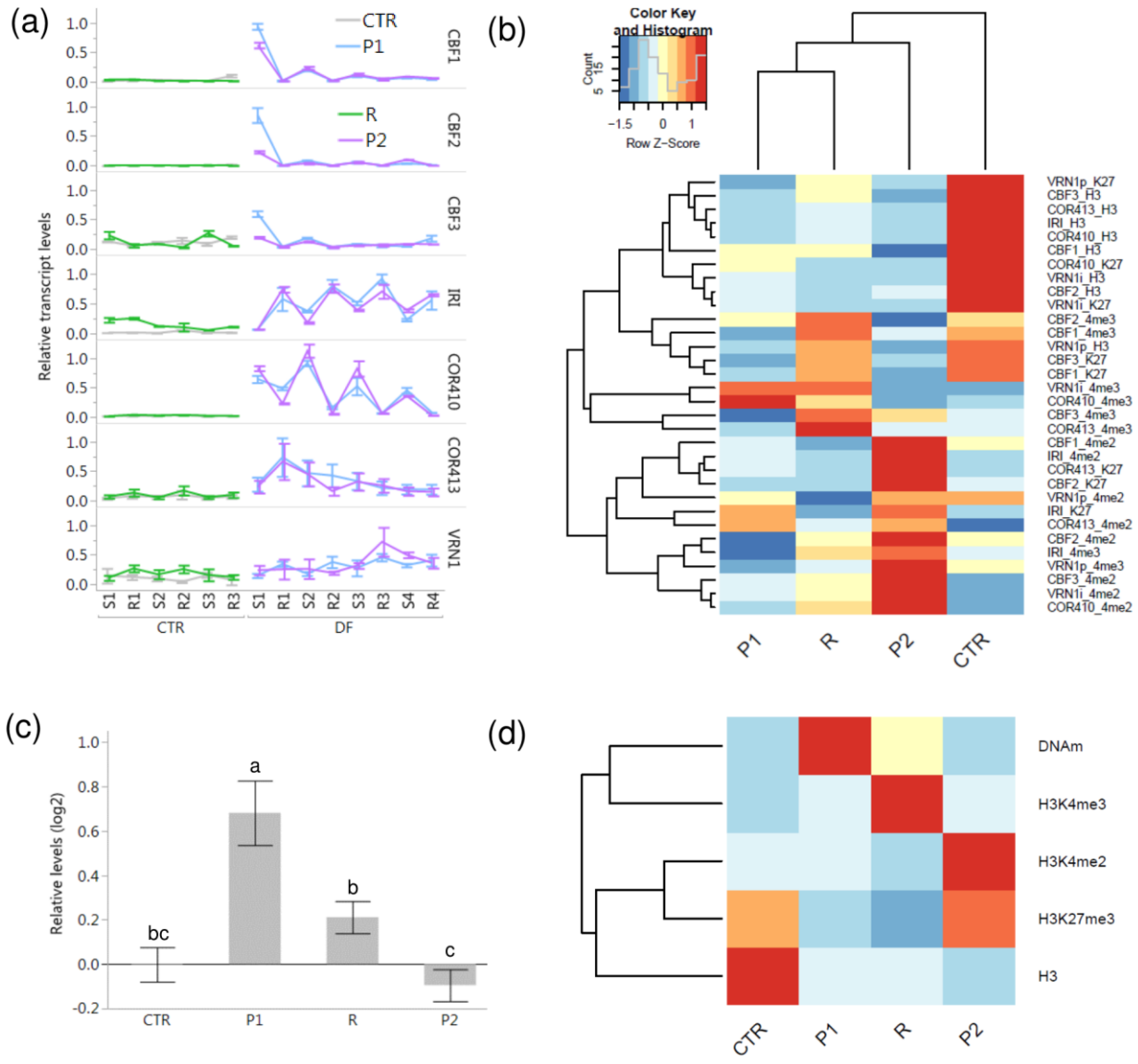

Figure 4.7: Repetitive priming in diurnal-freezing induced similar effects on transcription but distinct chromatin responses.

(a) Relative transcript levels during a first (P1) and second (P2) episode of priming under diurnal-freezing separated by a 3-day recovery from DF (R) in COR genes *CBFs*, *IRI*, *COR410-413* and *VRN1*. **(b)** Relative levels of epigenetic marks H3, H3K27me3 (k27), H3K4me2 (4me2) and H3K4me3 (4me3) in CTR, P1, R and P2. *Different letters indicate statistical difference; p<0.05.* **(c)** Relative global levels of DNA methylation in CTR, P1, R, and P2. **(d)** Summary of the epigenetic change on *COR* genes and global DNA methylation levels during repeated priming. *Error bars represent standard deviation between three biological replicates.*

4.10 Connecting text

Chapter 4 demonstrated that transcriptional memories are important mechanisms involved in the regulation of physiological, morphological and phenological phenotypic plasticity responses in *B. distachyon*. Hence, phenotypic plasticity is regulated by similar gene networks (*e.g.* through *VRN1*) and by similar mechanisms (*e.g.* transcriptional memory).

Hence, how can these responses evolve separately from one another? The previous studies show that the regulation of phenotypic responses can overlap. However, these responses should technically be able to evolve separately.

Chapter 5 investigates further the ways through which phenotypic plasticity can evolve, notably after hybridization and polyploidization. *B. hybridum*, the daughter hybrid species of *B. distachyon*, demonstrates the remarkable physiological and morphological plasticity that contribute to freezing tolerance observed in *B. distachyon*, but without the cold-mediated control of flowering time. The following study on *B. hybridum* demonstrates how the common regulation of physiological-morphological-phenological plasticity can be dissociated and provides an example of how this can contribute to species colonization into new territories.

5. DISSOCIATED FLOWERING AND COLD ACCLIMATION IN *B. HYBRIDUM* PROVIDE INSIGHTS INTO THE EVOLUTION OF TEMPERATE-CLIMATE ADAPTIVE PLASTICITY IN *BRACHYPODIUM*

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5.1 Abstract

Understanding how plants adapt to environmental change is increasingly critical for designing more resilient agricultural systems. Encompassing physiology, morphology and phenology, the phenotypic plasticity of temperate plants is a useful study system, in which cold acclimation leads to freezing tolerance and vernalization to flowering competence. Cold acclimation and vernalization are linked in the temperate grass *Brachypodium distachyon via VERNALIZATION1* and mechanisms of transcriptional memory. Finding *B. distachyon* accessions that undergo cold acclimation without simultaneous vernalization would facilitate the characterization of cold acclimation in temperate grasses but this, so far, has been elusive. Here, we report the isolation of a *B. hybridum* accession, an allotetraploid of *B. distachyon* and *B. stacei* parents, in which cold exposure confers high freezing tolerance but has no effect on flowering time. Results show that *B. hybridum* expresses the cold acclimation traits of *B. distachyon*, including the increase of *VERNALIZATION1* expression which has, however, no influence on *FLOWERING LOCUS-T* expression and flowering. Moreover, *B. hybridum* showed a high adaptability compared to *B. distachyon*, highlighting possible mechanisms for its radiation into new environments. Overall, this study reports a new model to study cold acclimation in cereals and provides insights on the evolution of phenotypic plasticity in plants.

5.2 Introduction

Given the current environmental crises, adopting regenerative and a more resilient agriculture is crucial. Unfortunately, the consequences of human activities will continue to affect the biosphere for many decades (Bathiany et al., 2018; Gray & Brady, 2016; USGCRP, 2017). Because the consequences of global warming have been difficult to predict, agriculture will benefit from plants that possess high plasticity and which are resilient to stress. Research focused on plant adaptation to environmental change highlight complex and yet largely unknown mechanisms that regulate stress response, development and phenology. Understanding how plants regulate their plasticity to adapt to their environment is hence critical for the agriculture of tomorrow.

The traits behind temperate-climate adaptation in plants represent a useful system to study how plants respond and adapt to their environment (Mayer et al., 2020; Mayer & Charron, 2020). Because temperate plants have had to adapt to stressful low temperature conditions and to seasonality, they have developed extensive phenotypic plasticity to continue functioning through

significant changes in atmospheric variables. Some annual plants avoid winter altogether by setting seeds before cold arrives and by germinating in spring, while others withstand freezing stress and can overwinter as vegetative structures (Körner, 2016). These plants have often acquired the ability to (i) change their physiology and morphology as temperature decreases to best survive upcoming freezing and (ii) adjust their phenology to delay flowering until the more clement conditions of spring (Chouard, 1960; Thomashow, 1999). These responses, respectively called cold acclimation (CA) and vernalization, are adaptive traits that ensure persistence in cold-rhythmed climates (Chouard, 1960; Thomashow, 1999).

The Mediterranean grass *Brachypodium distachyon* is a model plant related to main temperate cereals crops such as wheat and barley. Exposing the plant to cold induces a physiological and morphological cold acclimation response that leads to higher freezing tolerance (Colton-Gagnon et al., 2014; Mayer et al., 2020). Cold also shortens the growth time required to flower by inducing a vernalization response, which is often required for flowering in *B. distachyon* populations that grow in the coldest microclimates (Colton-Gagnon et al., 2014; Mayer et al., 2020; Ream et al., 2014). Flowering time and vernalization requirement are important characteristics that mark the distinction between populations of *B. distachyon*, more so than geography (Gordon et al., 2017; Lopez-Alvarez et al., 2015; Tyler et al., 2016). Recently, a link between cold acclimation and vernalization was discovered in the species, connecting physiology, plant morphology and development through the MADS-box transcription factor *VERNALIZATION1* (*VRN1*; Mayer et al., 2020). Another link, this time at the mechanistic level, unites cold acclimation and vernalization through transcriptional memories that regulate (i) the cold-induced activation of *VRN1* – which is central to the vernalization response – and (ii) the evolution of cold stress responses into a morphological response during cold acclimation (Mayer & Charron, 2020). Therefore, cold acclimation and vernalization are closely linked in *B. distachyon*, through both gene networks and regulatory mechanisms.

The interaction between the stress tolerance components and the phenological components of temperate-climate adaptative plasticity complicate the dissection of the mechanisms controlling winter survival and flowering. It was previously mentioned that a “true” spring accession in *B. distachyon*, analogous to wheat varieties sown in spring which show a reduced ability to cold acclimate, remains to be found (Colton-Gagnon et al., 2014). Indeed, all *B. distachyon* accessions studied so far show an almost identical ability to increase their freezing tolerance, and so,

independently of their vernalization requirement or geographical origin, unlike what is observed in wheat varieties (Colton-Gagnon et al., 2014; Ganeshan et al., 2008; Gordon et al., 2017; Mayer et al., 2020). This particularity of wheat is likely due to domestication and breeding and to fixed traits found in separate varieties, whereas the undomesticated *B. distachyon* shows more plasticity and variability, as the species grows in a range of environments as both winter and spring annual (Des Marais & Juenger, 2016; Mayer et al., 2020). Hence, finding a population of *B. distachyon* in which cold acclimation and flowering are independent has substantial research value, but has so far been elusive.

We isolated a line from *B. distachyon* accession PI639818 that flower readily without vernalization. Accession PI639818 was collected on the south of Crimea and is the source accession of winter habit *B. distachyon* line Bd29-1, which requires long vernalization times to flower (Colton-Gagnon et al., 2014; Ream et al., 2014). Seeds taken from PI639818 mostly yielded plants with strong vernalization requirement (*e.g.* Bd29-1) while a small percentage of seeds grew into rapidly flowering plants with or without cold treatment. This observation has prompted us to further investigate the origin and behavior of these outlier plants.

5.3 Results

5.3.1 Seed stock PI639818 yielded populations of *B. distachyon* and *B. hybridum*

Two contrasting plant populations were isolated from accession PI639818 seed stocks. Although both were clearly annual *Brachypodium*, these two groups showed contrasting morphologies and different vernalization requirements. Most plants (population 1or P1) required long vernalization to flower, while the smaller plant population flowered rapidly (population 2 or P2) and displayed morphological features similar to the polyploid *Brachypodium hybridum* rather than *B. distachyon*, according to an identification key (Catalán et al., 2016). *B. hybridum* is an allotetraploid resulting from a cross of diploid *B. distachyon* and *B. stacei* parents (Catalan et al., 2012). After one generation, the collected seeds of P2 were clearly larger than typical seeds of P1. Along with the identification key, larger seed size also indicated that P2 were likely polyploid Brachypodium (John P. Vogel et al., 2009). To confirm that these were seeds of *B. hybridum*, we performed whole genome sequencing on P1 and P2. Sequencing reads of P1 and P2 were aligned to the reference genomes of *B. distachyon*, *B. stacei* and *B. hybridum*. The highest number of P2 reads aligned on the *B. hybridum* reference genome (98.9%) compared to reference genomes of

B. distachyon (91.4%) and *B. stacei* (87.9%; Fig. 5.1A). Reads from P1 mapped to reference *B. hybridum* did not cover the chromosomes inherited from the *B. stacei* parent, indicating that the seeds of PI639818 contained seeds of *B. distachyon* (P1) and seeds of *B. hybridum* (P2; Fig. 5.1A). To determine the maternal parent of P2, reads were mapped to alleles of the plastome gene *rsp19*, which is present in a different version in *B. distachyon* and *B. stacei* (Sancho et al., 2018). Reads of P2 mapped to the *rsp19* allele of Bd21, suggesting that P2 descends from *B. distachyon* as the maternal parent (Fig. 5.1B).

5.3.2 Cold-response genes in *B. hybridum*

To determine whether *B. hybridum* also possesses the genes involved in cold acclimation as in *B. distachyon*, we searched for the homologs of *B. distachyon* cold-stress genes in *B. hybridum* using BLAST, including *C-REPEAT BINDING FACTOR 1*, *2* and *3* (*CBF1*, *CBF2*, *CBF3*), *ICE-RECRYSTALLIZATION INHIBITOR* (*IRI*), *COLD-REGULATED 410* and *413* (*COR410*, *COR413*), and *VERNALIZATION1* (*VRN1*) (Bredow et al., 2016; Colton-Gagnon et al., 2014; Mayer et al., 2020; Mayer & Charron, 2020; Ryu et al., 2014). Top hits in the *distachyon* and *stacei* subgenomes of *B. hybridum* (that respectively contain D and S genes) were used to perform a phylogenetic analysis along with *B. distachyon* query sequences (Fig. 5.1C; Table 5.1). Unsurprisingly, all D genes segregated with *B. distachyon* genes and have a S homolog, except for *IRI* that seemed at least by sequence unique to *B. distachyon* and D. These analyses revealed that Bd*IRI*s have no similar counterparts in the *B. stacei* subgenome of *B. hybridum*, suggesting that *B. stacei* was less adapted to cold than *B. distachyon* (Fig. 5.1D).

VRN1 alleles tend to be different in three main branches of *B. distachyon* populations (Gordon et al., 2017). We thus performed another sequence phylogenetic analysis as an attempt to identify the original population of the maternal *B. distachyon* parent of P2. Alleles from accessions that belong to the *B. distachyon* branches extremely delayed flowering (Bd29-1, Tek-2, BdTR7A), predominantly Spanish (ABR6, Bd30-1, ABR4) and predominantly Turkish (Bd21-3, Bd21, Bd18-1) were analyzed with BhD*VRN1* consensus sequence from P2 (Gordon et al., 2017). Results showed that P2*VRN1* tends to diverge early from the rest, segregating with extremely delayed flowering, like BdTR7A and Bd29-1, and predominantly Turkish Bd18-1 (Fig. 5.1D). This suggests that P2 may have originated from a maternal *B. distachyon* part of the extremely delayed

flowering/predominantly Turkish cluster in the eastern part of the Mediterranean region, like its Bd29-1 congener. P2 from this point on will be referred to as *B. hybridum*.

5.3.3 *B. hybridum* is less stressed by chilling compared to *B distachyon*

To determine the plasticity of *B. hybridum* in cold conditions, we investigated its response to chilling and diurnal-freezing, which induce cold acclimation in *B. distachyon* (Colton-Gagnon et al., 2014; Mayer et al., 2020). As controls, we measured morphological differences between *B. hybridum* and *B. distachyon* Bd21-3 grown in control conditions. Under long days at 22ºC, *B. hybridum* plants were more vigorous, grew taller structure with more tillers compared to *B. distachyon* (Fig. 5.2A). Under short-day conditions at 22ºC, *B. hybridum* plants grew higher stems and deeper roots for similar aboveground biomass compared to *B. distachyon* (Fig. 5.2B). It was previously shown that long-term chilling induces physiological stress in *B. distachyon*, which likely limits plant growth, as opposed to diurnal-freezing which allows *B. distachyon* to grow similar biomass as control plants (Mayer et al., 2020). Compared to *B. distachyon*, *B. hybridum* exposed to chilling grew taller structures and continued to grow vigorously, reaching the flowering stage after approximately 250 days (but did not produce seeds), suggesting that *B. hybridum* is less prone to chilling stress and chilling-induced growth inhibition than *B. distachyon* (Fig. 5.2C-D). Conversely, *B. hybridum* and *B. distachyon* grew very similar structures in response to diurnal-freezing (Fig. 5.2C-D). When transferred to flowering conditions, chilling-treated *B. distachyon* did not grow new shoots, while chilling-treated *B. hybridum* gained numbers of shoots comparable to both *B. distachyon* and *B. hybridum* exposed to diurnal-freezing (Fig. 5.2D). In addition, chlorophyll content did not decrease in chilling-exposed *B. hybridum*, and proline levels increased more moderately in *B. hybridum* compared to chilling-exposed *B. distachyon*, supporting that *B. hybridum* was less stressed by chilling than *B. distachyon* (Fig. 5.2E; Mayer et al., 2020). Hence, these results show that *B. hybridum* grew more vigorously under cold, was less stressed by chilling compared to *B. distachyon* and showed the same response ad *B. distachyon* in diurnal-freezing.

5.3.4 Flowering in *B. hybridum* is not affected by cold exposure

To determine the effect of cold on flowering in *B. hybridum*, we measured the number of days to heading in *B. distachyon* and *B. hybridum* treated for 7-56 days in chilling and diurnal-

freezing, then transferred to flowering conditions. Chilling and diurnal-freezing accelerated flowering in treated *B. distachyon* plants but had, surprisingly, no effect on *B. hybridum* (Fig. 5.3A-B). Regardless of the time spent in cold, *B. hybridum* flowered after around 45 days outside cold conditions (Fig. 5.3A-B). Interestingly, *B. distachyon* and *B. hybridum* accumulated similar levels of *VRN1* transcripts, whose activation is characteristic of vernalization in *B. distachyon* in response to both chilling and diurnal-freezing (Fig. 5.3C). Moreover, levels of *FLOWERING LOCUS-T* (*FT*) transcripts, a gene that induces flowering in *B. distachyon*, were similar in *B. hybridum* exposed to control, chilling and diurnal-freezing conditions. Conversely, *FT* levels in *B. distachyon* increased greatly in response to chilling, and moderately in response to diurnal-freezing (Fig. 5.3C). Hence, flowering and *FT* expression were unaffected by cold treatments in *B. hybridum*, even though *VRN1* expression increased as observed in *B. distachyon*. Overall, the pathway connecting the expression of *VRN1* and *FT* and flowering time in *B. distachyon* appear absent in *B. hybridum*, while the induction of *VRN1* expression by cold exposure is maintained. This observation indicates that the developmental responses of *B. distachyon* and *B. hybridum* to cold exposure are fundamentally different.

5.3.5 *B. hybridum* cold acclimated like *B. distachyon* but tended to tolerate freezing better

To investigate the cold acclimation capacity of *B. hybridum*, we performed whole-plant freeze test to measure the survival of *B. distachyon* and *B. hybridum* non-acclimated controls (CTR), cold-acclimated in diurnal-freezing for 7 and 28 days (DF7 and DF28) and cold acclimated in chilling for 28 days (CC28). Although *B. distachyon* and *B. hybridum* showed similar survival curves to freezing, CTR, DF7 and CC28 *B. hybridum* survived to higher numbers than *B. distachyon* when exposed to temperatures above ~ -10ºC (Fig. 5.4A). Interestingly, DF28 *B. distachyon* and *B. hybridum* displayed identical survival curves when exposed to freezing (Fig. 5.4A). As suggested by our previous results, *B. distachyon* and *B. hybridum* respond similarly to diurnal-freezing, although *B. hybridum* showed higher non-acclimated freezing tolerance than *B. distachyon* in whole-plant freeze test and electrolyte leakage assays (Fig. 5.4A-B). Interestingly, *B. hybridum* which was cold acclimated in chilling survived better to freezing than *B. distachyon*, supporting that *B. hybridum* was less stressed when exposed to chilling conditions. Nevertheless, cold acclimation in chilling led to limited freezing tolerance compared

to cold acclimation in diurnal-freezing in both *B. hybridum* and *B. distachyon*, suggesting that diurnal-freezing induces a conserved response in *B. hybridum* and *B. distachyon* (Mayer et al., 2020).

Over time, diurnal-freezing alters the response of cold-stress genes by inducing the establishment of transcriptional memories in *B. distachyon* (Mayer & Charron, 2020). Hence, we investigated the transcript levels of *B. hybridum* genes *CBF1*, *CBF2*, *CBF3*, *IRI*, *COR410* and *COR413* at 0, 5, 8, 12 and 16 hours at the first and fourth diurnal-freezing cycle (DF1 and DF4; Fig. 5.4C-D). All genes responded to diurnal-freezing and showed different transcript profiles at DF4 compared to DF1, indicating that they were also regulated by transcriptional memory in diurnal-freezing, as observed in *B. distachyon* (Mayer & Charron, 2020). A similar peak of transcripts for *CBF3*, along with earlier peaks for *CBF1*, *CBF2*, and *COR413* were observed, which may indicate a different regulation of cold-stress genes in *B. hybridum* compared to *B. distachyon* in diurnal-freezing (Fig. 5.4D). We further measured the transcript levels of COR genes at stress and recovery time-points in DF1, DF2, DF3 and DF4 (Fig. 5.4C). COR gene transcript levels oscillated between stress and recovery, with *CBF* transcripts decreasing after an initial peak at S1, *IRI* transcripts increasing over cycles of diurnal-freezing while *COR410* and *COR413* transcript remaining relatively stable (Fig. 5.4E). Hence, *B. hybridum* cold-stress genes were also regulated by transcriptional memory mechanisms during cold acclimation in diurnal-freezing.

5.3.6 DF-primed *B. hybridum* show no subgenome bias

As previously shown in *B. distachyon*, diurnal-freezing primes for freezing stress by gradually increasing freezing tolerance and by inducing the establishment of transcriptional memories (Mayer & Charron, 2020). A similar scenario occurs in *B. hybridum* under diurnal-freezing, where the expression of cold-stress genes is altered by exposure to diurnal-freezing (Fig. 5.4A-E). To determine if diurnal-freezing also induces the establishment of transcriptional memories in *B. hybridum*, we compared the transcriptome of primed and naïve *B. hybridum* exposed to chilling. Primed plants were previously exposed to 4 cycles of diurnal-freezing and transferred to control conditions for 1 day, while naïve plants were never exposed to diurnal-freezing. We compared RNA-seq samples of primed and naïve *B. hybridum* exposed to chilling for 3 hours to their respective controls, and both controls to one another to identify recovery genes.

The naïve response was characterized by a total of 1970 differentially expressed genes, the primed response by 1305, and the recovery response by 354 (Fig. 5.5A). The distribution of genes among these responses was divided into seven categories (categories A-G; Fig. 5.5A). In each of these categories, we investigated whether there was any expression bias towards D or S subgenomes. Interestingly, D and S genes each accounted for around half of expressed genes in each Venn diagram category and were expressed to similar levels (Fig. 5.5A). Moreover, the number of genes found on each *B. hybridum* chromosome and their equivalent in *B. distachyon* and *B. stacei* did not indicate a bias either, and transcript counts in control and cold conditions also indicated similar expression of D and S genes (Appendix 3 Fig. 1). Hence, there appeared to be no bias towards a specific subgenome in response to priming in *B. hybridum*.

5.3.7 Primed *B. hybridum* respond with fewer genes than naïve plants through transcriptional memory

To better characterize the effect of priming on gene expression in *B. hybridum*, we identified the following groups of response genes: *core*, *naïve-specific*, *primed-specific* or *recovery-specific* (Fig. 5.5B). *Core* genes were responsive in both naïve and primed responses, at similar or different expression levels, and hence remained responsive through cycles of diurnal-freezing. *Naïve-specific* and *primed-specific* genes were only responsive in naïve and primed respectively, and thus were the cold-responsive genes most affected by diurnal-freezing-induced priming. *Recovery-specific* genes were differentially expressed in primed plants transferred to control conditions but were not part of the naïve and primed responses (Fig. 5.5B). Gene ontology (GO) analysis revealed that: (i) the 884 *core* genes have the most significantly enriched GO terms relating to biosynthesis, the regulation of biological processes and response to stimulus, (ii) the 1086 *naïve-specific* genes were enriched in terms relating to protein modification and the regulation of transcription and (iii) the 421 *primed-specific* genes were enriched in localization and transport (Fig. 5.5C). The 209 *recovery-specific* genes were not significantly enriched in specific GO terms. Therefore, primed *B. hybridum* did not respond with transcriptional and post-translational regulation, but rather with more targeted structural responses, and fewer genes than the naïve response.

Genes that showed a different transcriptional response in primed compared to naïve were altered by priming in diurnal-freezing. Hence, because previous exposure changed their

transcriptional response to cold, these are memory genes. Hence, around 32% of the *core* response genes were memory genes, showing different transcript levels in naïve and primed (Fig. 5.5D). Around 28% of *core* memory genes were downregulated in after priming, compared to only 1.3% which were upregulated (Fig. 5.5D). Hence, most *core* memory genes were downregulated by priming.

The activation of *naïve-specific* genes in response to cold was silenced by diurnal-freezing, as they were not part of the primed response. Hence, *naïve-specific* genes are also memory genes. Together, *naïve-specific* genes and *core* memory genes account for a total of ~70% of the naïve-response genes (Fig. 5.5E). Therefore, the expression of most genes that comprise the naïve response were changed by diurnal-freezing through mechanisms of transcriptional memory. Of the recovery response genes, 60% were *recovery-specific*, 26% were memory genes, and 14% became cold-responsive after priming (Fig. 5.5E). Overall, 32% of primed-response genes were acquired, meaning they were not responsive prior to priming in diurnal-freezing, along with 22% whose expression changed with priming (memory genes), compared to a 46% which were unchanged by priming (*core* no memory genes). Therefore, most of chilling-responsive genes acquired transcriptional memories in diurnal-freezing.

5.3.8 Priming shapes the transcriptional responses of *B. hybridum* and *B. distachyon* similarly

Using previously published data with an identical experimental design, we analyzed, as we did for *B. hybridum,* the effects of diurnal-freezing-priming on the transcriptomic response of *B. distachyon* (Fig. 5.5). As in *B. hybridum*, priming of *B. distachyon* led to genes behaving as *core* with and without memory, *naïve-specific* (memory) and *primed-specific* (acquired) when comparing the response of naïve and primed plants to chilling. Although the response of *B. hybridum* to chilling elicited the response of more genes than *B. distachyon*, the two species display almost identical proportions of *core (no memory)* and *primed-specific (acquired)* genes at respectively 24% and 18% for *B. distachyon*, and 25% and 18% for *B. hybridum* (Fig. 5.6A). The proportion of *naïve-specific* genes, whose response was dampened after priming, occupied 55% of cold-responsive genes in *B. distachyon* compared to 45% in *B. hybridum*, thus leaving only 3% for *core* memory genes in *B. distachyon* and 12% in *B. hybridum*. Hence, although the proportion of memory genes was similar in *B. distachyon* and *B. hybridum* (58% in *B. distachyon*, 57% in

B. hybridum), there was 4 times the proportion of *core* memory genes in *B. hybridum*. Hence, there were fewer genes silenced by priming in *B. hybridum* than in *B. distachyon* (Fig. 5.6A). Memory genes (*core memory* and *naïve-specific*) were associated to over 7 times more GO in *B. hybridum* than in *B. distachyon*, while *primed-specific* and *core no memory* genes were represented by fewer GO terms (Appendix 3 Table 1). This result indicates that there is a larger overlap between the naïve and the primed response in *B. hybridum* than in *B. distachyon*, which means that *B. hybridum* underwent a milder priming response that had less of an effect on the cold-responsive transcriptome.

BhD cold-stress genes (cold-stress genes of the D subgenome) tended to behave like those of *B. distachyon*, *i.e. CBF1* as *core no memory*, *CBF2* and *CBF3* as *core memory*, and *IRI* as *recovery-specific* (Table 5.2). BhD*COR410* was however *naïve-specific* rather than *core no memory* like Bd*COR410*. Moreover, other than BhS*CBF1* and BhS*CBF3*, the cold-regulated *B. hybridum* genes of the S subgenome behaved differently compared to those of the D subgenome. Specifically, neither BhS*IRI* nor BhS*COR410* responded to chilling, and BhS*CBF2* was a *primed-specific/acquired* gene rather than *core memory* (Table 5.2). Therefore, although cold-stress genes generally behaved similarly in *B. hybridum* and *B. distachyon*, those of *B. hybridum* from the S subgenome responded differently to priming. Overall, priming in diurnal-freezing resulted in similar transcriptional responses in *B. hybridum* and *B. distachyon*, including of cold-stress genes from the BhD subgenome, but priming induced a more specialized response in *B. distachyon* than in *B. hybridum*.

5.3.9 Early priming affects global DNA methylation and *VRN1* levels differently in *B. hybridum*

To investigate the global epigenetic response of *B. distachyon* and *B. hybridum* to priming, we measured the global levels of DNA methylation in plants exposed to 1 and 4 cycles of diurnal-freezing, measured at S1, R3 and S4 (Fig. 5.6B). When grown in control conditions, *B. hybridum* plants displayed around 50% higher DNA methylation levels than *B. distachyon*. However, as priming progresses in diurnal-freezing, the levels of DNA methylation follow opposite trends in the two species, increasing in *B. distachyon* and decreasing in *B. hybridum* (Fig. 5.6B). The change in DNA methylation levels was larger for *B. hybridum*, reaching levels similar to those of *B. distachyon* control plants.

VRN1 is active in *B. hybridum* without cold exposure. Although levels were similar in *B. hybridum* and *B. distachyon* in response to chilling and diurnal-freezing, *VRN1* transcripts also accumulated in *B. hybridum* grown in short day conditions at 22ºC, with levels already higher in 2-week control *B. hybridum* plants compared to *B. distachyon* (Fig. 5.6C-D). In early exposure to chilling and diurnal-freezing, *VRN1* transcripts in *B. distachyon* and *B. hybridum* oscillated between stress and recovery time-points of diurnal-freezing, peaking at recovery R in diurnal-freezing similarly to *IRI* and *COR413*, but reached higher levels in *B. hybridum* compared to *B. distachyon* (Fig. 5.6C, 5.3E). Interestingly, after approximately 180 days, *B. hybridum* flowered and eventually produced viable seeds under short-day conditions (Appendix 3 Fig. 2). Hence, while *B. distachyon* and *B. hybridum* responded similarly to diurnal-freezing, *VRN1* is active without cold in *B. hybridum* and the change in global DNA methylation follow opposite trends. These differences suggest that although the main response of *B. distachyon* to diurnal-freezing are maintained in *B. hybridum*, this species shows nonetheless more dynamic chromatin responses and *VRN1* expression.

5.3.10 *B. hybridum* shows a higher adaptability *B. distachyon*

To assess the global response of *B. hybridum* to chilling and diurnal-freezing compared to *B. distachyon*, we measured in addition to *VRN1* transcript levels, yield (total weight of seeds per plant), the number of final leaves, number of spikes, biomass (dry weight), and final height in *B. distachyon* and *B. hybridum* plants grown for 14 or 56 days in short-day at 22ºC (CTR and CTR56), or exposed to chilling or diurnal-freezing for 56 days. Measurements were taken after plants went through flowering and senescence under long-day conditions. Diurnal-freezing produces *B. distachyon* plants that grow equivalent biomass and yield to control plants, but with smaller height, more leaves, and more spikes (Fig. 5.6D). *B. hybridum* plants grown for 56 days in control conditions grow more biomass, heavier seeds, more leaves and more spikes than plants exposed to other treatments, suggesting that short-day conditions do not limit growth as much as in *B. distachyon* (Fig. 5.6D). Cold exposure influences final height in *B. distachyon*, while *B. hybridum* grows to similar heights when grown at 22ºC, in chilling or diurnal-freezing. Therefore, unlike in *B. distachyon*, cold exposure generally had a negative effect on growth yield in *B. hybridum* compared to short-day at 22ºC. However, *B. hybridum* grew taller structures, but

had equivalent biomass than *B. distachyon* across treatments. Hence, the growth and development of *B. hybridum* is generally less restricted by photoperiod and temperature than *B. distachyon*.

5.4 Discussion

5.4.1 Isolation of an annual Brachypodium *B. hybridum*

We isolated *B. hybridum* from *B. distachyon* accession PI639818 (Fig. 5.1A). The distinction between *B. distachyon*, *B. stacei* and *B. hybridum* is relatively recent, each species presenting a different cytotype. They were previously thought to be successively derived autopolyploids and were grouped into *Brachypodium distachyon* (Catalan et al., 2012). *B. distachyon* and *B. stacei* are actually both diploids (respectively x=5, $2n$=10 and x=10, $2n$=20), and *B. hybridum* an alloteraploid descending from a cross between *B. distachyon* and *B. stacei* (x=5+10, $2n$=30; Catalan et al., 2012).

As *B. hybridum* can arise from bi-directional crosses of *B. distachyon* and *B. stacei*, plastome analysis indicated that our *B. hybridum* isolate descended from a maternal *B. distachyon*, unlike most previously studied *B. hybridum* (Fig. 5.1B; López-Alvarez et al., 2012; Sancho et al., 2018). *VRN1* sequence analysis suggested that this *B. distachyon* parent is closer to the eastern Mediterranean cluster of *B. distachyon* populations (Fig. 1E; Gordon et al., 2017). However, the intraspecific variations of *B. distachyon*, which probably gave rise to different *VRN1* alleles have occurred during the last 0.5 million years, while *B. hybridum* likely originated around 1 million years ago (Gordon et al., 2017; Sancho et al., 2018), making the comparison of *VRN1* alleles in *B. hybridum* and *B. distachyon* of limited use. Nevertheless, given the polyphyletic nature of *B. hybridum*, hybridization between *B. stacei* and *B. distachyon* was recurrent (López-Alvarez et al., 2012; Lopez-Alvarez et al., 2015). There is to our knowledge no mention of *B. stacei* and *B. hybridum* from Crimea, suggesting that this *B. hybridum* originates from elsewhere around the eastern Mediterranean basin. More advanced genome analyses are however necessary to further elucidate its origins and to evaluate our findings, in addition to comparing this *B. hybridum* isolate to other accessions.

5.4.2 Cold does not affect flowering time in *B. hybridum*

B. hybridum plants flowered readily and remained unaffected by cold exposure (Fig. 3A-B). Cold accelerates flowering in all studied *B. distachyon* accessions so far through increased

VRN1 expression (Colton-Gagnon et al., 2014; Mayer et al., 2020; Ream et al., 2014). While accessions of *B. hybridum* and *B. stacei* flower without vernalization (John P. Vogel et al., 2009), studies that investigate their flowering system and their response to cold are lacking.

In *B. hybridum*, flowering time and the expression of the flowering-inducing gene *FT* were not influenced by cold exposure, unlike in *B. distachyon* (Fig. 3C). Indeed, in spring-like accessions of *B. distachyon* such as Bd21-3, *FT* tends to be constitutively expressed, is less responsive to cold and its expression is regulated by photoperiod, where short-days seem to inhibit *FT* expression (Bettgenhaeuser et al., 2017; Ream et al., 2014; Woods et al., 2014). Here, *B. hybridum* showed similar signs of relatively high *FT* expression in short-day with or without cold exposure, but when grown in short-days at 22ºC, *B. hybridum* flowered and produced seeds, and in short-days at 4ºC, *B. hybridum* eventually flowered (Fig. 3C, 2C, Appendix 3 Fig. 2). Hence, these results indicate that *B. hybridum* can flower under what is considered non-inductive for *B. distachyon* and is also less sensitive to photoperiod. As *B. hybridum* and *B. stacei* can flower without vernalization, *B. hybridum* has likely inherited this spring-like flowering habit from *B. stacei*, although *B. hybridum* exhibits the same *VRN1* cold responsivity observed in *B. distachyon*.

5.4.3 Cold acclimation responses of B. distachyon are conserved in *B. hybridum*

Cold exposure induced the accumulation of *VRN1* transcripts in *B. distachyon* and *B. hybridum* alike (Fig. 5.3C). In *B. distachyon*, there is a positive feedback loop between *VRN1* and *FT*, which is likely behind vernalization in plants exposed to chilling, but not in plants exposed to diurnal-freezing (Mayer et al., 2020; Ream et al., 2014; Woods et al., 2016). Here, results show that flowering time and the expression of *FT* are independent from *VRN1* expression in *B. hybridum* (Fig. 5.3). In addition, there seems to be no difference between the responsivity of *FT* to chilling and to diurnal-freezing (Fig. 5.3). Therefore, the *VRN1-FT* regulatory loop, especially observed in chilling-induced vernalization, appears to be absent in *B. hybridum*. Because the cold-to-flowering link is a fundamental adaptive plasticity trait in temperate cereals and Brachypodium (McKeown et al., 2016), their dissociation in *B. hybridum* is a profound change in the expression of temperate-climate adaptive responses studied in *B. distachyon*. Indeed, *VRN1* in *B. hybridum* may, in response to cold, function in cold acclimation rather than in flowering.

B. hybridum successfully cold acclimated and gained high freezing tolerance. *B. hybridum* displayed similar survival curves to freezing, expressed the same molecular machinery in response to chilling and to diurnal-freezing than *B. distachyon* (Fig. 5.1C-D, Fig. 5.4A, D-E). Diurnal-freezing transcriptionally primed *B. hybridum* through the acquisition of transcriptional memories and new cold responsive genes, as was previously described in *B. distachyon* (Fig. 5.5, 5.6A: Mayer & Charron, 2020). *B. hybridum* also gained different morphologies under chilling and diurnal-freezing, with more spikes and leaves, and higher biomass and yield in *B. hybridum* exposed to diurnal-freezing compared to those exposed to chilling (Fig. 5.6D). In fact, chilling and diurnal-freezing induce contrasting morphological responses in *B. distachyon*, at least partly through the expression levels of *VRN1* (Mayer et al., 2020). Therefore, the main mechanisms behind cold acclimation in *B. distachyon* are conserved in *B. hybridum*.

5.4.4 *B. hybridum* has a wider adaptation range

B. hybridum also showed better adaptability than *B. distachyon*. First, *B. hybridum* had a survival edge over *B. distachyon* when non-acclimated and after early cold acclimation (Fig. 5.4A-B). Interestingly, early cold exposure induced a higher *VRN1* response in *B. hybridum* than in *B. distachyon*, indicating a faster response to cold (Fig. 5.6C). Second, *B. hybridum* was less stressed during and after exposure to chilling (Fig. 5.2E, 5.6D). Third, *B. hybridum* was more vigorous than *B. distachyon* overall, grew significantly taller in all conditions, had longer total root length, had more shoots/tillers in control and chilling conditions (Fig. 5.2A-D, 5.6D). Fourth, *B. hybridum* flowered under short-day and chilling (Fig. 5.2C, Appendix 3 Fig. 2). Fifth, a higher proportion of memory genes were in the *core* category, indicating that there is less gene response specialization during priming in *B. hybridum* (Fig. 5.6A). Overall, *B. hybridum* has a higher plasticity than *B. distachyon*.

B. hybridum is the only annual *Brachypodium* that is established outside of its native range. Indeed, *B. hybridum* successfully colonized parts of Europe, America, Africa and Oceania, and, although its growing range overlaps with *B. distachyon*, *B. hybridum* has not colonized the colder habitats of *B. distachyon* (Catalán et al., 2016; Lopez-Alvarez et al., 2015; John P Vogel, 2016). Hence, the plasticity of *B. hybridum* likely contributed to its spread, while its lack of vernalization response may prevent the colonization of colder regions occupied by *B. distachyon*. The polyploidy of *B. hybridum* likely contributed to its high ecological tolerance, as polyploidy

contributes to invasiveness (te Beest et al., 2012), although it may not have driven drought tolerance in *Brachypodium* (Penner et al., 2020).

5.4.5 Another step into the evolution of annual *Brachypodium*

Cyclic polyploidy-diploidy are increasingly recognised as common events in the evolution of plants, polyploidization being often associated to major events of adaptive speciation (Alix et al., 2017; Bird et al., 2018). The *B. stacei*-like flowering and *B. distachyon*-like cold acclimation, and no apparent gene expression bias between D and S suggest that the subgenomes of *B. hybridum* are in harmony (Fig. 5.5A-B, Appendix 3 Fig. 1; Bird et al., 2018). Increased genetic diversity leads to more variation in DNA methylation, which contributes to phenotypic diversity in *Brachypodium* (Eichten et al., 2020). Hence, the extensive DNA demethylation observed in *B. hybridum* during cold acclimation, opposite to the trend observed in *B. distachyon* along with the higher developmental flexibility displayed *B. hybridum*, support that temperate-climate adaptive plasticity has evolved in *B. hybridum* (Fig. 5.6C). Overall, the phenotype exhibited by the allopolyploid *B. hybridum* and its radiation into new environments highlight an important step of the evolution of Brachypodium annuals.

5.4.6 Conclusion

Unlike in *B. distachyon*, cold acclimation is a trait that is independent from phenology in *B. hybridum*. This dissociation likely allowed *B. hybridum* to colonize new environments, while restricting its persistence out of colder regions. Our results support that polyploidy and hybridity contribute to increasing phenotypic plasticity and provide insight into the evolution of traits in hybrids. Moreover, our results suggest that *VRN1*'s main function may lie in cold acclimation in Brachypodium annuals. Overall, this study reveals that *B. hybridum* is a valuable model to study cold acclimation independently from flowering in temperate grasses.

5.5 Methods

5.5.1 Seed stocks and plant growth

B. hybridum was isolated from seeds stocks of PI631898 ordered from the USDA National Plant Germplasm System (https://www.ars-grin.gov/). Information about the collection of PI631898 and development of Bd29-1 can be found on

https://www.ars.usda.gov/ARSUserFiles/1931/GarvinLabCoreBrachypodiumdistachyonLineSet(2).pdf. Prior to planting, seeds of *B. distachyon* Bd29-1 or Bd21-3 and *B. hybridum* were soaked for 2 hours and stratified in the dark at 4ºC for 1 week, then sown in 3X3 inch 0.5 L pots containing G2 Agromix (Fafard et Frères Ltd., Saint-Remi, QC, Canada), and grown in an environmental growth chamber (Conviron, Winnipeg, MB, Canada) under short days at 8/16 hours light/dark at 22°C, PAR intensity of 150 µmol m−2 s−1. Plants were kept equally watered throughout experiments. To induce flowering, plants were transferred to long-day conditions at 16/8 hours light/dark at 22°C, PAR intensity of 150 µmol m−2 s−1 on a growth bench at 25°C and maintained watered until senescence.

5.5.2 Genome analyses

Genomic DNA was extracted from sampled aerial tissue crushed with liquid nitrogen using a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and sequenced using HiSeqX PE150 (Illumina, San Diego, California, USA) by Le Centre d'Expertise et de Services Genome Québec. Adapters from reads were removed using Trimmomatic and aligned on the reference genomes of *B. hybridum*, *B. distachyon* and *B. stacei* (**Brachypodium hybridum** v1.1, DOE-JGI http://phytozome.jgi.doe.gov; **Brachypodium stacei** v1.1, DOE-JGI http://phytozome.jgi.doe.gov; **Brachypodium distachyon** Bd21v.3.1, DOE-JGI http://phytozome.jgi.doe.gov) using BWA-MEM and visualized with bam.iobio on Galaxy (Bolger et al., 2014; Li & Durbin, 2009; Miller et al., 2014). Reads were made available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA660191?reviewer=7ldt4jiluf3k9602hg4pv4otft (pre-release).

5.5.3 Sequence alignment and phylogenetic analyses

Plastome alignment was performed using BWA-MEM on sequences from (Sancho et al., 2018), after which reads of high quality were filtered (QC > 20; Li & Durbin, 2009). Consensus sequence of plastome gene *rps19* from putative *B. hybridum* was then obtained using Integrative Genome Viewer (Robinson et al., 2011), and aligned to other *rsp19* alleles using MUSCLE in the software MEGA5 (Kumar et al., 2018), and visualized using MView (Madeira et al., 2019). COR genes sequences were retrieved using the BLAST tool from phytozome (http://phytozome.jgi.doe.gov) and COR genes sequences from *B. distachyon* as queries

(accession numbers found in Table 5.1). Consensus sequence of *VRN1* from putative *B. hybridum* was obtained using Integrated Genomics Viewer (Robinson et al., 2011), and *VRN1* alleles from different *B. distachyon* populations were obtained from BrachyPan (http://brachypan.jgi.doe.gov/; Gordon et al., 2017). Phylogenetic analyses were performed in MEGA5 using MUSCLE for sequence alignment and bootstrap values of 1000 replicates for maximum likelihood phylogenetic trees (Kumar et al., 2018). Sequence similarity between *B. distachyon*, Bh D and Bh S genes were obtained using Clustal W (Larkin et al., 2007).

5.5.4 Cold and flowering treatments

Plants previously grown for 14 days in short-day control conditions were cold treated with constant-chilling (CC; at 4°C) in an environmental growth chamber (Conviron) or with diurnal-freezing (DF; Fig. 5.4C; temperature cycles as previously described (Mayer et al., 2020)) in a LT-36VL growth chamber (Percival Scientific, Perry, IA, USA). Plants treated under chilling or diurnal-freezing were exposed to short-days and lighting conditions identical to control conditions of 8/16 hours light/dark at 22°C, PAR intensity of 150 μmol m−2 s−1.

5.5.5 Chlorophyll and proline quantification

Total chlorophyll extraction was performed on fresh tissue pooled from 3 plants per biological triplicate and quantified as previously described (Ritchie, 2006). Proline extraction was performed on dehydrated aerial tissue pooled from 10 plants for each biological triplicate and quantified by spectrophotometry as previously described (Colton-Gagnon et al., 2014).

5.5.6 Phenotypic measurements

Age at heading encompassed the vegetative phase, including time spent in cold and in flower-inducing conditions until the first visible emergence of flowers (heads). Final height, dry weight, number of spikes, final leaf number, and seed weight (yield) were determined after senescence when seeds were filled and dry. Dry weight measurements were performed on total aerial tissue (without seeds) after thorough drying of plant tissues using a dehydrator (BioRad, Hercules, CA, USA). Measurements were performed on at least 9 plants per experiment, which was performed three times.

5.5.7 RNA extraction and RT-qPCR

Total RNA was extracted after liquid nitrogen grinding of a pool of aerial tissue from at least three plants for each biological triplicate using EZ-10 RNA kit (CAT#: BS82314, Bio Basic, New York, NY, USA) following the manufacturer's protocol. Prior to extraction, tissue was collected, flash-frozen in liquid nitrogen and stored at -80°C. Total RNA was used for cDNA synthesis using iScript™ (CAT# 1725037, BioRad), and subsequently for RT-qPCR using Green-2-Go (CAT# QPCR004, Bio Basic) performed on CFX Connect Real Time (BioRad) both following manufacturer's protocol. Relative transcript levels were analyzed following the ΔΔCT method using *UBC18* gene as reference (Hong *et al.*, 2008; Mayer *et al.*, 2020). Primer sequences can be found in Appendix 3 Table 2.

5.5.8 Measures of freezing tolerance

Survival to freezing was measured in whole-plant freeze tests (WPFT) where plants were gradually exposed to sub-zero temperatures ranging from -8 to -12°C as previously described (Mayer et al., 2020). Electrolyte leakage was measured from leaf tissue sampled in plants exposed to freezing as described in (Lee & Zhu, 2010).

5.5.9 RNA extraction for high-throughput sequencing

Primed *B. hybridum* were exposed to 4 days of diurnal-freezing, transferred back to control conditions (short-day at 22°C) when temperatures in the diurnal-freezing cycle reached 22°C and then transferred to chilling the next day at 12 pm. Naïve *B. hybridum* were grown in control conditions and transferred to chilling at the same time as diurnal-freezing-primed plants. A pool of three plants of naïve or primed plants were collected after 3 hours of exposure to chilling, along with primed or naïve controls that remained in control conditions. Tissue was flash frozen in liquid nitrogen and briefly stored at -80°C until total RNA extraction. This experiment was performed two times. A total of eight samples (four samples replicated twice) were extracted using RNeasy Kit (Qiagen) following the manufacturer's protocol. Sequencing libraries were built using NEBNext® Multiplex Oligos for Illumina® (CAT#E7600S, New England Biolabs, Ipswich, Massachusetts, USA) and sequenced using NovaSeq 6000 (Illumina) by Le Centre d'Expertise et de Services Genome Québec.

5.5.10 Transcriptome analyses

Using Galaxy, FASTQ reads (pre-released data available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA660287?reviewer=s9852cjl3i8di8t5248oqlaapj), were analysed using FastQC and adapters removed using Trimmomatic (Andrews, 2010; Bolger *et al.*, 2014; Afgan *et al.*, 2018). Trimmed reads were mapped to *B. hybridum* v1.1 genome obtained on phytozome (**Brachypodium hybridum** v1.1 DOE-JGI, http://phytozome.jgi.doe.gov) using RNA-STAR (Dobin et al., 2013). Reads were counted using FeatureCounts (Liao et al., 2014). Using DEseq2 with cold/priming and replicates as factors, fold-change was calculated for naïve and primed responses to chilling (N and P) by comparing chilling-treated with their respective non-stress controls, and for recovery-responsive genes by comparing primed controls to naïve controls (Anders & Huber, 2010). Genes that displayed significant differential expression (p-adj < 0.05) and an absolute fold change > 2 (FC > 2) were selected for further analysis. Comparison to RNA-seq data from *B. distachyon* was performed on previously published data from (Mayer & Charron, 2020) publically available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA629906?reviewer=g0dlh6m54716vpjpa333uf1tpr (pre-released data).

5.5.11 Gene ontology enrichment analysis

Gene ontology (GO) analyses were performed using the tool agriGO 2.0 (Tian et al., 2017) with FDR adjusted p-value of 0.05, and annotated *B. hybridum* gene list from phytozome. Treemaps were visualized using the package *treemap* in R (R Core Team, 2013).

5.5.12 Global DNA methylation

Global DNA methylation assay was performed using an Imprint Methylated-DNA Quantification Kit (Sigma-Aldrich Corp., St. Louis, MO, USA) following the manufacturer's protocol. Genomic DNA from a pool of aerial tissue from three plants was extracted by standard phenol-chloroform extraction from two experiments replicated in time. Each sample was measured in technical quadruplicate using a Microplate Reader (BioRad).

5.5.13 Statistical analyses

All experimental data were analyzed by one-way ANOVA followed by Tukey HSD, which were performed in JMP (SAS Institute, Cary, NC, USA). Statistical significance was determined with $p<0.05$ on at least three independent biological replicates, including fold values for qPCR data.

5.6 Supplementary information

Supplementary Fig. 1: Genomic differences between subgenomes of *B. hybridum* and the parental genomes.

Supplementary Fig. 2: *B. hybridum* producing filled and viable seeds in short-day (8/16 hours light/dark) conditions.

Supplementary Table 1: Gene ontology analysis of cold-responsive genes divided intro core memory, core no memory, naïve-specific (memory) and primed-specific (acquired), and comparison between *B. distachyon* and *B. hybridum*.

Supplementary Table 2: Primers used in this study

5.7 Author contribution

BFM and JBC designed the research, BFM performed the experiments and analyses, LO contributed laboratory work, BFM and JBC wrote the manuscript.

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Gene	*B. distachyon*	*B. hybridum*	Parent genome	Similarity of cds (%)	Similar nt
CBF1	Bradi3g51630	Brahy.D03G0712700	*B. distachyon*	99.7	682 / 684
		Brahy.S04G0111900	*B. stacei*	99.3	630 / 675
CBF2	Bradi1g49560	Brahy.D01G0665100	*B. distachyon*	99.8	662 / 663
		Brahy.S07G0034300	*B. stacei*	91.8	592 / 645
CBF3	Bradi4g35650	Brahy.D04G0515900	*B. distachyon*	99.8	716 / 717
		Brahy.S05G0207600	*B. stacei*	93.8	667 / 711
IRI	Bradi5g27350	Brahy.D05G0363300	*B. distachyon*	83.3	580 / 696
		Brahy.D05G0363400	*B. distachyon*	84.9	609 / 717
		Brahy.D05G0363500	*B. distachyon*	99.2	830 / 837
		Brahy.S06G0263200	*B. stacei*	58.0	448 / 773
COR410	Bradi3g51200	Brahy.D03G0707100	*B. distachyon*	99.5	761 / 765
		Brahy.S04G0117200	*B. stacei*	96.0	729 / 759
COR413	Bradi1g07441	Brahy.D01G0093200	*B. distachyon*	99.7	631 / 633
	.	Brahy.S02G0343700	*B. stacei*	97.5	617 / 633
VRN1	Bradi1g08340	Brahy.D01G0104700	*B. distachyon*	100	732 / 732
		Brahy.S02G0334300	*B. stacei*	96.7	708 / 732
UBC18	Bradi4g00660	Brahy.D04G0006200	*B. distachyon*	100	588 / 588
		Brahy.S10G0005700	*B. stacei*	97.0	545 / 562
FT	Bradi1g48830.1	Brahy.D01G0654200.1	*B. distachyon*	98.5	526/534
		Brahy.S07G0045400.	*B. stacei*	100	534/534

Table 5.1: Cold-stress genes homologs in *B. hybridum*

	B. distachyon		B. hybridum	
Gene	**ID**	**Category**	**ID**	**Category**
CBF1	Bradi3g51630	Core, no memory	BrahyD03G0712700	Core, no memory
			BrahyS04G0111900	Core, no memory
CBF2	Bradi1g49560	Core, memory	BrahyD01G0665100	Core, memory
			BrahyS07G0034300	Primed-specific/acquired
CBF3	Bradi4g35650	Core, memory	BrahyD04G0515900	Core, memory
			BrahyS05G0207600	Core, memory
IRI	Bradi5g27350	Recovery-specific	BrahyD05G0363400	Recovery-specific
			BrahyD05G0363500	Recovery-specific
			Brahy.S06G0263200	N/A
COR410	Bradi3g51200	Core, no memory	BrahyD03G0707100	Naïve-specific/ memory
			Brahy.S04G0117200	N/A

Table 5.2: Distribution of cold-stress genes in naïve, primed and recovery responses in *B. distachyon* and *B. hybridum*

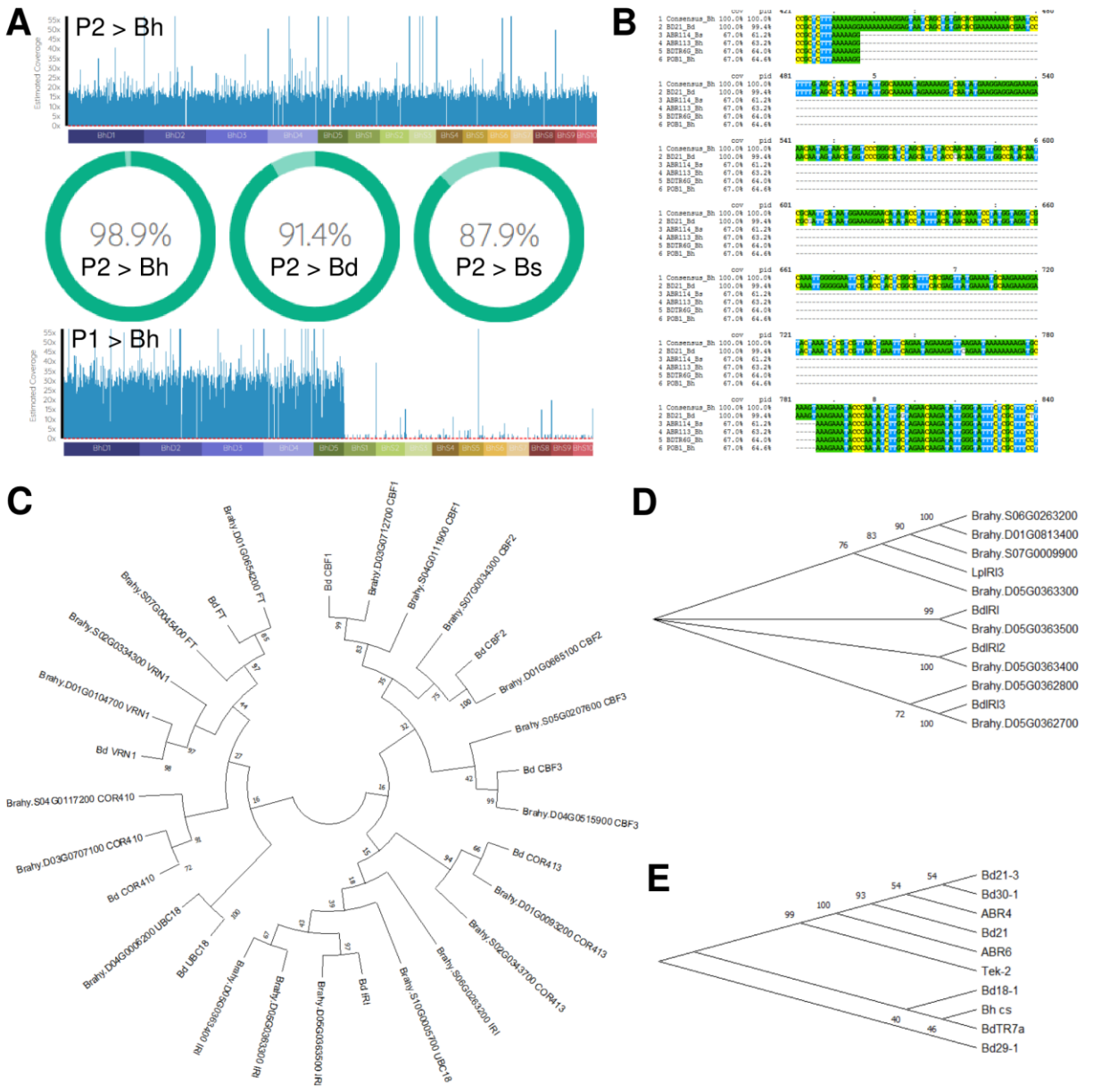

Figure 5.1: Genome sequencing and cold acclimation genes in *B. hybridum*.

(**A**) Coverage of putative *B. hybridum* (P2) mapped to the reference genome of *B. hybridum* (Bh), *B. distachyon* (Bd) and *B. stacei* (Bs), and of congener seeds P1 mapped to the reference genome of B. hybridum (P1 > Bh). (**B**) Multiple sequence alignment of plastome gene *rsp19* alleles from Bd (Bd21), Bs (ABR114_Bs), previously studied Bh accessions (ABR113_Bs, BDTG6G_Bh and POB1_Bs) and pBh *rsp19* consensus sequence (Consensus_Bh). (**C**) Phylogenetic analysis of selected Bd COR gene homologs in Bh. (**D**) Phylogenetic analysis of COR gene ICE RECRISTALLIZATION INHIBITOR homologs in Bh, BdIRI1-3 and *Lolium perenne* (LpIRI).

(**E**) Phylogenetic analysis of VRN1 alleles in selected Bd accessions and pBh consensus sequence (Bh cs). *Phylogenetic trees were built from bootstrap values (>50%) on 1000 replicates.*

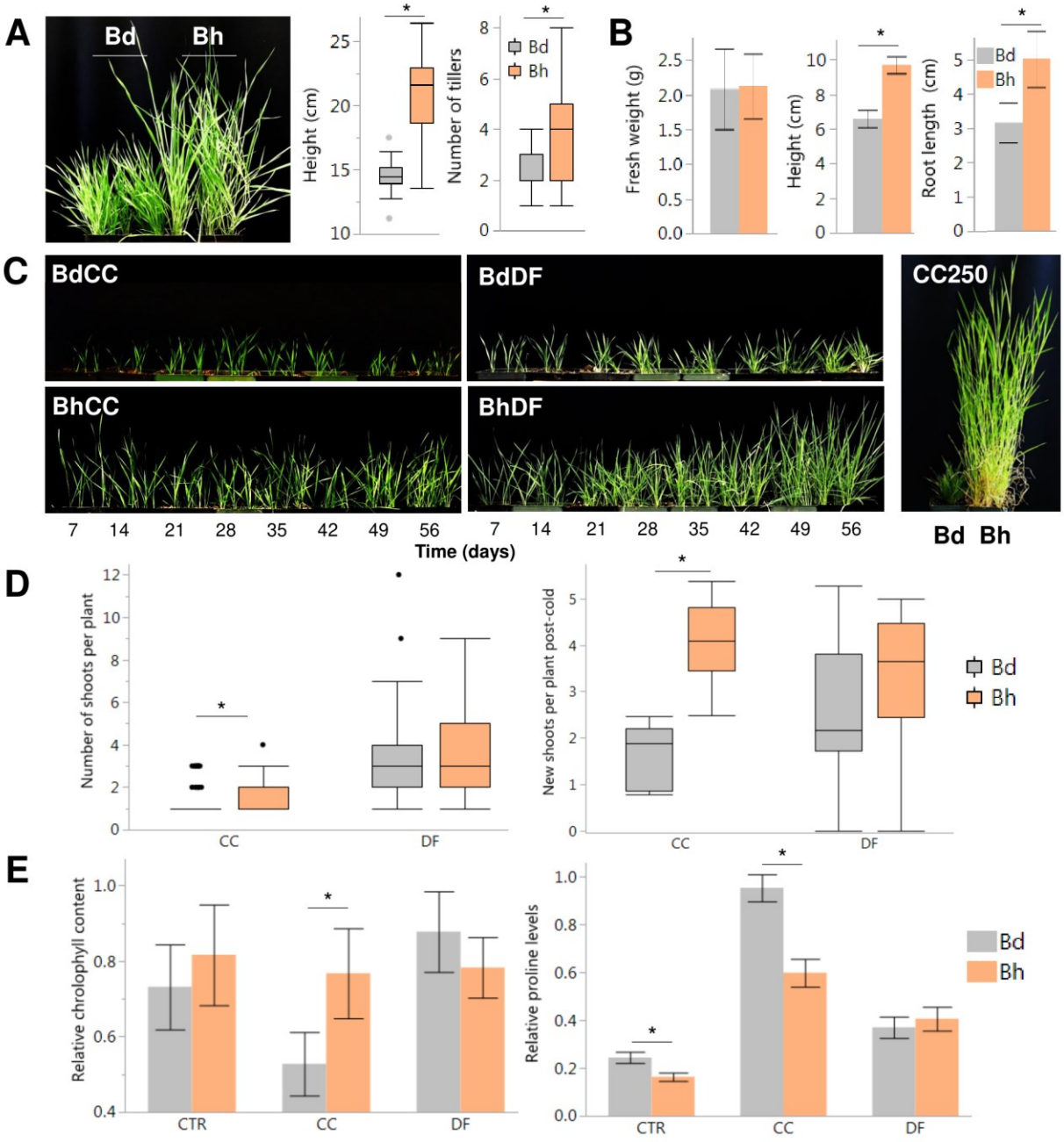

Figure 5.2: *B. hybridum* is less stressed by chilling compared to *B. distachyon*.

(**A**) Height and number of tillers of *B. distachyon* (Bd) and *B. hybridum* (Bh) grown for 4 weeks in long-day conditions. (**B**) Fresh biomass, shoot and root length in plants grown for 2 weeks in vitro in short-day conditions. **C** 2-week old Bd or Bh grown in short-day and transferred to 7-56 days in constant-chilling (CC) or diurnal-freezing (DF), or 180 days in CC. (**D**) Number of shoots in Bd and Bh grown for 7-56 days in CC or DF, in cold and post-cold exposure. (**E**) Relative chlorophyll and proline content in Bd and Bh exposed to CC or DF for 14 days. * *indicate*

statistical difference $p < 0.05$, error bars show standard deviation from the mean from three biological replicates.

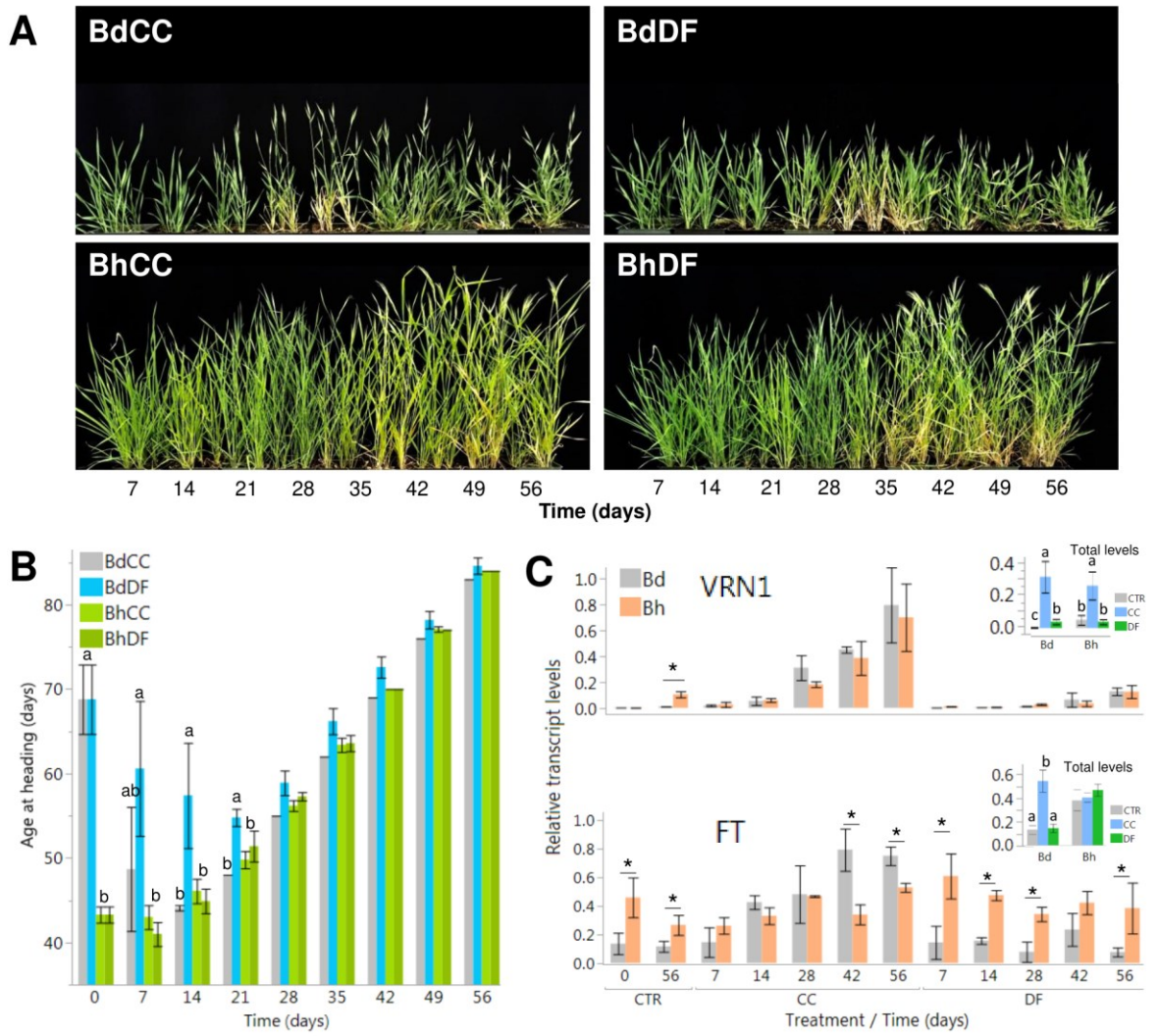

Figure 5.3: Flowering in *B. hybridum* is not responsive to vernalization.

(**A**) Flowering response of *B. distachyon* (Bd) and *B. hybridum* (Bh) exposed for 7-56 days in chilling (CC) or diurnal-freezing (DF) for 7-56 days and transferred to flowering conditions for 14 days. (**B**) Age of Bd and Bh plants at heading after 0-56 of exposure to CC or DF. (**C**) Relative transcript levels of vernalization gene VRN1 and flowering gene FT in Bd and Bh plants grown in control conditions (CTR) and exposed for 7-56 days to CC or DF. Small graphs indicate the relative levels of transcripts overall in CTR, CC and DF. * *and letters indicate statistical difference* $p < 0.05$*; error bars show standard deviation from the mean from three biological replicates.*

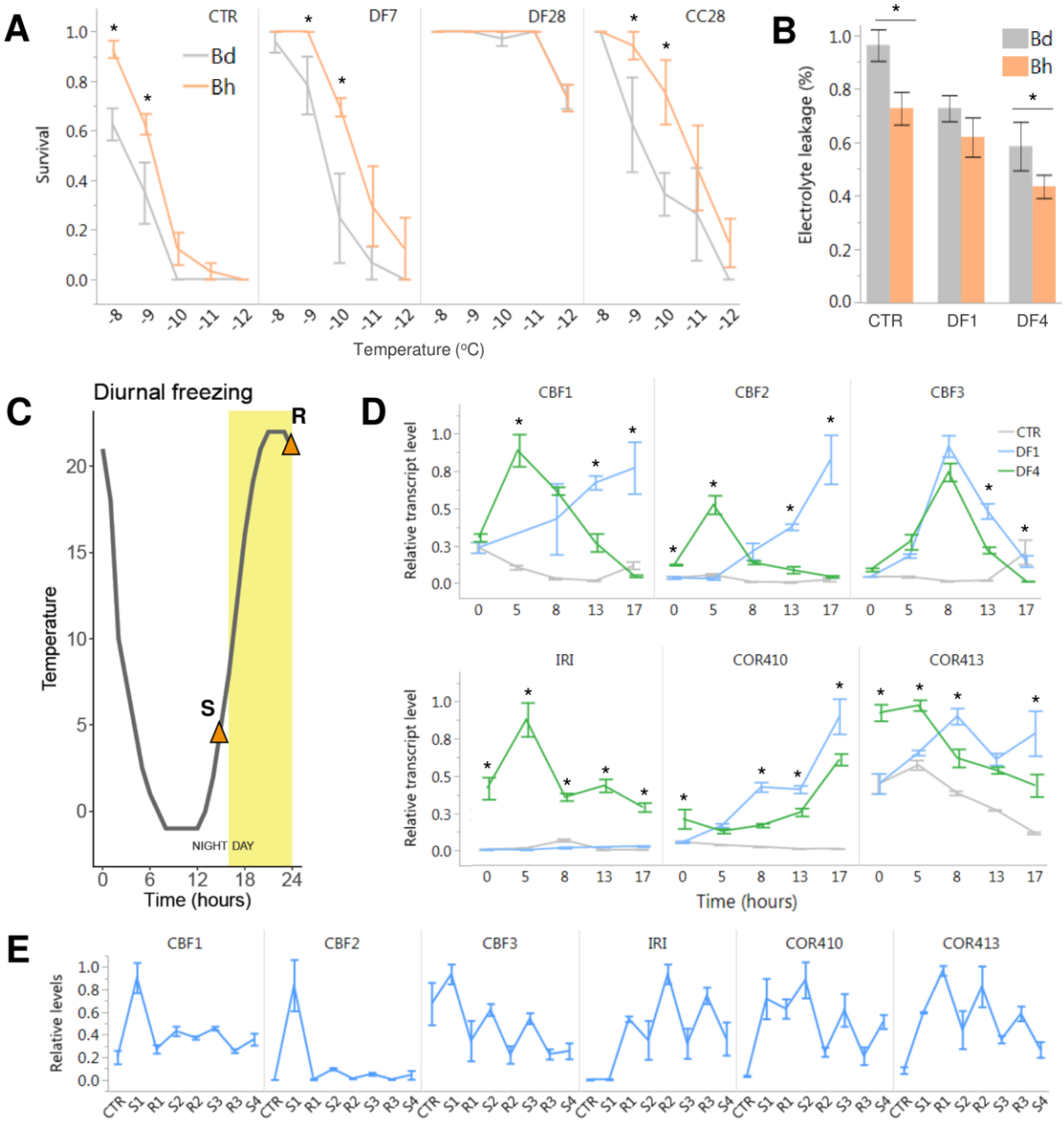

Figure 5.4: Freezing tolerance and transcriptional cold acclimation responses of *B. hybridum*.

(**A**) Survival to freezing of *B. distachyon* (Bd) and *B. hybridum* (Bh) grown at 22ºC (CTR) and exposed to diurnal-freezing (DF) for 7 or 28 days (DF7, DF28) or constant-chilling (CC) for 28 days (CC28). (**B**) Tissue damage induced by freezing measured by electrolyte leakage in Bd and Bh exposed to one or four cycles of DF (DF1, DF4). (**C**) Temperature cycle of diurnal-freezing over 24 hours, S and R time-points. (**D**) Transcript levels of Bh cold-stress genes in response to one or four cycles of DF (DF1, DF4). (**E**) Transcript levels of BhCOR genes in response to 1-4

cycles of DF at S and R time-points. * *indicate statistical difference $p < 0.05$; in D between DF1 and DF4; error bars show standard deviation from the mean from three biological replicates.*

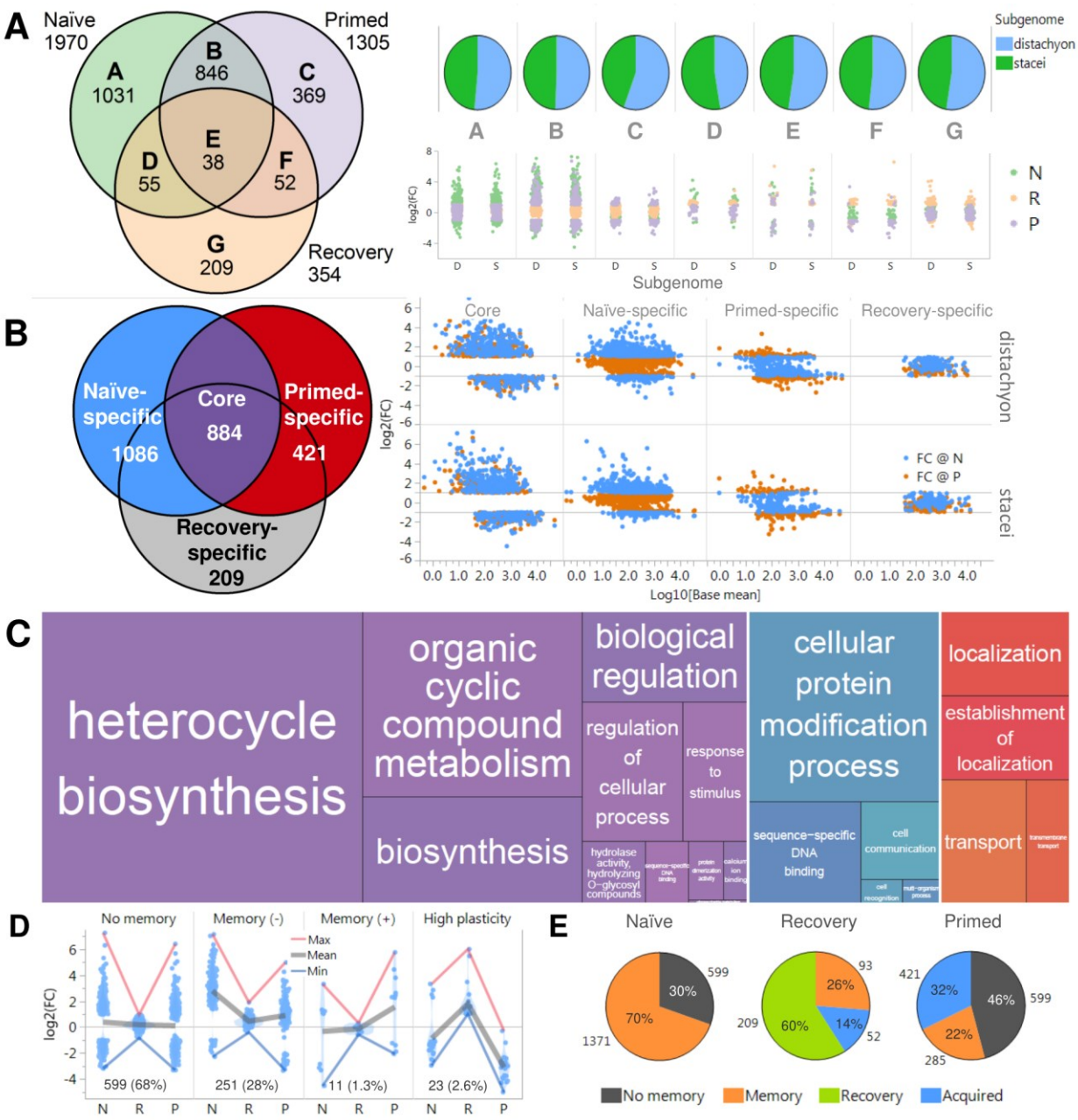

Figure 5.5: Transcriptome of naïve and DF-primed *B. hybridum* in response to chilling.

(**A**) Distribution of responsive genes during the naïve and primed responses to constant-chilling (CC), and during recovery from DF (*left panel)* and their location on distachyon or stacei sub-genomes *(right panel*). (**B**) Transcript levels of genes activated in both naïve and primed responses to CC (core) or specific to naïve, primed, and recovery responses. (**C**) Gene ontology analysis of core (purple), naïve-specific (blue) and primed-specific genes (red). (**D**) Genes of the core response that exhibit no memory (same response in both naïve N and primed P responses), or memory with different expression in P than in N, either negative or positive (- or +), or with high plasticity

(upregulated during recovery R). (**E**) Number and proportions of gene in specific profiles (no memory, memory, recovery and acquired) in the naïve (N), recovery (R) and primed (P) responses.

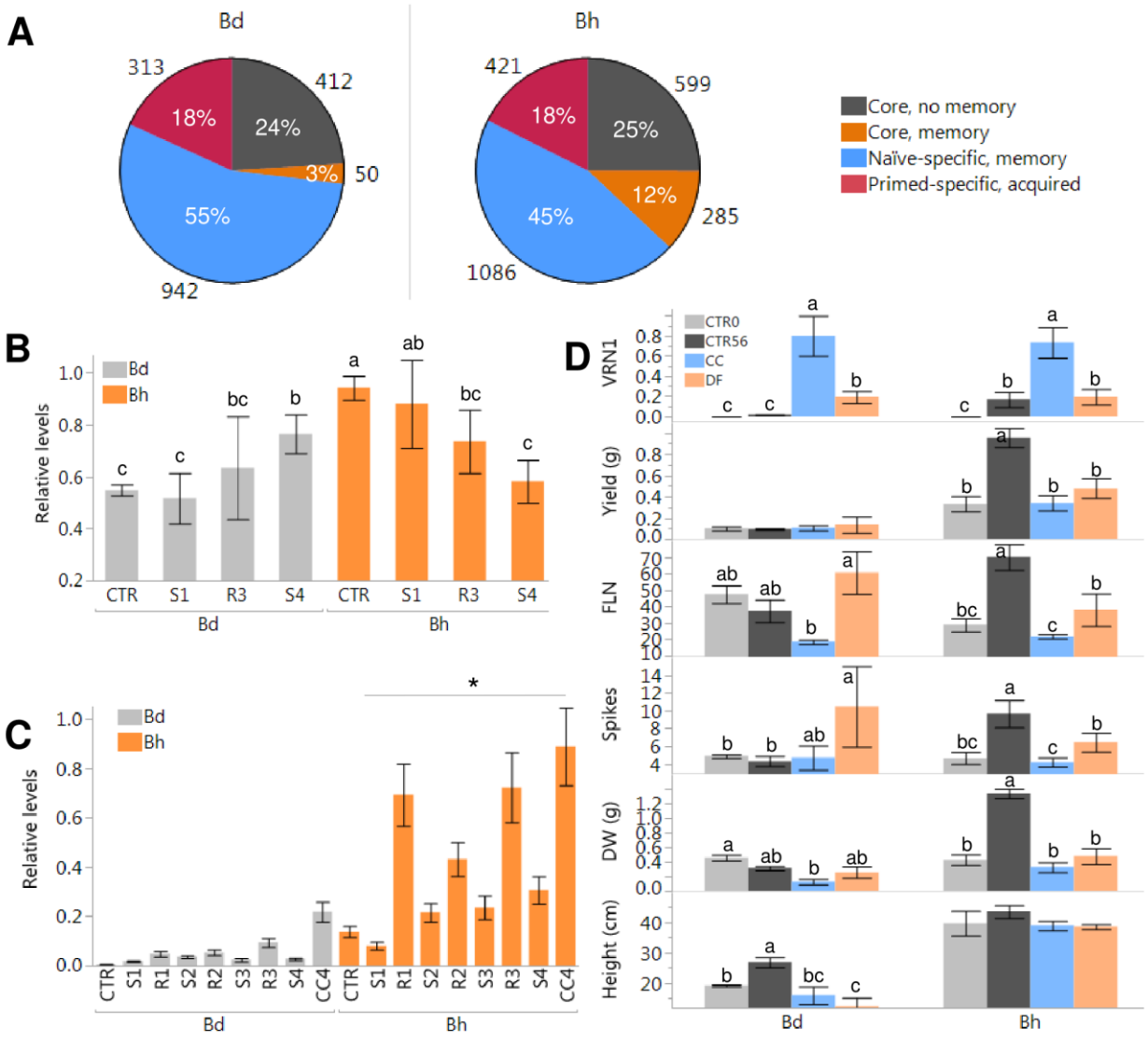

Figure 5.6: Contrasting cold-adaptation responses of *B. distachyon* and *B. hybridum*.

(**A**) Transcriptional priming in *B. distachyon* (Bd) and *B. hybridum* (Bh). (**B**) Relative global DNA methylation in Bd and Bh exposed to DF. (**C**) *VRN1* levels in early exposure to cycles of DF (DF1 to DF4 at S and R) and to four days of CC (CC4) in Bd and Bh. (**D**) Final height post-flowering (Height), dry weight (DW), number of spikes (Spikes), final leaf number (FLN), weight of seeds per plant (Yield), and relative *VRN1* levels in non-stressed controls CTR0 and CTR56, CC and DF-exposed (7-56 days) Bd and Bh. * *and letters indicate statistical difference $p < 0.05$; error bars show standard deviation from the mean from three biological replicates.*

5.10 Connecting text

Vernalization is characterized by the establishment of a transcriptional memory through chromatin change on *VRN1*. Chapter 4 demonstrated that physiological and morphological cold acclimation of *B. distachyon* also concurs with the establishment of transcriptional memories and important chromatin responses. Chapter 5 showed that the plastic *B. hybridum* also displayed the establishment of transcriptional memories during cold acclimation but had contrasting differences in the change in global DNA methylation in response to diurnal-freezing. These results suggest that chromatin, regulating transcriptional memories, is involved in regulating phenotypic plasticity in Brachypodium.

Chapter 6 presents a perspective on the role that chromatin could play in regulating phenotypic plasticity in plants and in facilitating the influence of behavior on adaptation, by drawing examples from the phenotypic plasticity of temperate grasses, including Chapter 3-5.

6. CHROMATIN: A PLATFORM FOR PHENOTYPIC PLASTICITY AND A PATH TO ADAPTATION

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6.1 Abstract

Enhancing plant adaptability has become increasingly important to sustain agricultural production through the consequences of global warming. Scientific studies have highlighted the implication of chromatin regulation in plant stress response, growth and development. Chromatin integrates signals that reflect the biological environment from growth to biochemistry, can store latent transcriptional information and influence genome evolution events. When passed on through cell division and reproduction, chromatin changes that become epigenetic can shape phenotypes and ultimately influence adaptation. Encompassing growth, stress response and development, the epigenetically-regulated plasticity traits that support the persistence of temperate grasses in cold climates provide a useful system to study the regulation of phenotypic plasticity. We propose that epigenomes mediate the plasticity of stress responses and development, help process and encode the complexity of environments and, as such, may be a processing platform for the expression of phenotypes in plants.

6.2 Introduction

The serious and unexpected outcomes of global warming have precipitated the need to understand how plants adapt to environmental change. The field of epigenetics has brought new perspectives on the interaction between environments and genomes, and recent work has highlighted the implication of chromatin in several aspects of plant growth, development and stress response (Chinnusamy and Zhu, 2009; Dowen et al., 2012; Engelhorn et al., 2014; Guo et al., 2015; Lämke and Bäurle, 2017; Narsai et al., 2017; Vergara and Gutierrez, 2017; Lee and Seo, 2018). Although much remains to be understood, evaluating the function of epigenomes in connecting phenotypic plasticity, *i.e.* the ability of organisms to produce different phenotypes in response to their environment, to adaptation, *i.e.* long-term evolutionary mechanisms, has become critical.

For the past 50 years or so, human activities have greatly perturbed on a global scale the natural environments to which recent biodiversity has adapted. Indeed, the consequences of global warming will challenge the growth and development of plants and their persistence in the environment (Gray and Brady, 2016; Bathiany et al., 2018). Plants respond to stressful environmental perturbations in different ways, but these generally range from rapid responses to alleviate physiological stress to long-term developmental responses that shape morphology and

determine their life cycle (Chouard, 1960; Ciarmiello et al., 2011; Liu and Su, 2016; Klem et al., 2019; Mayer et al., 2020). In this article, we present the perspective that chromatin can coordinate the expression of phenotypic plasticity by mediating stress and developmental responses, that it provides the means to shape future responses and that it connects behavior to adaptative evolution. We hence propose that chromatin constitutes a processing platform for phenotypic expression that integrates complex information associated with genetics, the environment, developmental programs and stress responses. We also discuss the possibility that the cold-adaptive plasticity of temperate grasses is an example of chromatin-mediated adaptation.

6.3 Chromatin regulates development and stress response

The eukaryotic genome is packaged in a DNA-protein complex called chromatin. Essentially made of DNA wrapped around histones, the structure and composition of chromatin can regulate gene expression (Hendrich and Willard, 1995; Felsenfeld et al., 1996; Shilatifard, 2006). Modifications of chromatin, such as DNA methylation and various chemical modifications of histones, influence transcription by regulating the bonds between DNA and histone proteins which affect the accessibility of genes and by directly influencing the binding of regulatory proteins to DNA (Hendrich and Willard, 1995; Felsenfeld et al., 1996; Shilatifard, 2006). Chromatin composition can also determine the identity of mRNA transcripts by influencing alternative splicing events (van Bakel, 2011; Naftelberg et al., 2015; Wang et al., 2016; Pajoro et al., 2017). When transmitted over cell division and reproduction, chromatin changes then become epigenetic. Studies of epigenetically-different but genetically identical recombinant inbred lines have shown that chromatin information can account for the heritability of complex traits, and inherited independently from genetics (Cortijo et al., 2014; Kooke et al., 2015; Zhang et al., 2018). Hence, regulating gene expression, chromatin can determine the expression of complex traits.

Chromatin is involved in the regulation of development and stress responses to the environment. When transmitted over cell division, chromatin composition safeguards the combination of expressed genes to preserve the identity and integrity of cells, and by changing over the course of growth and development, regulates important processes like in seed dormancy and germination, cell differentiation and reproduction (Engelhorn et al., 2014; Guo et al., 2015; Narsai et al., 2017; Lee and Seo, 2018). Moreover, the regulation of cell de-differentiation and pluripotency is connected to chromatin, receiving signals from the environment (Xiao et al., 2017).

Indeed, responses to environmental stress are connected to changes in the chromatin composition of stress-responsive genes, to the function of specific chromatin modifiers and to changes in epigenetic landscapes (Charron et al., 2009; Chinnusamy and Zhu, 2009; Dowen et al., 2012; Lämke and Bäurle, 2017; Mayer and Charron, 2020b). For example, chromatin responses are linked to profound phenological, physiological and morphological change, as observed for example in stress-induced flowering and shade avoidance (Bastow et al., 2004; Yaish et al., 2011; Miao et al., 2017; Peng et al., 2018; Zhang et al., 2019; Mayer et al., 2020). Hence, chromatin composition can determine development and responses to the environment.

6.4 Chromatin integrates extrinsic and intrinsic signals to regulate phenotypic expression

Signals linked to development and those that induce stress responses converge on chromatin. For instance, chromatin can directly sense environmental change, as highlighted in *Arabidopsis* where levels of H2A.Z, a variant of histone 2A, rapidly change with temperature (Kumar and Wigge, 2010). Moreover, the activity of chromatin regulating enzymes, *e.g.* DNA and histone methyltransferases and demethylases, histone acetylases and deacetylases, is influenced by concentrations and regulation of substrate and/or co-factors such as s-adenosylmethionine and acetyl-CoA (Groth et al., 2016; Chen et al., 2017; Meng et al., 2018). The regulation of chromatin structure and of plant growth and development also have common regulatory molecules, including various metabolites and hormones (Gray, 2004; Obata and Fernie, 2012; Verma et al., 2016; Yamamuro et al., 2016). Furthermore, chromatin is responsive to energy levels in the cell (Nicolaï et al., 2006). Hence, chromatin is responsive to signals form the physiological and biochemical environment connected to stress and developmental responses.

Chromatin responses that are triggered by extrinsic signals (consequences of environmental and biochemical change) are simultaneously influenced by intrinsic signals (the pre-stress chromatin structure), which are referred to as the *chromatin context* or *chromatin environment* (Liu et al., 2014; Wiles and Selker, 2017). Indeed, chromatin marks can interact and influence one another's regulation by having contrasting functions and by assembling into modules (Wang et al., 2008). This is best exemplified by the interaction between tri-methylation of lysine 27 and lysine 4 of histone 3 (H3K27me3 and H3K4me3). The transcriptionally repressive H3K27me3 and activating H3K4me3 can function antagonistically in plant development, for

example in vernalization or cold-induced flowering (Bastow et al., 2004; Pien et al., 2008; Liu et al., 2010). During vernalization, levels of H3K27me3 increase on the flowering repressor *FLOWERING LOCUS C (FLC*) during its transcriptional repression in *Arabidopsis* and decrease on the flowering activator *VERNALIZATION1* (*VRN1*) during its transcriptional activation in barley and *Brachypodium* (Bastow et al., 2004; Oliver et al., 2009; Woods et al., 2017; Mayer et al., 2020). In *Arabidopsis*, barley and *Brachypodium*, H3K4me3 levels change antagonistically to H3K27me3 levels on *AtFLC*, *HvVRN1* and *BdVRN1* during vernalization (Finnegan et al., 2005; Oliver et al., 2009; Huan et al., 2018; Mayer and Charron), during germination in *Arabidopsis* (Molitor et al., 2014), and during cold-stress response in potato (Zeng et al., 2019).

The co-regulation of H3K27me3 and H3K4me3, however, was not observed in all contexts. For instance, the activation of the wheat *VRN1* is associated with an increase in H3K4me3 but with no change in H3K27me3 (Diallo et al., 2012), and H3K27me3 levels are independent of H4K4me3-associated expression during flower morphogenesis and during drought stress in *Arabidopsis* (Liu et al., 2014; Engelhorn et al., 2017). Interestingly, H3K27me3 and H4K4me3 are independently recognized by a single histone reader whose binding is mutually exclusive for H3K27me3 and H3K4me3, indicating that their regulation can be chromatin-dependent (Qian et al., 2018). Indeed, the mechanisms regulating the editing of H3K27me3, carried by the conserved chromatin-regulation complex Polycomb Repressive Complex 2 (PRC2), depend on the composition and structure of chromatin they bind to, hence of the chromatin context (Wiles and Selker, 2017). Therefore, chromatin changes that occur in responses to a given stress depend on its current state, or context.

6.5 Chromatin regulates transcriptional memories

Studies that investigated stress recovery, responses to repetitive stress, and stress priming have revealed the existence of stress memories that are linked to changes in chromatin (Ding et al., 2012; Crisp et al., 2016; Lamke et al., 2016; Yeung et al., 2018; Mayer and Charron, 2020b). Called transcriptional memories, these can shape gene expression during stress recovery and regulate transcriptional response to subsequent stress exposure (Ling et al., 2018; Bäurle and Trindade, 2020). Also important in development, transcriptional memories established on genes *FLC* and *VRN1* during vernalization are maintained long after cold exposure and only reset at reproduction, which ensures that flowering occurs when conditions are right and that a

vernalization requirement is maintained in the population (Pien et al., 2008; Chen and Dubcovsky, 2012; Huan et al., 2018). In *Brachypodium*, the transcriptional memories linked to freezing tolerance are quickly reversible compared to the memory established on *VRN1*, likely because cold-stress traits are no longer necessary outside of cold unlike flowering competence (Mayer and Charron, 2020b).

In addition to their variable stability, transcriptional memories can have delayed and context-specific effects on gene expression. Unlike the chromatin and expression states of *FCL* and *VRN1* which are stably maintained long after cold exposure, the expression of stress-responsive genes can return to non-stress levels during stress recovery but carry new epigenetic identities that, in turn, shape subsequent stress responses (Ding et al., 2012; Mayer et al., 2015; Feng et al., 2016; Lamke et al., 2016; Ling et al., 2018; Mayer and Charron, 2020b). In this way, chromatin can store latent transcriptional information which affects gene expression in response to subsequent exposure to stimuli. Overall, chromatin mediates gene expression with high complexity and allows developmental and stress-induced changes to intertwine which can shape future behavior. Thus, chromatin can be described as an updatable template that channels the expression of phenotypes.

6.6 Chromatin generates variation for evolution

Chromatin holds updatable transcriptional information that can be maintained or erased depending on the context. Hence, in extreme cases, plants could remain unaffected for long periods by their environment or, on the opposite, may never completely return to a naïve state. The mechanisms that determine which changes stick and which are quickly forgotten remain, to this day, poorly understood. It was proposed that changes in chromatin composition could be passively lost over cell divisions, or actively removed by chromatin modifiers (Avramova, 2015; Bäurle and Trindade, 2020). It is possible that the maintenance of chromatin changes is connected to energy costs, allowing that plants break dormancy, silence costly stress responses when these are no longer necessary, or modulate responses to optimize their performance in the environment (van Hulten et al., 2006). For example, *B. distachyon* transitions from the expression of physiological responses typical of cold acclimation to morphological responses to tolerate freezing, which is linked to changes in chromatin (Mayer and Charron, 2020b). As chromatin regulation is connected to homeostatic changes, it likely receives feedback as responses unfold to modulate gene

expression (Nicolaï et al., 2006; Gowans et al., 2018; Suganuma and Workman, 2018). Thus, chromatin offers the means to shape responses based on their outcome, or in other words, informed adjustments to responses. It is important to note that not all phenotypic responses are adaptive, as they do not necessarily improve fitness (the contribution of genetic information to the next generation; Van Kleunen and Fischer, 2005; Ghalambor et al., 2007; Acasuso-Rivero et al., 2019). Hence, maintaining or losing chromatin-encoded memories is ultimately determined by natural selection and complements the driving role of phenotypic plasticity in evolution (Gilbert et al., 2015; Skúlason et al., 2019). Therefore, the succession of events in an individual's lifetime and the associated behaviors provide a source of variation for natural selection, which can contribute to evolution when linked to chromatin.

In addition to affecting adaptation through behavior, chromatin changes also have direct effects on genetics. Studies focusing on different organisms and contexts have shown that chromatin can influence evolution. For instance, methylcytosine induces dynamic changes to DNA, leading to faster mutation rates (Shen et al., 1994; Nabel et al., 2012). Moreover, cross-over events are influenced by epigenetic marks, as shown in *Arabidopsis* (Shilo et al., 2015). Transposable elements, which are strongly controlled by DNA methylation, can induce genetic change when active, and influence the long-term expression of neighbouring genes as highlighted in soybean (El Baidouri et al., 2018). Importantly, epigenomes, or passed-on chromatin information, can provide stable and substantial variation for evolution, as shown with the study of epigenetically-different recombinant inbred lines (Cortijo et al., 2014; Kooke et al., 2015; Zhang et al., 2018). A study of the classical Darwin's finches suggest that epigenomes can provide higher variation for natural selection than genomes (Skinner et al., 2014), while habitat adaptation in freshwater snail has been attributed to environmentally-induced epigenetic change (Thorson et al., 2017). It was argued that, because epigenetic change can generate adaptive phenotypes faster than genetic change, clonal plants which are already relying on epigenetic variability for phenotypic plasticity will easily adapt to global environmental change (Dodd and Douhovnikoff, 2016). Overall, chromatin enables rapid change and can generate variability for evolution.

6.7 The evolution of cold-adaptive phenotypic plasticity in temperate grasses

Temperate-climate adaptive plasticity in temperate grasses (Pooideae), linked to improved winter survival and timely flowering, is regulated by mechanisms of transcriptional memory linked

to changes in chromatin (Oliver et al., 2009; Chen and Dubcovsky, 2012; Oliver et al., 2013; Woods et al., 2017; Huan et al., 2018; Mayer and Charron, 2020b). While vernalization is an example of phenological plasticity, cold acclimation is generally defined as an example of physiological plasticity. Morphological plasticity induced by cold exposure, known to be important for winter survival in arctic species, was recently described in *B. distachyon* (Körner, 2016; Mayer et al., 2020). Interestingly, cold-induced phenological, physiological and morphological responses are regulated by *VRN1* and by transcriptional memories in *B. distachyon* (Mayer et al., 2020; Mayer and Charron, 2020b). Hence, temperate-climate adaptive plasticity is regulated by *VRN1* which, as mentioned in the previous section, is itself regulated by a transcriptional memory linked to levels of H3K4me3 and H3K27me3 (Oliver et al., 2009; Woods et al., 2017; Huan et al., 2018).

VRN1 is regulated by an environment-responsive epigenetic mechanism that involves the chromatin modifying complexes PRC2 and Trithorax-group proteins (TrxG), conserved in the regulation of temperature-controlled flowering (Alvarez-Venegas, 2010; Diallo et al., 2012; Bratzel and Turck, 2015). PRC2 and TrxG are important regulators of plant stress and developmental responses (Kleinmanns and Schubert, 2014) and recent progress on the conserved PRC has highlighted the importance of this epigenetic modifying complex in conferring plasticity to organisms facing environmental change (Marasca et al., 2018). Hence, have *VRN1* and its central function in cold adaptation evolved from initial cold-stress responses? The vernalization response is a conserved trait in Pooideae that has radiated from tropical to temperate climates (McKeown et al., 2016). At the core of this radiation, studies have highlighted the occurrence of gene duplication events at the origin of cold acclimation and vernalization (Sandve et al., 2008; Sandve and Fjellheim, 2010; Zhong et al., 2018). Indeed, cold-stress genes have diverged from stress-responsive ancestors (Zhong et al., 2018), and *VRN1*, a relative of the *APETALA1/FRUITFULL* (*AP1/FUL*) family of MADS box transcription factors that mainly function in inflorescence and floral development, has likely duplicated along with its *FUL2* paralog (Preston and Kellogg, 2006; 2008). Such duplication events and the evolution of paralogs can also be driven and mediated by chromatin (Rodin and Riggs, 2003; Zheng, 2008; Keller and Yi, 2014). Although it is difficult to show whether *VRN1* may have arisen through epigenetically-driven gene duplication and functional change, its origin, the regulation of its expression by a

transcriptional memory, and its involvement in stress response and phenotypic plasticity suggest that the evolution of *VRN1* is closely connected to chromatin.

Recent work in *Brachypodium hybridum*, a polyploid species that resulted from a hybrid cross between *B. distachyon* and *B. stacei*, indicates that although *VRN1* expression is increased by cold exposure the expression of *VRN1* has no influence on flowering time (Mayer and Charron, 2020a). This work suggests that the function of *VRN1* in cold-adaptive phenotypic plasticity in *B. hybridum* mainly functions in regulating freezing tolerance, and indeed, growth and development in *B. hybridum* is less restricted by cold and photoperiod, which likely explains its radiation into various environments (Mayer and Charron, 2020a). It is increasingly recognized that cycles of diploidy-polyploidy are important steps in the evolution of plant phenotypes, polyploidization providing high genetic variation potential for evolution and diploidization occurring along with specialization, which are both regulated by epigenetic processes (Chen and Ni, 2006; Dodsworth et al., 2016; Alix et al., 2017; Bird et al., 2018; Hajheidari et al., 2019). Therefore, chromatin plays a role in shaping the expression of genomes, the outcome of genetic material in polyploids and the evolution of phenotypic plasticity.

6.8 Chromatin and the regulation of plasticity

Chromatin is an updatable information storage unit that can respond based on developmental stage and past experiences behaving like a decision-making platform. The interaction between chromatin and genomes indicate a route where environments can influence genetics, and which can drive adaptation. Therefore, chromatin and epigenomes are the convergence point between environmental and genetics, offering the possibility to imprint tuned gene regulation. It is important to note, however, that each tissue and cell tend to possess resilient epigenetic identities that are not largely affected by environmental signals. Instead, it is the consensus of cell colonies in each plant that can induce a strong phenotypic response (Berry et al., 2015). These can individually carry environmentally-induced epigenetic memory and maintain a high communication which is unsurprisingly important for development and stress response (Van Norman et al., 2011; Berry et al., 2015). Hence, understanding the mechanisms of environmentally-induced plasticity is a complex problem that likely involves a network of cell-specific epigenomes and their consensus. This network is at least an immensely complex source of information that ultimately coordinates responses into coherent phenotypes.

6.9 Future directions and outstanding questions

Studies that have focused on stress responses and memories have elucidated the immediate effects of stress-induced change. However, it will be interesting to test if chronic stress conditions can, over generations, drive change in stress responses networks or offset environmentally-cues development, especially as global warming will continue to destabilize environments and to challenge plant populations (Cook et al., 2012). Then, could we breed crops for high plasticity and maintain food production without costly trade-offs on fitness? Specifically, will it be possible to untie linked developmental and stress responses without the extensive genomic change observed in *B. hybridum* (Mayer and Charron, 2020a)? Addressing these questions becomes critical if we are to build an agricultural system that is flexible enough to continue producing under the consequences of climate change.

6.10 Author contributions

BFM drafted the manuscript, JBC contributed critical comments, BFM and JBC prepared the manuscript.

6.11 References

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7. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

The work presented in this thesis highlights the role of transcriptional memories in regulating cold acclimation and vernalization in Brachypodium grass. The study presented in Chapter 3 showed that *VRN1* is involved in the regulation of vernalization and of physiological and morphological cold acclimation. Importantly, the expression of *VRN1*, crucial for the acquisition of a winter-hardy and a flowering-competent phenotype, is regulated by a transcriptional memory. Moreover, the work presented in Chapter 4 show that the physiological and morphological responses of cold acclimation are regulated by transcriptional memories that modulate the progression of cold-stress responses. Therefore, transcriptional memories regulate cold-induced phenotypic plasticity in *B. distachyon*.

Transcriptional memories regulate the expression of phenotypic traits in response to the environment, in this case the traits that lead to freezing tolerance and flowering. However, the work presented in Chapter 4 demonstrates that transcriptional memories also modulate cold-stress responses over time, specifically that they are involved in the process of perceiving and responding differently to diurnal-freezing cycles when these are repeatedly applied. Therefore, transcriptional memories regulate both the expression of phenotypic traits and the plasticity of responses, *i.e.* the capacity of responses to change over time in the environment.

This observation raises an interesting question: when responses change over time, are transcriptional memories established following a pre-determined sequence of events, or do transcriptional memory mechanisms modulate responses based on the current environment? Pre-determined events can simply follow one another, for example early-response genes inducing the expression of secondary genes and so on. Over time, however, the growth environment is likely influencing how responses unfold, perhaps functioning like a feedback mechanism or through stress-responses. Hence, transcriptional memory mechanisms likely function both ways, by regulating the expression of pre-determined traits and by adjusting gene expression depending on the environment. Although this has not been directly tested in this thesis, the results presented in Chapter 4 support this dual function by showing that the establishment of some but not all transcriptional memories are conserved between plants exposed to diurnal-freezing and to chilling.

As presented in Chapter 5, the establishment of transcriptional memories was also observed in *B. hybridum* during cold acclimation, but induced a less specialized primed response. Notably, *B. hybridum* showed no vernalization response and its growth was less restricted by photoperiod and chilling than *B. distachyon*. Hence, the cold-induced phenotypic plasticity observed in *B. distachyon* has evolved in *B. hybridum* through the dissociation of flowering from temperate-climate adaptive plasticity, along with partly maintained transcriptional memory mechanisms. Chapter 6 hence presented a discussion on the possible role of chromatin in regulating transcriptional memories and phenotypic plasticity, and in linking plasticity to adaptation.

Overall, cold acclimation and vernalization are both regulated by mechanisms of transcriptional memory.

7.2 Future directions

7.2.1 Chromatin and phenotypic plasticity

The regulation of transcriptional memories by chromatin indicated that chromatin was likely involved in regulating and coordinating phenotypic plasticity. The experiments performed in this thesis, however, did not directly test the role of chromatin in regulating phenotypic plasticity in Brachypodium grass. Instead, this work has laid the foundations for attempting to answer the following question: does chromatin regulate phenotypic plasticity in Brachypodium grass?

A first step to deepen the analysis of transcriptional memories and chromatin changes is to perform chromatin immunoprecipitation followed by sequencing (ChIP-seq) on the memory marks H3K27me3, H3K4me2/3 and bisulfite conversion of genomic DNA followed by sequencing (MethylC-seq) to obtain a map of these chromatin marks during cold acclimation and vernalization of Brachypodium in diurnal-freezing. These results will likely highlight differentially regulated regions of the genome and will provide new insights on the regulation of transcriptional memories, especially if ChIP-seq and MethylC-seq data are layered onto RNA-seq data. Another useful avenue is to attempt to find the regulators of transcriptional memories. A genetic screen could be performed to identify these, by mutagenesis on a transgenic background that can report the establishment of transcriptional memories. For example, the transfer DNA can contain a reporter gene fused to the promoter of a memory gene which show rapid transcriptional change (*e.g. CBF*s as identified in Chapter 4).

To test whether chromatin is directly involved in regulating cold-induced phenotypic plasticity in Brachypodium, one can treat plants with chromatin-modifying compounds (*e.g.* azacytidine or zebularine that inhibit the maintenance of DNA methylation) and investigate the cold acclimation and vernalization responses of treated plants to diurnal-freezing, including the establishment of transcriptional memories. It is important to note, however, that extensive chromatin changes can be connected to genome structure events, and that both are connected to phenotypic plasticity. For instance, significant genome events like hybridization and polyploidization, which gave rise to *B. hybridum*, usually allow plants to escape evolutionary bottlenecks (Chen 2007). In turn, environmental stress accelerates the divergence between the regulation of duplicate genes in polyploids, more so than internal developmental signals, which seems to facilitate sub-functionalization, neo-functionalization and adaptive stress-response mechanism that occur in pair with changes in chromatin (Lynch et al. 2000, Lynch et al. 2001, Seoighe et al. 2004, Ha et al. 2007, Springer et al. 2016). Therefore, novel phenotypes are often the result of the interaction between genome-chromatin-environment. Treating with chromatin-modifying compounds, like DNA methylation inhibitors, can hence also induce genomic change, for example through the activity of transposable elements silenced by DNA methylation. Hence, to investigate the effects of chromatin on phenotypic plasticity one has to also monitor changes in genome structure, especially during long-term experiments.

Chromatin can also encode traits which are inherited, hence epigenetic. Studying chromatin change and phenotypic plasticity over generations can also provide useful insights on their contribution to adaptation.

7.2.2 Generating phenotypic variation

As transcriptional memories and chromatin regulate phenotypic plasticity, their regulatory mechanisms can be used to generate phenotypic variation in plants. A first step to facilitate the generation of phenotypic variation would be to induce changes in chromatin structure which can be done by treating plants with chromatin-modifying compounds or by transferring plants into tissue culture through callus formation (undifferentiated plant tissue) which typically induces substantial changes in chromatin and genome structure (M Lee et al. 1988). Called somaclonal variation, the phenotypic variation produced by tissue culture has been useful for crop improvement and variety development.

Phenotypic variation can then be encouraged, in a second step, by exposing plants to specific signals or challenging conditions. Typically, phenotypic variation produced from somaclonal variation occurs in standard culture conditions. Hence, inducing variation under environmental stress is not typically attempted, even though stress responses facilitate the evolution of adaptive mechanisms and of new gene functions (Lynch et al. 2000, Lynch et al. 2001, Seoighe et al. 2004, Chen 2007). Therefore, exposing plants to challenging conditions after destabilizing their chromatin structures can generate phenotypic variation that could be adaptive, especially at the proximate (immediate) level. Nonetheless, this would have to be determined experimentally. Complementarily to this approach, the potential for phenotypic variation can be further enhanced or depressed, for instance by overexpressing writers or by knocking down/out erasers of transcriptional memories. The outcome of generating phenotypic plasticity can also be tested using transgenics such as these to infer the function of specific chromatin modifiers.

A third possible step could consist in fixing the newly generated phenotypes. This can be done by repeating the stress treatment in subsequent generations. For instance, experiments performed in insects showed that heat shock can generate novel phenotypes. Through "genetic assimilation" or "genetic accommodation", these phenotypes became fixed (expressed without the need of a stimulus) after several generations through artificial selection and by repeating the inductive stimulus (Waddington 1953, Suzuki et al. 2006). This can also occur in plants, as phenotypic plasticity contributed to invasiveness of Jerusalem artichoke and likely to the domestication of maize, both through genetic accommodation (Lorant et al. 2017, Bock et al. 2018). Although novel phenotypes would likely be random as observed with somaclonal variation, there is a possibility that phenotypes that are also adaptive can be generated (Bateson 2017). The meaning of the adjective "adaptive" here is especially important, as what is adaptive in a natural context may not be so in an agricultural context, and vice-versa. Hence, these phenotypes may provide an advantage in agricultural settings, but not in nature. For example, this discrepancy is especially important for using group selection in breeding, which is likely very rare in the natural environment but is useful for agriculture (Weiner et al. 2010, Weiner et al. 2017).

A specific experiment can be designed following the approach explained above on *B. hybridum*. As an allopolyploid, *B. hybridum* presents a high potential for phenotypic variation. Hence, *B. hybridum* can be treated with chromatin-modifying compounds and/or placed into tissue culture for calli generation, then exposed to various stress conditions to induce phenotypic

variation. Preliminary experiments have shown that as calli, *B. hybridum* can withstand chilling and diurnal-freezing and can easily regenerate into plants while under diurnal-freezing, whereas similar attempts performed with *B. distachyon* have repeatedly failed. *B. hybridum* hence shows a remarkable vigorousness when faced with stress conditions, indicating that it is a promising candidate for stress-induced variation experiments. It would be interesting to determine if a vernalization requirement can be imprinted onto *B. hybridum*, as the molecular machinery associated with the transcriptional memory of *VRN1* is likely still present, as indicated in Chapter 5.

Determining whether such an *in situ* generation of phenotypic variation provides advantages or can accelerate the process of adaptive evolution, especially in agricultural settings, will be important if there is a possibility that it can contribute to sustainable intensification, to the recruitment of new crops and to participatory plant breeding. Nonetheless, studying the use of phenotypic plasticity to induce variation or to breed plants in a specific context presents an interesting avenue towards understanding the mechanisms of evolution in plants.

8. APPENDICES

Appendix 1 Supplementary information for Chapter 3

Appendix 1 Figure 1. Freezing tolerance in CC-treated *B. distachyon* and associated chilling stress.

Appendix 1 Figure 2. *VRN1* transcript levels in relation to days to heading in CC and DF treated vernalization-requiring Bd18-1.

Appendix 1 Figure 3. Expression of *VRN1* and *FT* in non-vernalized, vernalized in CC and DF, and flowering post-vernalization *VRN1* transgenic lines.

Appendix 1 Figure 4. The compact plant structure produced by DF may better insulate crown tissues.

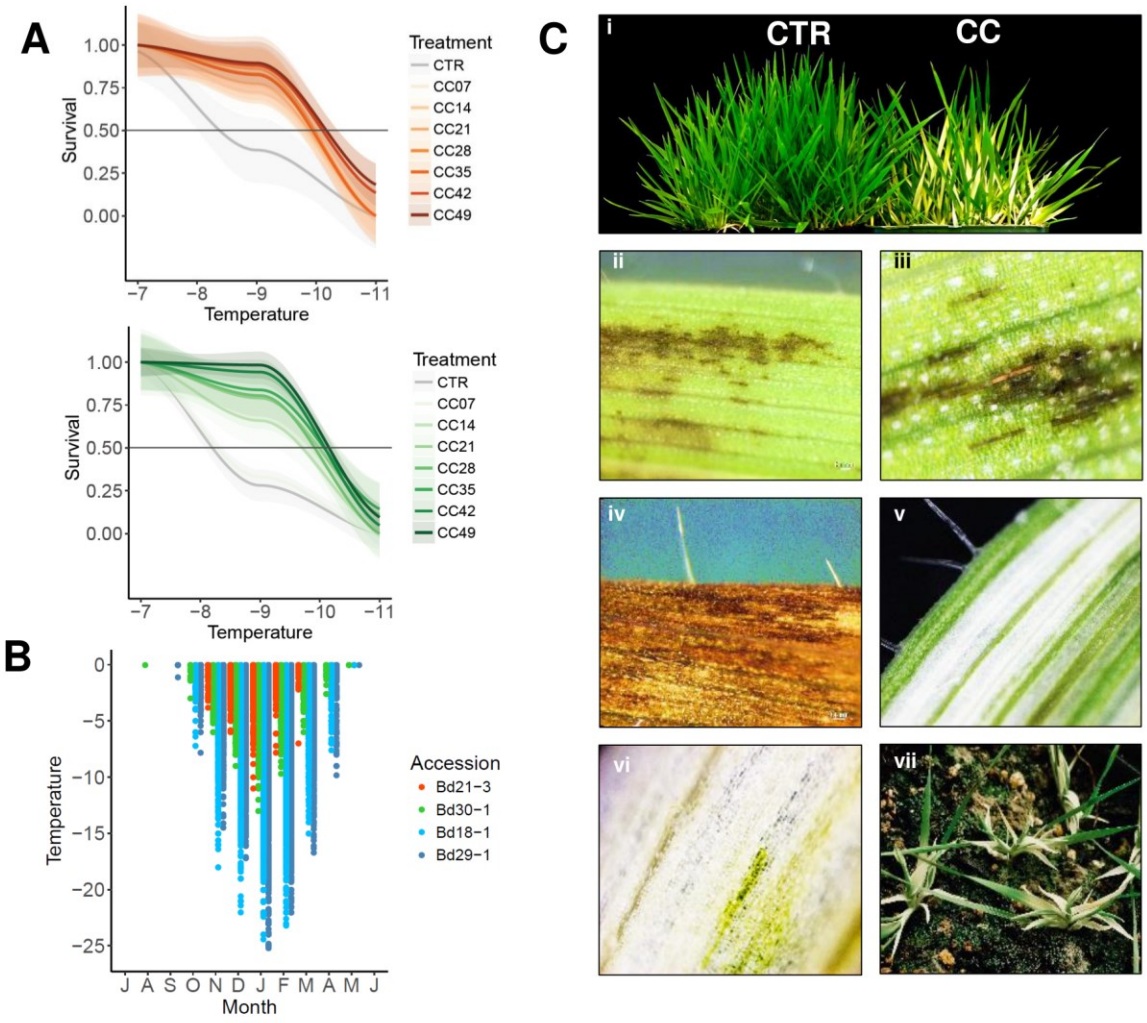
Appendix 1 Figure 5. Phenotype of Bd21-3 and Bd18-1 in response to CC and DF at 7-56 days of exposure.

Appendix 1 Figure 6. Phenotype of DF56 *VRN1* transgenic plants at senescence.

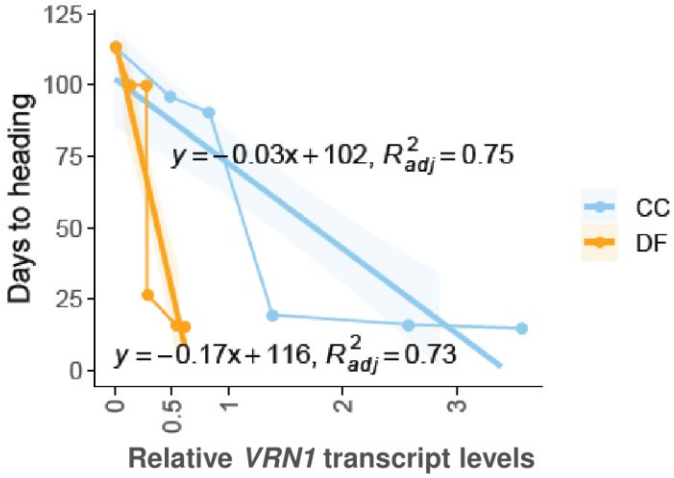
Appendix 1 Table 1. Summary of the dataset on habitats H1-4 and the diurnal-freezing treatment (DF), and the detailed temperature and light cycles of DF.

Appendix 1 Table 2. Accessions selected for this study, the corresponding geographic location of their parental seed collection site and associated climate.

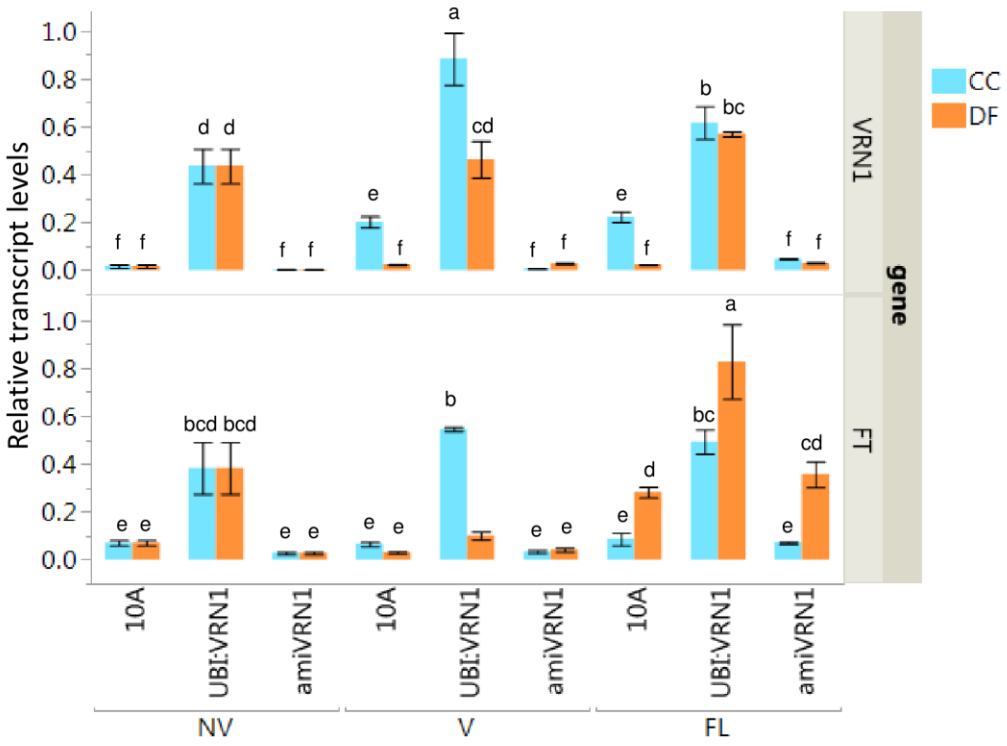
Appendix 1 Table 3. Primers used in this study.



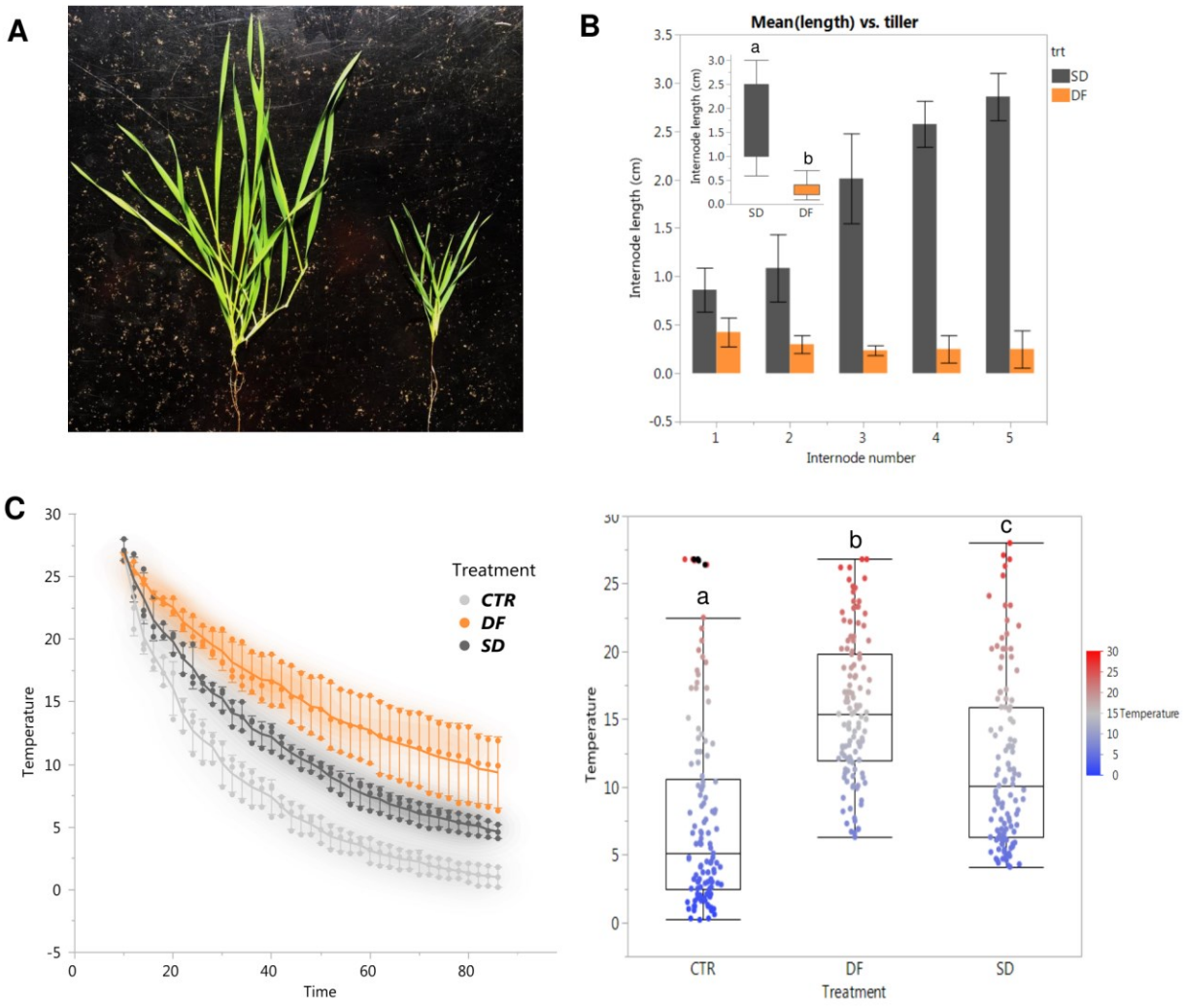
Appendix 1 Figure 1: Freezing tolerance in CC-treated *B. distachyon* and associated chilling stress. (**A**) LT50 obtained from whole-plant freeze test in Bd21-3, Bd30-1, Bd18-1 and Bd29-1 under CC at 7-49 days of exposure after cold acclimation under short-day (8/16 hours light/dark, top panel) or long-day (16/8 hours light/dark, bottom panel) photoperiod regimes. (**B**) Freezing temperatures observed at the parental collection sites of Bd21-3, Bd18-1, Bd30-1 and Bd29-1. (**C**) Visible signs of chilling stress under CC in Bd30-1 (ii, v-vii) and Bd21-3 (ii-iv).



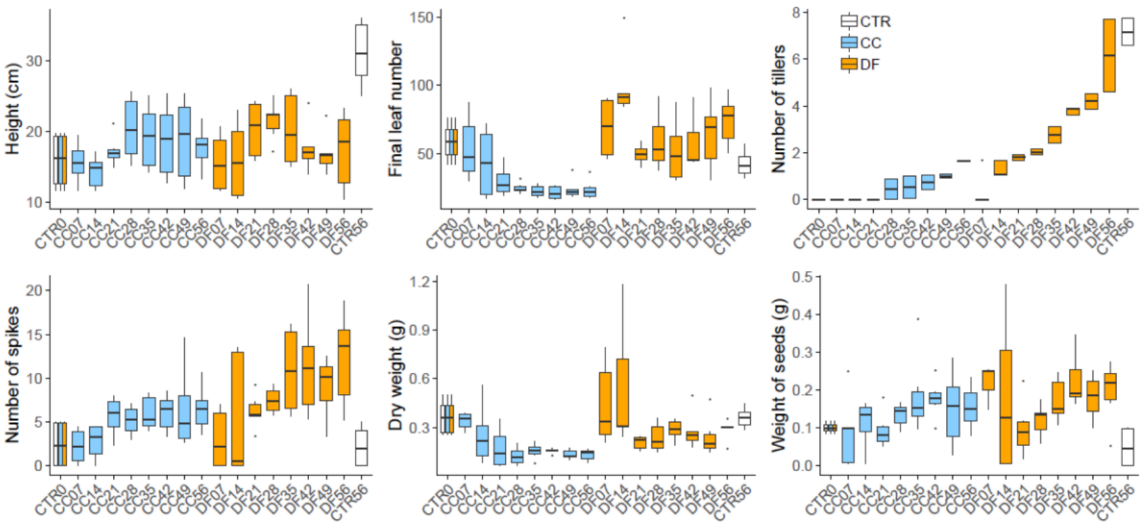
Appendix 1 Figure 2: *VRN1* transcript levels in relation to days to heading in CC and DF treated vernalization-requiring Bd18-1. Linear regression of the form *y=mx+b* was fitted on both CC and DF-induced days to heading (*y*) relative to VRN1 levels (*x*) under both CC and DF.



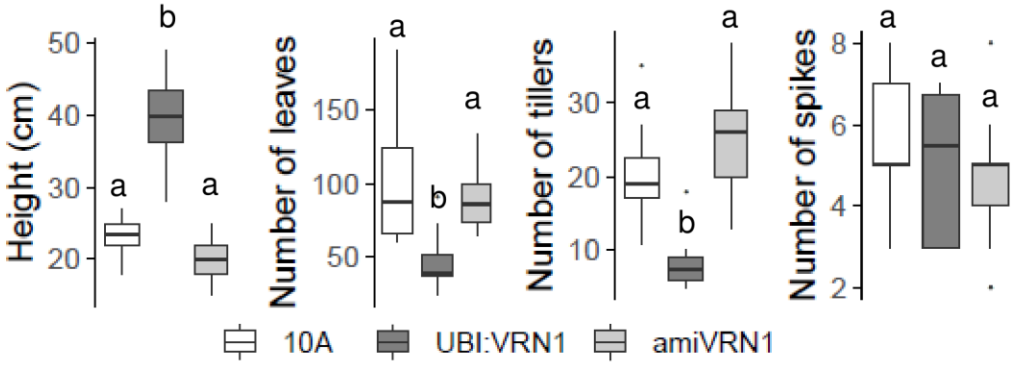
Appendix 1 Figure 3: Expression of *VRN1* and *FT* in non-vernalized, vernalized in CC and DF, and flowering post-vernalization *VRN1* transgenic lines. Relative transcript levels of *VRN1* and *FT* in transgenic lines empty-vector pANIC 10A control (10A), *VRN1* overexpressor (UBI:*VRN1*) and *VRN1* knock-down (ami*VRN1*) grown under non-inductive control (non-vernalized; NV), vernalized under CC or DF (vernalized; V), or after a week under flowering conditions (flowering; FL) after a vernalization treatment.



Appendix 1 Figure 4: The compact plant structure produced by DF may better insulate crown tissues. (**A**) Internode length between tillers in control plants (short-day 22ºC; SD) and DF-treated plants (DF). (**B**)Temperature measured on the surface of crown tissues when exposed to a cold temperatures of SD and DF plants. CTR is the decrease in temperature of the exposed temperature probe. Time in seconds.



Appendix 1 Figure 5: Phenotype of Bd21-3 and Bd18-1 in response to CC and DF at 7-56 days of exposure. CTR: control plants at 0 and 56 days of age (CTR0 and CTR56), CC: constant-chilling (CC7-56). DF: diurnal freezing (DF7-56).



Appendix 1 Figure 6: Phenotype of DF56 *VRN1* transgenic plants at senescence. DF56 plants were moved to flowering conditions (22°C 16 hours lights) until scenescence. Empty vector control (10A), overexpressor (UBI:VRN1) and VRN1 knock-down (amiVRN1) transgenic lines.

	Habitats H1-4				Diurnal freezing
	Mean temperature (°C)	Diurnal temperature range (°C)	Daily hours of light (h)	Frequency of frost days (days/month)	Temperature (°C)
Min.	-6.00	4.20	8.90	0.00	-1.00
1st Qu.	8.60	9.00	10.39	0.00	0.00
Median	14.30	10.80	12.19	0.39	6.25
Mean	14.71	11.25	12.19	5.01	8.71
3rd Qu.	21.10	13.20	14.02	7.55	18.25
Max.	36.90	21.20	15.48	29.23	22.00

Diurnal freezing (DF) 24 hour cycle	Hourly change in temperature (°C)	18	10	8	5	3	1	0	-1	-1	-1	-1	-1	0	2	5	8	12	16	19	21	22	22	22	21
	Hours of light																							

Appendix 1 Table 1: Summary of the dataset on habitats H1-4 and the diurnal-freezing treatment (DF), and the detailed temperature and light cycles of DF.

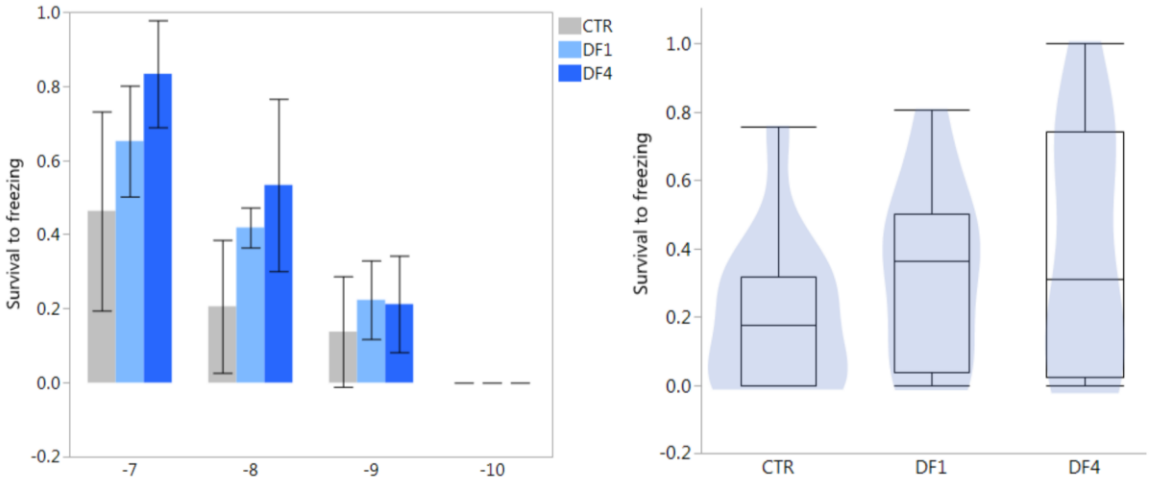
Accession				Geographic location					Climate
Line	Original seed stock	Growth habit	Meteo station	Country	Latitude	Longitude	Elevation (m)	Distance from seed collection site (km)	Climate (Köppen-Geiger classification)
Bd21-3	PI254867*	Facultative		Iraq	**35.814034**	**42.926296**	**200**		**Warm semi-arid (BSh)**
			Al-Hasakah	Syria	36.5	40.75	308	210	Warm semi-arid (BSh)
			Deir ez-Zor		35.317	40.15	212	250	Desert (BWh)
			Al Bukamal		34.417	40.917	182	240	Desert (BWh)
Bd30-1		Facultative		Spain	**36.990489**	**-3.558733**	**1200**		**Hot-summer Mediterreanean (Csa)**
			Granada		37.183	-3.783	570	20	Hot-summer Mediterreanean (Csa)
			Cordoba		37.85	-4.85	92	100	Hot-summer Mediterreanean (Csa)
			Gibraltar		36.15	-5.35	5	180	Hot-summer Mediterreanean (Csa)
			Cuidad Real		38.983	-3.917	629	220	Hot-summer Mediterreanean (Csa)
Bd18-1	245730	Winter		Turkey	**39.353038**	**33.749648**	**1000**		**Warm-summer Mediterreanean/Cold semi-arid (Csb/Bsk)**
			Etimesgut		39.95	32.683	806	105	Cold semi-arid (Bsk)
			Corum		40.55	34.95	776	170	Warm-summer Mediterreanean (Csb)
			Konya		37.967	32.549	1031	185	Cold semi-arid (Bsk)
			Kayseri/Erkilet		38.817	35.433	1054	150	Cold semi-arid (Bsk)
Bd29-1	639818*/UKR-99-081*	Winter		Ukraine	**44.515278**	**33.556389**	**260**		**Humid subtropical/Oceanic (Cfa/Cfb)**
			Yalta		44.483	34.167	72	48	Humid subtropical (Cfa)
			Simferopol		44.683	34.133	181	65	Oceanic (Cfb)
			Yevpatoria		45.183	33.367	6	75	Humid subtropical (Cfa)

Appendix 1 Table 2: Accessions selected for this study, the corresponding geographic location of their parental seed collection site and associated climate. The meteorological stations in bold were selected for Fig. 2A-C and Fig. S1C from the presented stations in *HadISD: Global sub-daily, surface meteorological station data, 1931-2017, v2.0.2.2017f.*

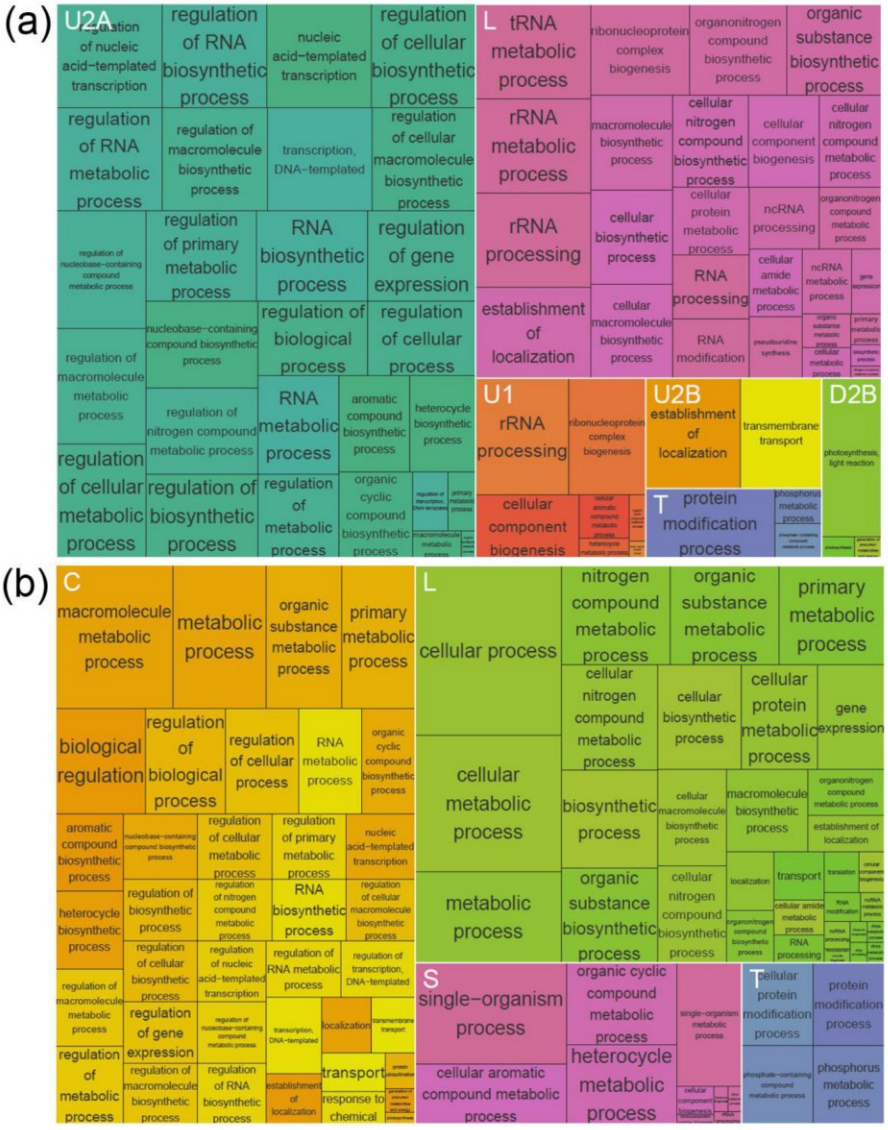
Primer	Analysis	Primer sequence	Reference
UBC18_F	RT-qCPR	GTCACCCGCAATGTCTGTAAGTTC	(*18*)
UBC18_R	RT-qCPR	TTGTCTTGCGGACGTTGCTTTG	(*18*)
VRN1_F	RT-qCPR	GCTCTGCAGAAGGAACTTGTGG	(*18*)
VRN1_R	RT-qCPR	CTAGTTTGCGGGTGTGTTTGCTC	(*18*)
CBF1_F	RT-qCPR	ACCCGTACTACGAGATGGGC	(*19*)
CBF1_R	RT-qCPR	ATCGGAGGAGGGTCAATGAG	(*19*)
CBF2_F	RT-qCPR	GTGGCGCAGTCGTCTTCTT	(*19*)
CBF2_R	RT-qCPR	GCTGGTCCTGCPAGTCACAC	(*19*)
CBF3_F	RT-qCPR	TCGTCCTCCCTCACTGACAA	(*19*)
CBF3_R	RT-qCPR	GCGTAGTAGAGGTCCCAGCC	(*19*)
IRI(Bradi5g27350)_F	RT-qCPR	TCTGGGACCTACCATGTCGT	
IRI(Bradi5g27350)_R	RT-qCPR	CGGACATGAGCTTCGTCAGT	
COR410(Bradi3g51200)_F	RT-qCPR	AGCAAAAGCCACAAGCCAAG	
COR410(Bradi3g51200)_R	RT-qCPR	GTCAAAGAGGCCCCTATCCG	
VRN1_I1_F	ChIP-qPCR	TACGCACGCCTACGCTTAAG	(*8*)
VRN1_I1_R	ChIP-qPCR	GAAATGGAGCAGACAGGCAAG	(*8*)
VRN1_I5_F	ChIP-qPCR	GCACGGACGTGTAGGTTAAAGT	(*8*)
VRN1_I5_R	ChIP-qPCR	CACTGCCTGTGTGCATCTTC	(*8*)
VRN1_I6_F	ChIP-qPCR	GCAGGCAGCAAATAGGAGAAG	(*8*)
VRN1_I6_R	ChIP-qPCR	GAGCCAGTAGTAGCAAGGTGAGC	(*8*)
VRN1_CArG_F	ChIP-qPCR	CGACAACGGATATGCTCCAGACC	(*8*)
VRN1_CArG_R	ChIP-qPCR	GAAGAGAGCCGGAGAGTGGGT	(*8*)
CBF1_R1_F	ChIP-qPCR	CAAGAGCAGAGTAGCCCAGC	
CBF1_R1_R	ChIP-qPCR	GGCGTTAACTGGGTCGGAAC	
CBF1_R2_F	ChIP-qPCR	GGAAAAGGTGGTCCACAGGT	
CBF1_R2_R	ChIP-qPCR	TGGTCAGCCTTTTCGCTCAT	
CBF2_R1_F	ChIP-qPCR	TTTGGCGGGATCTCTTGCAT	
CBF2_R1_R	ChIP-qPCR	CGGGATTGCTATGCGTGTTG	
CBF2_R2_F	ChIP-qPCR	CCATGGGTCCAGCTTAGCAA	
CBF2_R2_R	ChIP-qPCR	GGTTTGCACCTGTCGGTCTA	
CBF3_R1_F	ChIP-qPCR	CGGTTGTACGGTATGTCGCT	
CBF3_R1_R	ChIP-qPCR	AAATCTCCGCTGGAGGAACC	
CBF3_R2_F	ChIP-qPCR	TTCGGCACTGTACGGTCACT	
CBF3_R2_R	ChIP-qPCR	ACTGAAAGCGGTGACGTCCT	

Appendix 1 Table 3: Primers used in this study

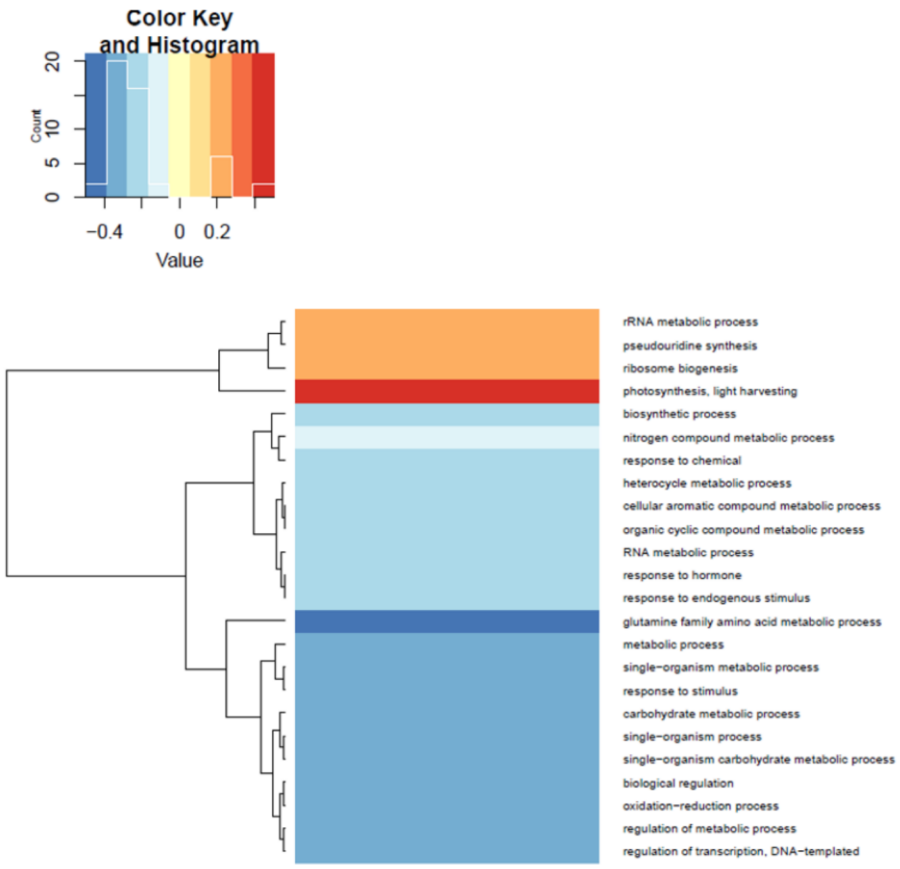
Appendix 2 Supplementary information for Chapter 4



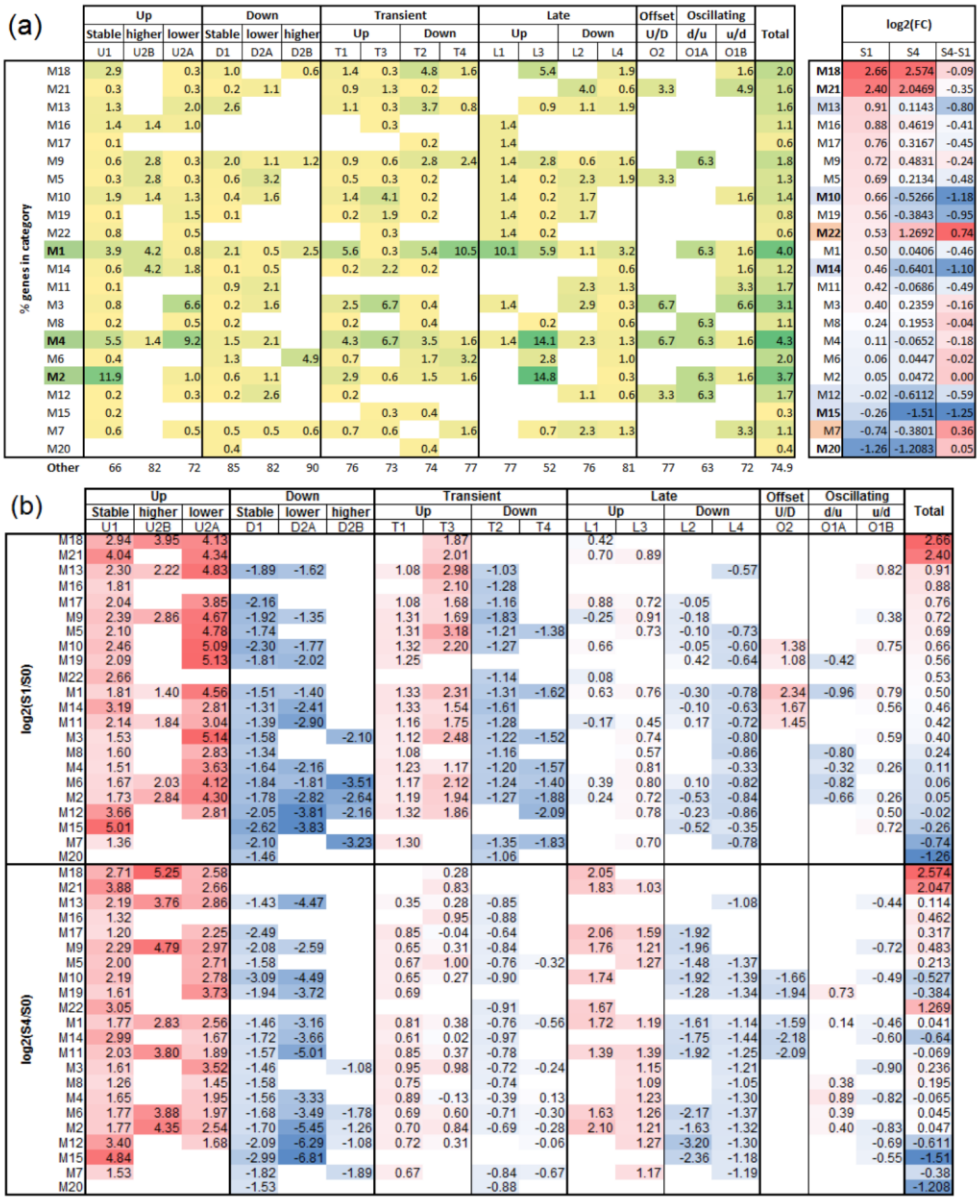
Appendix 2 Figure 1: Whole-plant freeze tests performed on plants exposed to 1 and 4 cycles of diurnal-freezing compared to non-acclimated plants



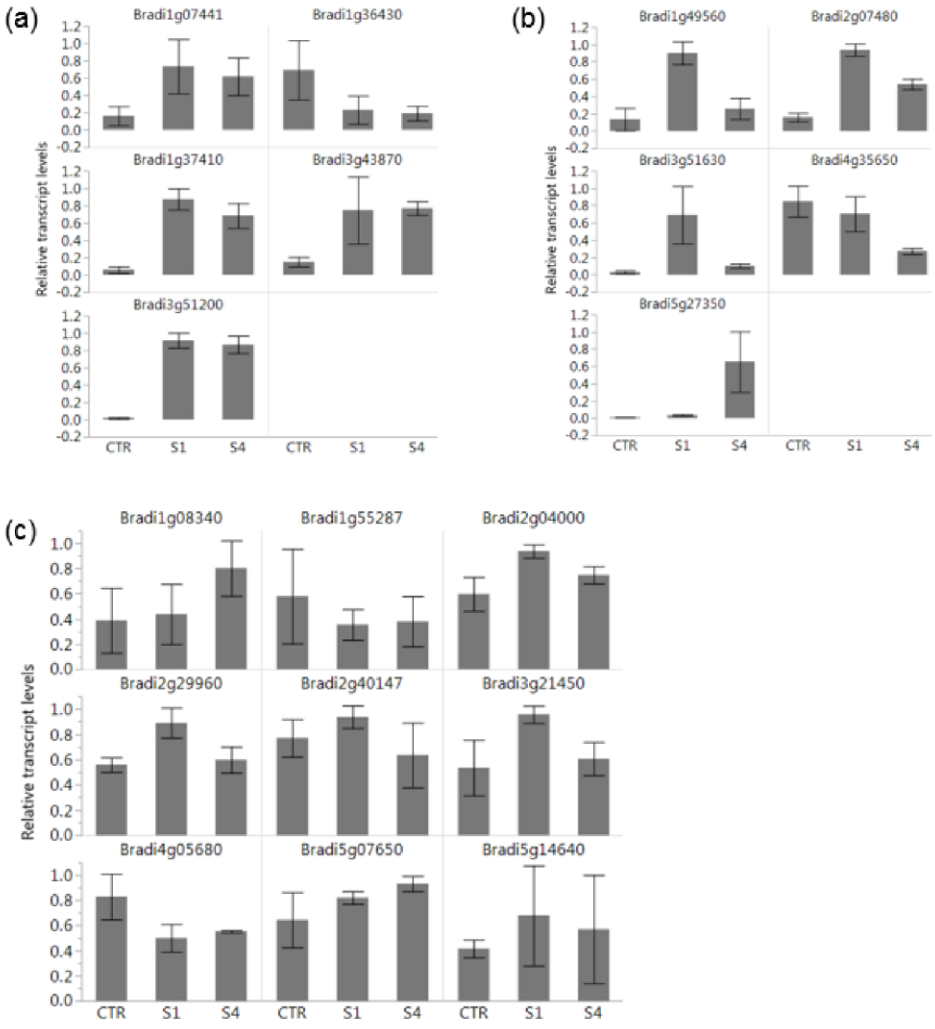
Appendix 2 Figure 2: Significantly enriched GO terms in the 17 categories regrouped into 6 expression profiles identified in diurnal-freezing responsive genes. (**a**) U2A, L (L1-4), U1, 2B, D2B, T(T1-4) are part of the 17 gene categories described in Table 1. (**b**) C (complex-convergent and complex-divergent), L (late-responsive), S (stable) and T (transient) are part of the 6 transcriptional profiles in DFRG. NB categories that are not represented returned no significantly enriched terms.



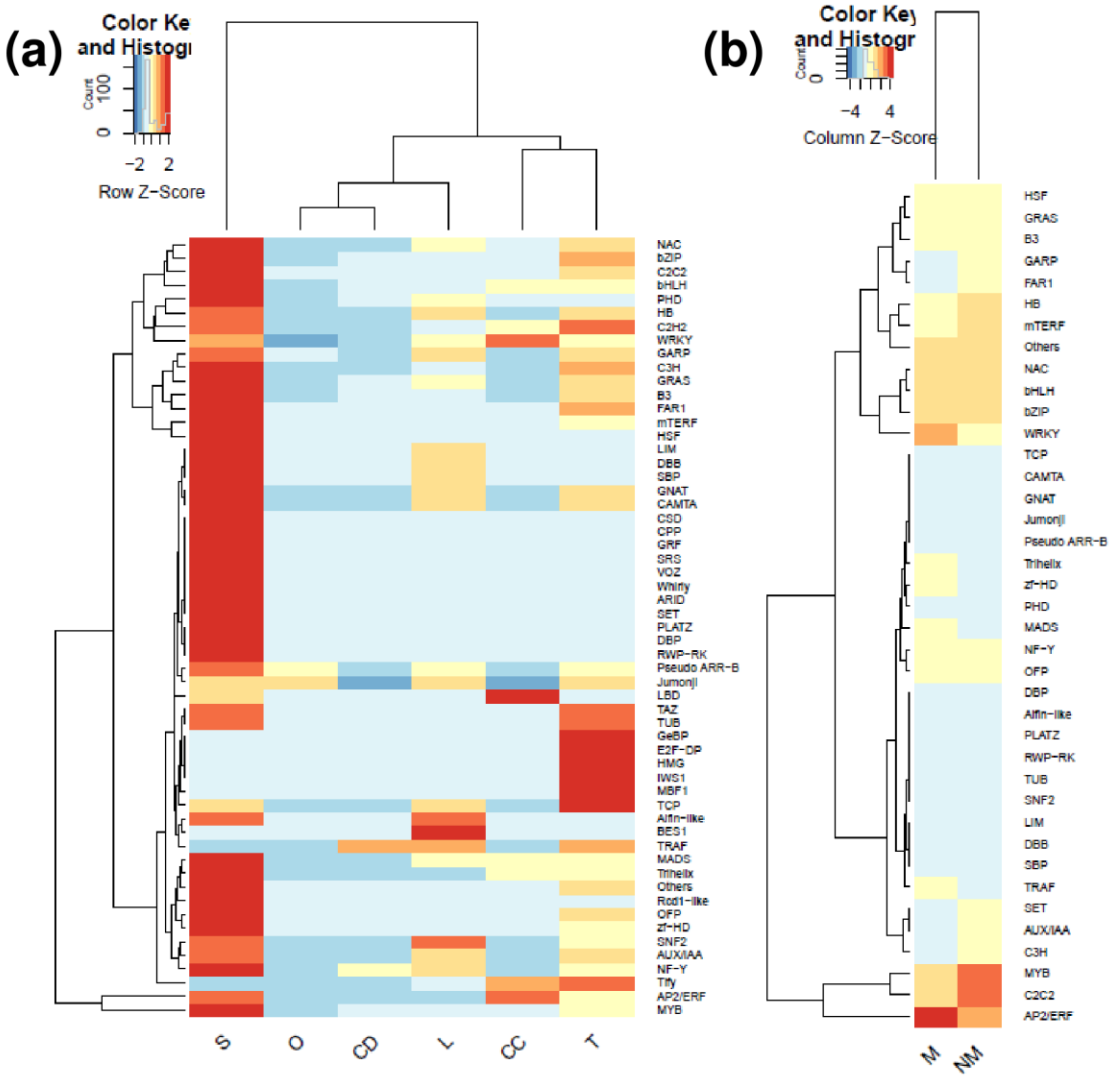
Appendix 2 Figure 3: Transcript levels of genes whose expression change in diurnal-freezing (S4/S1) that are associated with significantly enriched GO terms



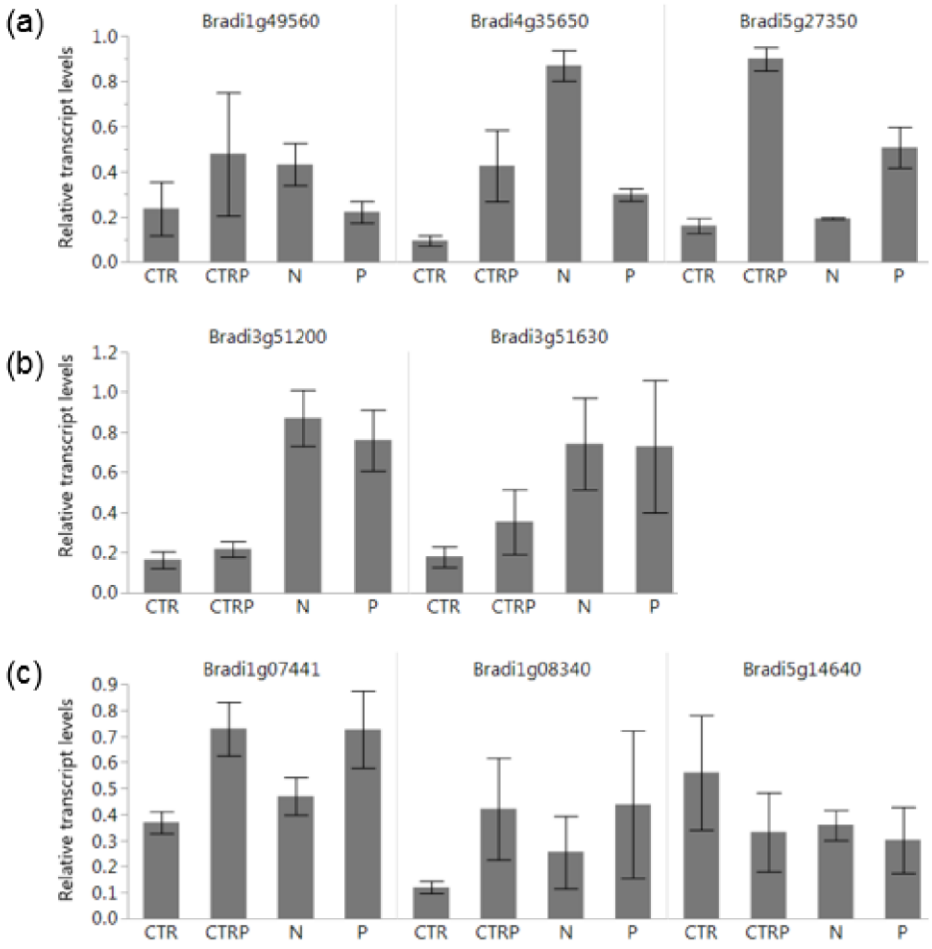
Appendix 2 Figure 4: Distribution and differential expression of diurnal-freezing responsive genes in abiotic stress response modules. (**a**) Percent of 17 diurnal-freezing responsive gene categories which belong to previously identified modules present (in yellow to green) and total fold-change associated with each module in S1 and S4 responses (in blue to red). (**b**) Fold change associated with each DFRG category x gene module. Modules were identified in Priest et al., 2014 https://doi.org/10.1371/journal.pone.0087499.



Appendix 2 Figure 5: RT-qPCR validation of RNA-seq analysis of plants exposed to diurnal-freezing. Genes identified in the RNA-seq analysis in response to one (S1) or 4 (S4) cycles of diurnal-freezing that were responsive but showed no memory (**a**), whose transcript levels changed over time in diurnal-freezing (**b**) or that were not found to be responsive to the treatment (fold change lower than 2; **c**). *This analysis was performed on three biological replicates from experiments replicated in time, error bars show standard deviation between these.*



Appendix 2 Figure 6: Families of transcription factors in the 6 expression profiles identified in diurnal-freezing. (**a**) Distribution of transcription factor and transcriptional regulator families in stable (S), complex-convergent (CC), complex-divergent (CD), transient (T), late-responsive (L) and offset/oscillating (O) genes. (**b**) Distribution of transcription factor and transcriptional regulator families in memory (CCo, CD and strong T) and non-memory genes (S, L, O and weak T).

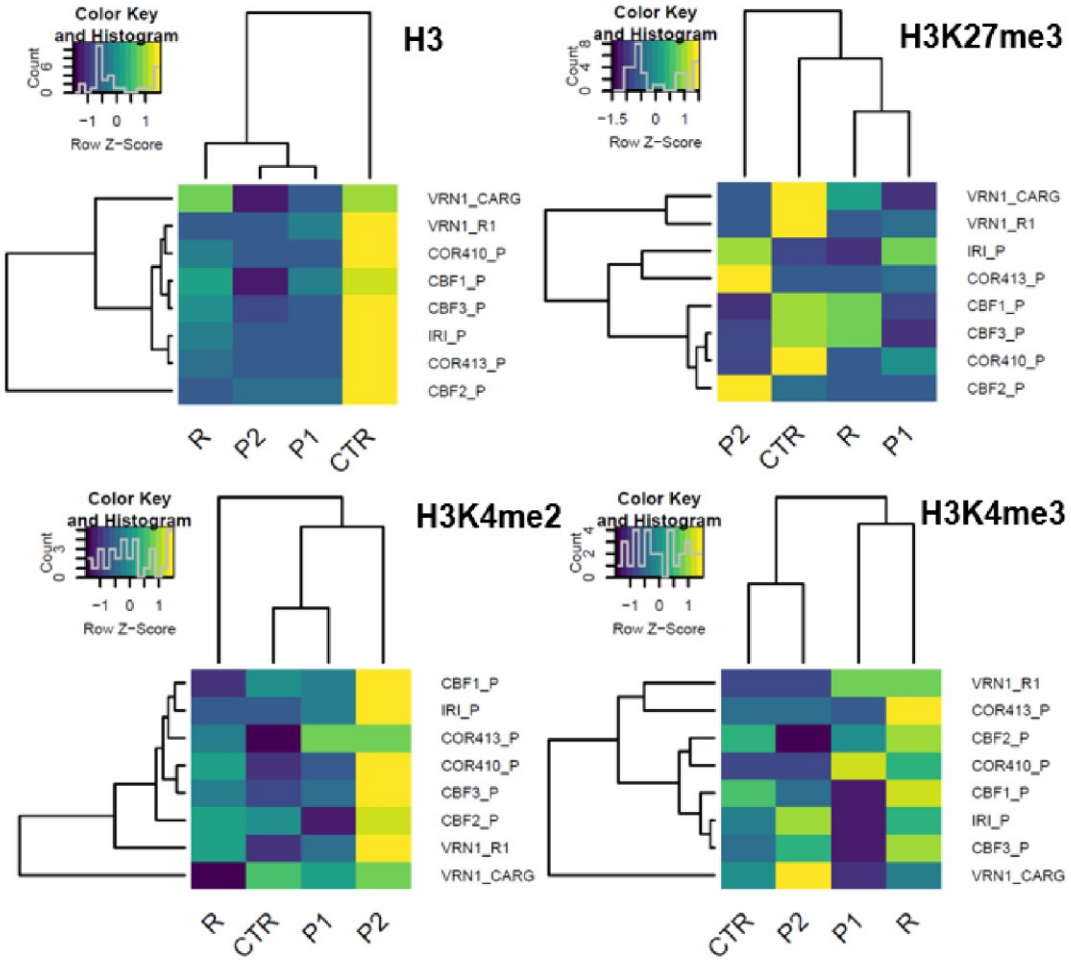


Appendix 2 Figure 7: RT-qPCR validation of RNA-seq analysis of primed plants exposed to chilling. Genes identified in the RNA-seq analysis in plants primed in diurnal-freezing and exposed to chilling (P) compared to naïve plants (N) and non-stressed primed plants (CTRP) and non-stressed control plants (CTR) that showed, according to the RNA-seq analysis, the establishment of memory in response to diurnal-freezing (a), or showed no change in response to chilling after priming in diurnal-freezing (no memory; b), or that showed no response to chilling (fold change lower than 2; c). This analysis was performed on three biological replicates from experiments replicated in time, error bars show standard deviation between these.

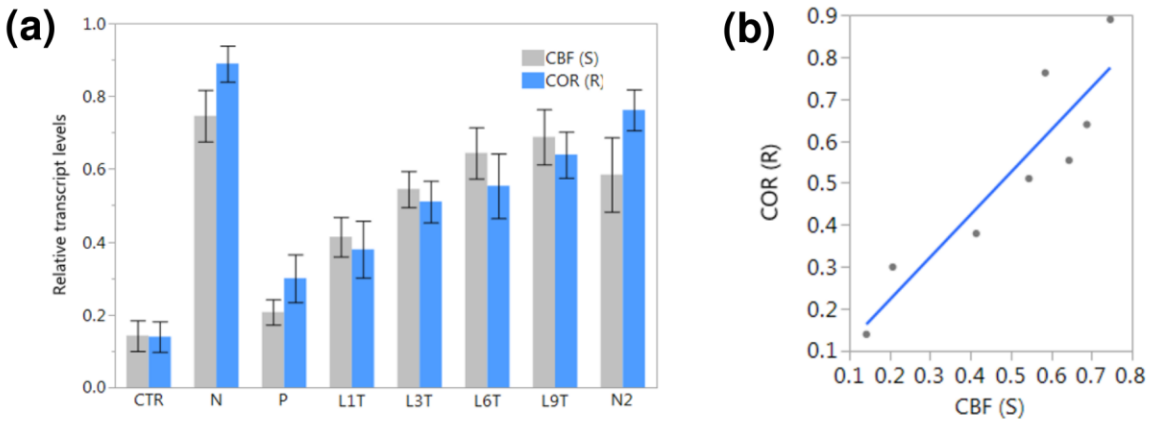
by identity to modules	uniN	uniP	shared
1	51	7	14
2	18	8	41
3	7	0	0
4	1	4	4
5	11	1	4
6	4	0	2
7	6	1	3
8	0	0	0
9	7	3	2
10	2	7	11
11	2	0	0
12	16	25	16
14	2	2	2
15	0	1	0
16	6	13	1
17	12	2	7
18	12	2	14
19	14	1	3
20	8	14	0
21	0	0	1
22	11	8	2

Appendix 2 Figure 8: B. distachyon gene modules identified in abiotic stress response and their distribution in in chilling-responsive genes

NB: *Number of genes unique to the naïve response (uniN), to the primed response (uniP) or that are found in both naïve and primed responses (shared). Modules were identified in Priest et al., 2014 https://doi.org/10.1371/journal.pone.*0087499. *Stress-response transcription factors AP2/ERF, bHLH, WRKY and C2C2 are found in module 18.*



Appendix 2 Figure 9: Chromatin marks at the loci of genes involved in cold acclimation in response to repeated priming in diurnal-freezing. Levels of histone H3 relative to input DNA (top left), H3K27me3 (top right), H3K4me2 (bottom left) and H3K4me3 (bottom right) relative to H3 on promoters of *CBFs*, *IRI*, *COR410-413* and *VRN1* and within the first intron of *VRN1* (VRN1_R1) in CTR, P1, R and P2.



Appendix 2 Figure 10: Transcript levels of CBF1-3 at stress S and COR410/413 at recovery R are positively correlated. (**a**) Relative transcript levels of averaged CBF1-3 sampled at the stress time-point in diurnal-freezing (CBF (S)) and COR410-COR413 sampled at the recovery time-point (COR (R)) in naïve (N, N2, DF1), primed (P, DF4), primed in lag for 1, 3, 6 and 9 days and triggered by 1 cycle of DF (L1-9T). NB *at all time points, CBF (S) and COR (R) were not found to be statistically different.* (**b**) Linear regression between CBF (S) and COR (R). COR = 0.019517 + 1.0116828*CBF; R^2_{adj}=0.812; Prob > F = 0.0014.

Multivariate target=CBF1_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	0.8130	-0.5908	0.5192
ip.k27	0.8130	1.0000	-0.6295	0.9198
ip.k4me2	-0.5908	-0.6295	1.0000	-0.5221
ip.k4me3	0.5192	0.9198	-0.5221	1.0000

Multivariate target=COR413_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	-0.3387	-0.9246	-0.2078
ip.k27	-0.3387	1.0000	0.4869	-0.2940
ip.k4me2	-0.9246	0.4869	1.0000	-0.1787
ip.k4me3	-0.2078	-0.2940	-0.1787	1.0000

Multivariate target=CBF2_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	-0.1950	-0.0358	0.1843
ip.k27	-0.1950	1.0000	0.7772	-0.9365
ip.k4me2	-0.0358	0.7772	1.0000	-0.5108
ip.k4me3	0.1843	-0.9365	-0.5108	1.0000

Multivariate target=IRI_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	-0.6501	-0.4670	-0.0446
ip.k27	-0.6501	1.0000	0.7573	-0.0287
ip.k4me2	-0.4670	0.7573	1.0000	0.6252
ip.k4me3	-0.0446	-0.0287	0.6252	1.0000

Multivariate target=CBF3_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	0.8018	-0.7379	-0.1059
ip.k27	0.8018	1.0000	-0.4899	0.4658
ip.k4me2	-0.7379	-0.4899	1.0000	0.4790
ip.k4me3	-0.1059	0.4658	0.4790	1.0000

Multivariate target=VRN1_CARG

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	0.8563	-0.4901	-0.3979
ip.k27	0.8563	1.0000	-0.0416	0.0141
ip.k4me2	-0.4901	-0.0416	1.0000	0.4107
ip.k4me3	-0.3979	0.0141	0.4107	1.0000

Multivariate target=COR410_P

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	0.9172	-0.6832	-0.4602
ip.k27	0.9172	1.0000	-0.8349	-0.2392
ip.k4me2	-0.6832	-0.8349	1.0000	-0.3131
ip.k4me3	-0.4602	-0.2392	-0.3131	1.0000

Multivariate target=VRN1_R1

Correlations

	ip.h3	ip.k27	ip.k4me2	ip.k4me3
ip.h3	1.0000	-0.4237	-0.7762	-0.4940
ip.k27	-0.4237	1.0000	0.8723	-0.5673
ip.k4me2	-0.7762	0.8723	1.0000	-0.1590
ip.k4me3	-0.4940	-0.5673	-0.1590	1.0000

Appendix 2 Figure 11: Correlation R^2adj between epigenetic marks at COR gene loci

(a)

		CTR-14		C1-14		R-3		C2-7	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
CBF1	H3	2.257	± 0.07 ^{b}	0.93	± 0.34 ^{c}	1.119	± 0.29 ^{c}	4.02	± 0.32 ^{a}
	H3K27me3	6.135	± 1.28 ^{ab}	8.292	± 1.46 ^{a}	4.881	± 1.52 ^{b}	1.193	± 0.15 ^{c}
	Mock	0.874	± 0.27	0.337	± 0.58	0.823	± 0.16	0.296	± 0.05
COR410	H3	9.549	± 1.25 ^{a}	1.64	± 0.29 ^{c}	4.997	± 0.16 ^{b}	6.098	± 0.3 ^{b}
	H3K27me3	7.726	± 0.56 ^{a}	5.866	± 1.29 ^{a}	3.575	± 0.45 ^{b}	1.137	± 0.26 ^{c}
	Mock	0.379	± 0.11	0.694	± 0.19	1.582	± 0.08	0.142	± 0.1
IRI	H3	11.24	± 0.86 ^{a}	2.933	± 0.47 ^{b}	9.467	± 0.67 ^{c}	19.13	± 0.56 ^{d}
	H3K27me3	10.9	± 1.22 ^{a}	4.383	± 1.45 ^{b}	4.892	± 0.4 ^{b}	1.348	± 0.05 ^{c}
	Mock	0.281	± 0.08	0.637	± 0.09	0.825	± 0.18	0.036	± 0.02
VRN1	H3	10.21	± 0.6 ^{a}	2.534	± 0.34 ^{b}	5.762	± 1.23 ^{c}	20.09	± 1.05 ^{d}
	H3K27me3	29.87	± 3.23 ^{a}	11.59	± 1.26 ^{c}	19.55	± 1.52 ^{b}	12.76	± 0.27 ^{c}
	Mock	0.333	± 0.01	2.612	± 0.64	1.724	± 0.05	0.324	± 0.11

(b)

		CTR (3h)		C1 (3h)		R (3h)		C2 (3h)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
CBF1	H3	5.62	± 0.16 ^{a}	3.20	± 0.35 ^{c}	4.49	± 0.54 ^{b}	2.33	± 0.29 ^{c}
	H3K27me3	1.75	± 0.73 ^{a}	1.82	± 0.33 ^{a}	2.63	± 0.4 ^{a}	1.71	± 0.36 ^{a}
	H3K4me2	0.95	± 0.15 ^{ab}	0.57	± 0.13 ^{b}	1.38	± 0.4 ^{a}	1.05	± 0.28 ^{ab}
	H3K4me3	13.08	± 0.9 ^{c}	17.89	± 0.51 ^{bc}	24.33	± 2.18 ^{a}	19.23	± 2.8 ^{b}
	Mock	0.67	± 0.2	0.35	± 0.49	0.18	± 0.08	0.17	± 0.16
COR410	H3	11.92	± 1.26 ^{a}	11.20	± 1.15 ^{a}	7.05	± 1.09 ^{b}	6.78	± 0.7 ^{b}
	H3K27me3	1.14	± 0.39 ^{a}	1.10	± 0.29 ^{a}	0.63	± 0.08 ^{ab}	0.36	± 0.09 ^{b}
	H3K4me2	1.39	± 0.18 ^{ab}	0.64	± 0.35 ^{b}	1.76	± 0.31 ^{a}	0.79	± 0.37 ^{b}
	H3K4me3	20.44	± 3.79 ^{b}	14.49	± 2.45 ^{b}	57.13	± 7.58 ^{a}	12.70	± 0.89 ^{b}
	Mock	0.67	± 0.3	0.30	± 0.07	0.29	± 0.33	0.26	± 0.17
IRI	H3	12.49	± 0.9 ^{a}	12.63	± 1.58 ^{a}	10.65	± 1.16 ^{ab}	8.84	± 1.07 ^{b}
	H3K27me3	5.28	± 1.11 ^{a}	5.94	± 0.86 ^{a}	2.61	± 0.63 ^{b}	2.04	± 0.28 ^{b}
	H3K4me2	0.29	± 0.15 ^{b}	0.30	± 0.09 ^{b}	0.78	± 0.24 ^{a}	0.30	± 0.14 ^{b}
	H3K4me3	5.16	± 1.39 ^{ab}	4.83	± 0.95 ^{ab}	5.78	± 0.3 ^{a}	3.43	± 0.36 ^{b}
	Mock	0.53	± 0.16	0.27	± 0.09	0.03	± 0.03	0.12	± 0.1
VRN1	H3	17.74	± 1.04 ^{a}	15.97	± 1.81 ^{ab}	14.73	± 0.3 ^{b}	9.99	± 0.36 ^{c}
	H3K27me3	17.33	± 0.34 ^{a}	15.11	± 1.49 ^{ab}	15.71	± 1.24 ^{ab}	10.48	± 0.62 ^{b}
	H3K4me2	2.16	± 0.6 ^{ab}	2.12	± 0.16 ^{ab}	3.63	± 0.23 ^{a}	1.84	± 1.11 ^{b}
	H3K4me3	42.85	± 4.84 ^{b}	29.06	± 4.21 ^{c}	88.81	± 5.86 ^{a}	44.93	± 3.28 ^{b}
	Mock	0.54	± 0.13	0.28	± 0.12	0.15	± 0.05	0.16	± 0.01

Appendix 2 Table 1: ChIP-qPCR signals and statistical difference. (**a**) Results presented in a heatmap in Fig. 1d. (**b**) Results presented in a heatmap in Fig. 2b. SD: standard deviation; different letters refer to statistically significant differences between treatments. Signals relative to H3, except for H3 relative to input.

	GO term	Description	Input	BG/Ref	p-value	FDR
Shared, DE	GO:0001071	F nucleic acid binding transcription factor activity	5	478	0.0022	0.028
	GO:0003700	F transcription factor activity, sequence-specific DNA binc	5	478	0.0022	0.028
Naïve only	GO:0001071	F nucleic acid binding transcription factor activity	32	478	2.50E-05	0.0053
	GO:0003700	F transcription factor activity, sequence-specific DNA binc	32	478	2.50E-05	0.0053
Unique to primed	GO:0044710	P single-organism metabolic process	48	1366	1.50E-07	5.20E-05
	GO:0044699	P single-organism process	62	2340	2.20E-07	5.20E-05
	GO:0043436	P oxoacid metabolic process	13	322	0.0001	0.014
	GO:0055114	P oxidation-reduction process	28	1159	0.00012	0.014
	GO:0006082	P organic acid metabolic process	13	358	0.00027	0.026
	GO:0044763	P single-organism cellular process	32	1544	0.00058	0.046
	GO:0003824	F catalytic activity	97	6501	0.00018	0.026
	GO:0016491	F oxidoreductase activity	30	1274	0.00011	0.026
Shared, same expression	GO:0031326	P regulation of cellular biosynthetic process	38	1085	6.10E-07	2.30E-05
	GO:0050789	P regulation of biological process	47	1470	2.90E-07	2.30E-05
	GO:2000112	P regulation of cellular macromolecule biosynthetic proces	38	1085	6.10E-07	2.30E-05
	GO:0019219	P regulation of nucleobase-containing metabolic process	38	1072	4.60E-07	2.30E-05
	GO:0009889	P regulation of biosynthetic process	38	1085	6.10E-07	2.30E-05
	GO:0050794	P regulation of cellular process	47	1437	1.50E-07	2.30E-05
	GO:1903506	P regulation of nucleic acid-templated transcription	38	1058	3.30E-07	2.30E-05
	GO:2001141	P regulation of RNA biosynthetic process	38	1058	3.30E-07	2.30E-05
	GO:0051252	P regulation of RNA metabolic process	38	1059	3.40E-07	2.30E-05
	GO:0006355	P regulation of transcription, DNA-templated	38	1058	3.30E-07	2.30E-05
	GO:0010556	P regulation of macromolecule biosynthetic process	38	1085	6.10E-07	2.30E-05
	GO:0051171	P regulation of nitrogen compound metabolic process	38	1092	7.10E-07	2.40E-05
	GO:0065007	P biological regulation	47	1527	8.60E-07	2.50E-05
	GO:0010468	P regulation of gene expression	38	1101	8.70E-07	2.50E-05
	GO:0031323	P regulation of cellular metabolic process	38	1110	1.00E-06	2.70E-05
	GO:0080090	P regulation of primary metabolic process	38	1110	1.00E-06	2.70E-05
	GO:0060255	P regulation of macromolecule metabolic process	38	1130	1.60E-06	3.80E-05
	GO:0019222	P regulation of metabolic process	38	1136	1.80E-06	4.10E-05
	GO:0097659	P nucleic acid-templated transcription	39	1195	2.30E-06	4.70E-05
	GO:0006351	P transcription, DNA-templated	39	1195	2.30E-06	4.70E-05
	GO:0032774	P RNA biosynthetic process	39	1198	2.50E-06	4.80E-05
	GO:0034654	P nucleobase-containing compound biosynthetic proces:	40	1272	4.10E-06	7.60E-05
	GO:0018130	P heterocycle biosynthetic process	40	1336	1.30E-05	0.00022
	GO:0019438	P aromatic compound biosynthetic process	40	1335	1.20E-05	0.00022
	GO:1901362	P organic cyclic compound biosynthetic process	40	1387	2.90E-05	0.00048
	GO:0016070	P RNA metabolic process	40	1495	0.00015	0.0023
	GO:0043170	P macromolecule metabolic process	87	4365	0.00052	0.0078
	GO:0044238	P primary metabolic process	101	5315	0.00076	0.011
	GO:0071704	P organic substance metabolic process	104	5571	0.0012	0.016
	GO:0044260	P cellular macromolecule metabolic process	77	3862	0.0013	0.018
	GO:0044271	P cellular nitrogen compound biosynthetic process	42	1799	0.0016	0.021
	GO:0090304	P nucleic acid metabolic process	40	1708	0.0019	0.025
	GO:0008152	P metabolic process	127	7223	0.0022	0.028
	GO:0007154	P cell communication	11	280	0.0033	0.04
	GO:0001071	F nucleic acid binding transcription factor activity	26	478	1.70E-06	2.30E-06
	GO:0003700	F transcription factor activity, sequence-specific DNA binc	26	478	1.70E-06	2.30E-06
	GO:0043167	F ion binding	45	1701	6.70E-05	0.0053
	GO:0043169	F cation binding	42	1601	0.00015	0.0079
	GO:0046872	F metal ion binding	42	1594	0.00014	0.0079
	GO:0005509	F calcium ion binding	7	98	0.0008	0.035

Appendix 2 Table 2: Gene ontology analysis of chilling-responsive genes.

	Hypersensitive TM	Hyposensitive TM	**Total**	in diurnal-freezing
complex-convergent (U2A)	2	14	**18**	memory (59%)
transient (T3)	1	6	**7**	
complex-divergent (U2B, D2A)	1	1	**2**	
stable (U1, D1)	8	9	**17**	no memory (41%)
late-response (L1)	1	0	**1**	
NA / chilling-specific	4	2	**6**	
Total	**17**	**33**	**50**	
	34%	66%		

Appendix 2 Table 3: Chilling-responsive genes common to naïve and primed responses that show transcriptional memory (TM) and their categorization as diurnal-freezing responsive genes.

GeneID	log2NC'	log2PC'	response	comments	arabi-defline	rice-defline
Bradi2g24820	1.593	-4.26	strong.hypo	serine-type endopeptidase inhibitor activity // pe		BBTI11 - Bowman-Birk type bran trypsin inhibitor precursor, putative, expressed
Bradi4g44000	2.748	-3.34	strong.hypo	NAC domain contair	NAC domain containing pro	NAC domain transcription factor 47
Bradi3g55850	-2.48	1.389	strong.hyper	Peroxidase; Remov	Peroxidase superfamily pro	peroxidase precursor, putative, expressed
Bradi2g12105	-2.23	2.757	strong.hyper	UKN		
Bradi4g08320	-1.42	2.134	strong.hyper	The gene encodes a	S-adenosyl-L-methionine-	SAM dependent carboxyl methyltransferase, putative, expressed
Bradi2g39290	-1.39	1.66	strong.hyper	trypsin inhibitor activ	Serine protease inhibitor, pc	expressed protein
Bradi4g16093	-1.33	1.9	strong.hyper	Anthranilate O-meth	S-adenosyl-L-methionine-	SAM dependent carboxyl methyltransferase, putative, expressed
Bradi5g02010	-1.13	1.377	strong.hyper	Encodes a plasma m	white-brown complex homc	white-brown complex homolog protein 11, putative, expressed
Bradi1g42397	-3.67	-1.45	hypo		cysteine-rich RLK (RECEP1	TKL_IRAK_DUF26-lo.26 - DUF26 kinases have homology to DUF26 containing loci, expressed
Bradi3g10037	-2.96	-2.29	hypo		Subtilase family protein	OsSub17 - Putative Subtilisin homologue, expressed
Bradi1g15190	-2.76	-1.31	hypo		cytochrome P450, family 71	cytochrome P450, putative, expressed
Bradi3g51520	-1.79	-1.62	hypo			expressed protein
Bradi1g23390	1.953	1.753	hypo			expressed protein
Bradi1g70700	2.427	2.185	hypo		farnesylated protein 6	heavy metal-associated domain containing protein, expressed
Bradi1g72390	2.627	1.652	hypo		cold regulated gene 27	expressed protein
Bradi5g18830	2.786	2.059	hypo		MATE efflux family protein	MATE efflux family protein, putative, expressed
Bradi3g43150	2.883	2.634	hypo	Inositol-tetrakisphos	Inositol 1,3,4-trisphosphate	Inositol 1, 3, 4-trisphosphate 5/6-kinase, putative, expressed
Bradi2g57332	2.941	2.241	hypo			ARGOS, putative, expressed
Bradi2g27050	3.409	1.954	hypo		EF hand calcium-binding pi	OsCML10 - Calmodulin-related calcium sensor protein, expressed
Bradi2g60660	3.651	2.682	hypo		EF hand calcium-binding pi	OsCML10 - Calmodulin-related calcium sensor protein, expressed
Bradi3g57130	3.678	1.742	hypo		Subtilase family protein	OsSub22 - Putative Subtilisin homologue, expressed
Bradi3g09100	3.903	2.219	hypo		Peroxidase superfamily pro	peroxidase precursor, putative, expressed
Bradi5g14320	3.922	2.51	hypo		S-adenosyl-L-methionine-	expressed protein
Bradi3g40600	4.111	2.023	hypo		OSBP(oxysterol binding pro	oxysterol-binding protein, putative, expressed
Bradi5g26650	4.183	2.629	hypo	CCR4-NOT TRANSC	Polynucleotidyl transferase	CAF1 family ribonuclease containing protein, putative, expressed
Bradi1g20350	4.219	2.704	hypo		calmodulin-binding family p	calmodulin-binding protein, putative, expressed
Bradi2g14470	4.404	2.72	hypo	Cytokinin riboside 5'-	lysine decarboxylase family	uncharacterized protein PA4323, putative, expressed
Bradi3g49270	4.898	2.195	hypo		PHE ammonia lyase 1	phenylalanine ammonia-lyase, putative, expressed
Bradi4g28280	4.938	2.797	hypo	WRKY	WRKY DNA-binding protein	WRKY74, expressed
Bradi2g55310	5.233	2.866	hypo		Hs1pro-1 protein	hs1, putative, expressed
Bradi2g19900	5.636	4.85	hypo		Arabidopsis thaliana gibber	gibberellin 2-beta-dioxygenase, putative, expressed
Bradi4g07100	5.736	3.9	hypo	PTHR12537:SF75 -	pumilio 7	pumilio-family RNA binding repeat domain containing protein, expressed
Bradi5g21290	5.767	2.985	hypo	Coexpressed with genes in cold stress specific c		late embryogenesis abundant protein, group 3, putative, expressed
Bradi3g28210	7.062	4.035	hypo	Glycosyl hydrolase f	beta-amylase 1	beta-amylase, putative, expressed
Bradi2g61030	7.218	4.141	hypo	*UKN*		expressed protein
Bradi1g49560	7.584	3.862	hypo	AP2 domain (AP2) -	dehydration response elem	dehydration-responsive element-binding protein, putative, expressed
Bradi4g35650	7.716	6.398	hypo	AP2 domain (AP2) -	C-repeat/DRE binding facto	dehydration-responsive element-binding protein, putative, expressed
Bradi5g11970	7.875	4.15	hypo	*UKN*		expressed protein
Bradi1g49570	11.56	10.16	hypo	AP2/ERF->AP2/ERI	dehydration response elem	dehydration-responsive element-binding protein, putative, expressed
Bradi3g57350	1.157	2.14	hyper		Plant protein of unknown fu	plant protein of unknown function domain containing protein, expressed
Bradi1g03207	1.196	1.851	hyper		Glycosyl hydrolase superfai	OsMan05 - Endo-Beta-Mannanase, expressed
Bradi4g41377	1.206	1.987	hyper		O-fucosyltransferase family	growth regulator related protein, putative, expressed
Bradi2g49120	1.292	2.565	hyper		UDP-glucosyl transferase 8	anthocyanidin 5,3-O-glucosyltransferase, putative, expressed
Bradi1g33827	1.299	1.81	hyper	Xyloglucan endotrai	xyloglucan endotransglyco	glycosyl hydrolases family 16, putative, expressed
Bradi4g16635	1.351	2.002	hyper			
Bradi3g37210	2.107	2.592	hyper		PEP1 receptor 1	receptor-like protein kinase precursor, putative, expressed
Bradi1g21097	2.128	3.681	hyper		Protein of unknown functior	DUF1645 domain containing protein, putative, expressed
Bradi2g18101	2.542	2.834	hyper		Late embryogenesis abund	late embryogenesis abundant protein, group 3, putative, expressed
Bradi3g57862	3.311	5.016	hyper	AP2 domain (AP2)	Integrase-type DNA-bindin	AP2 domain containing protein, expressed
Bradi2g18095	3.965	4.121	hyper		Late embryogenesis abund	late embryogenesis abundant protein, group 3, putative, expressed

Appendix 2 Table 4: Annotated chilling-responsive memory genes common to naïve and primed responses

Primer	Analysis	Primer sequence	Reference
UBC18_F	RT-qCPR	GTCACCCGCAATGTCTGTAAGTTC	Ream et al. 2014
UBC18_R	RT-qCPR	TTGTCTTGCGGACGTTGCTTTG	
VRN1_F	RT-qCPR	GCTCTGCAGAAGGAACTTGTGG	Ream et al. 2014
VRN1_R	RT-qCPR	CTAGTTTGCGGGTGTGTTTGCTC	
CBF1_F	RT-qCPR	ACCCGTACTACGAGATGGGC	Ryu et al. 2014
CBF1_R	RT-qCPR	ATCGGAGGAGGGTCAATGAG	
CBF2_F	RT-qCPR	GTGGCGCAGTCGTCTTCTT	Ryu et al. 2014
CBF2_R	RT-qCPR	GCTGGTCCTGCPAGTCACAC	
CBF3_F	RT-qCPR	TCGTCCTCCCTCACTGACAA	Ryu et al. 2014
CBF3_R	RT-qCPR	GCGTAGTAGAGGTCCCAGCC	
IR_F	RT-qCPR	TCTGGGACCTACCATGTCGT	Mayer et al. 2020
IRI_R	RT-qCPR	CGGACATGAGCTTCGTCAGT	
COR410_F	RT-qCPR	AGCAAAAGCCACAAGCCAAG	Mayer et al. 2020
COR410_R	RT-qCPR	GTCAAAGAGGCCCCTATCCG	
COR413_F	RT-qCPR	AGGTTGGTTGCTGGATTGCGTTC	Colton-Gagnon et al. 2014
COR413_R	RT-qCPR	TCCAGCCAATCAGGAAAGTGGCG	
VRN1_CArG_F	ChIP-qPCR	CGACAACGGATATGCTCCAGACC	Woods et al. 2017
VRN1_CArG_R	ChIP-qPCR	GAAGAGAGCCGGAGAGTGGGT	
VRN1_I1_F	ChIP-qPCR	TACGCACGCCTACGCTTAAG	Woods et al. 2017
VRN1_I1_R	ChIP-qPCR	GAAATGGAGCAGACAGGCAAG	
CBF1_R1_F	ChIP-qPCR	CAAGAGCAGAGTAGCCCAGC	Mayer et al. 2020
CBF1_R1_R	ChIP-qPCR	GGCGTTAACTGGGTCGGAAC	
CBF2_R1_F	ChIP-qPCR	TTTGGCGGGATCTCTTGCAT	Mayer et al. 2020
CBF2_R1_R	ChIP-qPCR	CGGGATTGCTATGCGTGTTG	
CBF3_R1_F	ChIP-qPCR	CGGTTGTACGGTATGTCGCT	Mayer et al. 2020
CBF3_R1_R	ChIP-qPCR	AAATCTCCGCTGGAGGAACC	
IR_R1_F	ChIP-qPCR	TGCCCACTCCATACAACACC	This paper
IRI_R1_R	ChIP-qPCR	TGCAAAGTTAGTAGCGAAGGAGT	
COR410_R1_F	ChIP-qPCR	TGGAGGTAACGGATAGGGGC	This paper
COR410_R1_R	ChIP-qPCR	TTCACCGTCACGAGGTTAGTA	
COR413_R1_F	ChIP-qPCR	GCATCCTGAAGGCTGAATCC	This paper
COR413_R1_R	ChIP-qPCR	AATTCCGGGGGTAAACGTCG	

Appendix 2 Table 5: Primers used in this study

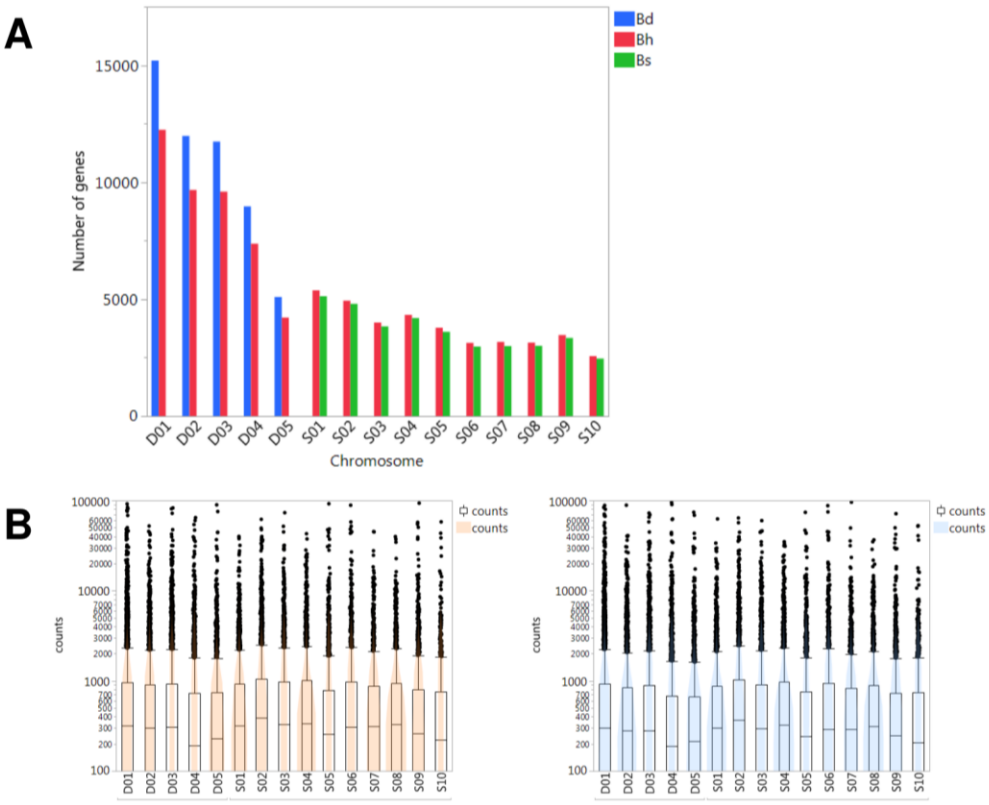
Appendix 3 Supplementary information for Chapter 5

Appendix 3 Figure 1: Genomic differences between subgenomes of *B. hybridum* and the parental genomes.

Appendix 3 Figure 2: *B. hybridum* producing filled and viable seeds in short-day (8/16 hours light/dark) conditions.

Appendix 3 Table 1: Gene ontology analysis of cold-responsive genes divided intro core memory, core no memory, naïve-specific (memory) and primed-specific (acquired), and comparison between *B. distachyon* and *B. hybridum*.

Appendix 3 Table 2: Primers used in this study



Appendix 3 Figure 1: Genomic differences between subgenomes of B. hybridum and the parental genomes. (**A**) Number of genes found on *B. distachyon* (Bd) chromosomes (D01-05) and *B. stacei* (Bs) chromosomes (S01-10) compared to their equivalent in *B. hybridum* (Bh). (**B**) Average transcript counts of genes present in each chromosome of Bh in control (left; samples N and P controls) or cold (right; samples N and P) conditions.

Appendix 3 Figure 2: *B. hybridum* producing filled and viable seeds in short-day (8/16 hours light/dark) conditions.

	B. distachyon							B. hybridum						
	GO term	Ont.	Description	Input	BG/Ref	p-value	FDR	GO term	Ont.	Description	Input	BG/Ref	p-value	FDR
Naïve-specific	GO:0001071	F	nucleic acid binding transcription factor activity	32	478	2.50E-05	0.0053	GO:0043565	F	sequence-specific DNA binding	34	499	2.10E-10	4.30E-08
	GO:0003700	F	transcription factor activity, sequence-specific DNA binding	32	478	2.50E-05	0.0053	GO:0003677	F	DNA binding	34	899	0.00011	0.012
	GO:0043565	F	sequence-specific DNA binding	21	263	6.50E-05	0.0093	GO:0043565	F	sequence-specific DNA binding	5	499	0.00073	0.011
Primed-specific	GO:0044710	P	single-organism metabolic process	48	1966	1.50E-07	5.20E-05	GO:0055085	P	transmembrane transport	6	997	0.001	0.029
	GO:0044699	P	single-organism process	62	2940	2.20E-07	5.20E-05	GO:0006810	P	transport	6	1396	0.0051	0.039
	GO:0043436	P	oxoacid metabolic process	13	322	0.0001	0.014	GO:0051179	P	localization	6	1408	0.0053	0.039
	GO:0055114	P	oxidation-reduction process	28	1159	0.00012	0.014	GO:0051234	P	establishment of localization	6	1407	0.0053	0.039
	GO:0006082	P	organic acid metabolic process	13	358	0.00027	0.026							
	GO:0044763	P	single-organism cellular process	32	1544	0.00058	0.046							
	GO:0003824	F	catalytic activity	97	6501	0.00018	0.026							
	GO:0016491	F	oxidoreductase activity	30	1274	0.00011	0.026							
Core, memory	GO:0001071	F	nucleic acid binding transcription factor activity	5	478	0.0022	0.028	GO:0019438	P	aromatic compound biosynthetic process	22	1516	0.00012	0.0018
	GO:0003700	F	transcription factor activity, sequence-specific DNA binding	5	478	0.0022	0.028	GO:0065007	P	biological regulation	24	1823	0.00025	0.0032
								GO:0009058	P	biosynthetic process	28	2560	0.0014	0.015
								GO:0044249	P	cellular biosynthetic process	28	2506	0.001	0.012
								GO:0044237	P	cellular metabolic process	46	5035	0.0027	0.028
								GO:0044271	P	cellular nitrogen compound biosynthetic process	24	2198	0.0028	0.028
								GO:0018130	P	heterocycle biosynthetic process	22	1508	0.00011	0.0018
								GO:0090304	P	nucleic acid metabolic process	22	1846	0.0014	0.015
								GO:0097659	P	nucleic acid-templated transcription	22	1389	3.50E-05	0.00066
								GO:0034654	P	nucleobase-containing compound biosynthetic process	22	1453	6.60E-05	0.0011
								GO:0006139	P	nucleobase-containing compound metabolic process	22	2030	0.0043	0.042
								GO:1901362	P	organic cyclic compound biosynthetic process	22	1537	0.00014	0.0021
								GO:1901576	P	organic substance biosynthetic process	28	2499	0.001	0.012
								GO:0050789	P	regulation of biological process	24	1810	0.00023	0.003
								GO:0009889	P	regulation of biosynthetic process	22	1205	4.30E-06	0.00014
								GO:0031326	P	regulation of cellular biosynthetic process	22	1205	4.30E-06	0.00014
								GO:2000112	P	regulation of cellular macromolecule biosynthetic process	22	1205	4.30E-06	0.00014
								GO:0031323	P	regulation of cellular metabolic process	22	1221	5.20E-06	0.00014
								GO:0050794	P	regulation of cellular process	24	1755	0.00015	0.0021
								GO:0010468	P	regulation of gene expression	22	1245	7.00E-06	0.00017
								GO:0010556	P	regulation of macromolecule biosynthetic process	22	1205	4.30E-06	0.00014
								GO:0060255	P	regulation of macromolecule metabolic process	22	1262	8.60E-06	0.0002
								GO:0019222	P	regulation of metabolic process	22	1270	9.50E-06	0.0002
								GO:0051171	P	regulation of nitrogen compound metabolic process	22	1215	4.90E-06	0.00014
								GO:1903506	P	regulation of nucleic acid-templated transcription	22	1192	3.60E-06	0.00014
								GO:0019219	P	regulation of nucleobase-containing compound metabolic process	22	1209	4.50E-06	0.00014
								GO:0080090	P	regulation of primary metabolic process	22	1220	5.20E-06	0.00014
								GO:2001141	P	regulation of RNA biosynthetic process	22	1192	3.60E-06	0.00014
								GO:0051252	P	regulation of RNA metabolic process	22	1194	3.70E-06	0.00014
								GO:0006355	P	regulation of transcription, DNA-templated	22	1192	3.60E-06	0.00014
								GO:0032774	P	RNA biosynthetic process	22	1395	3.70E-05	0.00066
								GO:0016070	P	RNA metabolic process	22	1656	0.00038	0.0047
								GO:0006351	P	transcription, DNA-templated	22	1389	3.50E-05	0.00066
Core, no memory	GO:0019438	P	aromatic compound biosynthetic process	40	1335	1.20E-05	0.00022	GO:0019438	P	aromatic compound biosynthetic process	29	1516	0.00058	0.0095
	GO:0065007	P	biological regulation	47	1527	8.60E-07	2.50E-05	GO:0065007	P	biological regulation	43	1823	3.30E-07	0.00013
	GO:0005509	F	calcium ion binding	7	98	0.0008	0.035	GO:0018130	P	heterocycle biosynthetic process	29	1508	0.00054	0.0091
	GO:0043169	F	cation binding	42	1601	0.00015	0.0079	GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	6	56	1.60E-05	0.0012
	GO:0007154	P	cell communication	11	280	0.0033	0.04	GO:0097659	P	nucleic acid-templated transcription	29	1389	0.00015	0.0029
	GO:0044260	P	cellular macromolecule metabolic process	77	3862	0.0013	0.018	GO:0034654	P	nucleobase-containing compound biosynthetic process	29	1453	0.0003	0.0054
	GO:0044271	P	cellular nitrogen compound biosynthetic process	42	1799	0.0016	0.021	GO:1901362	P	organic cyclic compound biosynthetic process	29	1537	0.00071	0.011
	GO:0018130	P	heterocycle biosynthetic process	40	1336	1.30E-05	0.00022	GO:0050789	P	regulation of biological process	41	1810	1.50E-06	0.0002
	GO:0043167	F	ion binding	45	1701	6.70E-05	0.0059	GO:0009889	P	regulation of biosynthetic process	29	1205	1.30E-05	0.00044
	GO:0043170	P	macromolecule metabolic process	87	4365	0.00052	0.0078	GO:0031326	P	regulation of cellular biosynthetic process	29	1205	1.30E-05	0.00044
	GO:0008152	P	metabolic process	127	7223	0.0022	0.028	GO:2000112	P	regulation of cellular macromolecule biosynthetic process	29	1205	1.30E-05	0.00044
	GO:0046872	F	metal ion binding	42	1594	0.00014	0.0079	GO:0031323	P	regulation of cellular metabolic process	29	1221	1.70E-05	0.00044
	GO:0001071	F	nucleic acid binding transcription factor activity	26	478	1.70E-08	2.30E-06	GO:0050794	P	regulation of cellular process	41	1755	7.30E-07	0.00014
	GO:0090304	P	nucleic acid metabolic process	40	1708	0.0019	0.025	GO:0010468	P	regulation of gene expression	29	1245	2.40E-05	0.00058
	GO:0097659	P	nucleic acid-templated transcription	39	1195	2.30E-06	4.70E-05	GO:0010556	P	regulation of macromolecule biosynthetic process	29	1205	1.30E-05	0.00044
	GO:0034654	P	nucleobase-containing compound biosynthetic process	40	1272	4.10E-06	7.60E-05	GO:0060255	P	regulation of macromolecule metabolic process	29	1262	3.00E-05	0.00069
	GO:1901362	P	organic cyclic compound biosynthetic process	40	1387	2.90E-05	0.00048	GO:0019222	P	regulation of metabolic process	29	1270	3.40E-05	0.00073
	GO:0071704	P	organic substance metabolic process	104	5571	0.0012	0.016	GO:0051171	P	regulation of nitrogen compound metabolic process	29	1215	1.50E-05	0.00044
	GO:0044238	P	primary metabolic process	101	5315	0.00076	0.011	GO:1903506	P	regulation of nucleic acid-templated transcription	29	1192	1.10E-05	0.00044
	GO:0050789	P	regulation of biological process	47	1470	2.90E-07	2.30E-05	GO:0019219	P	regulation of nucleobase-containing compound metabolic process	29	1209	1.40E-05	0.00044
	GO:0009889	P	regulation of biosynthetic process	38	1085	6.10E-07	2.30E-05	GO:0080090	P	regulation of primary metabolic process	29	1220	1.70E-05	0.00044
	O:0031326	P	regulation of cellular biosynthetic process	38	1085	6.10E-07	2.30E-05	GO:2001141	P	regulation of RNA biosynthetic process	29	1192	1.10E-05	0.00044
	GO:2000112	P	regulation of cellular macromolecule biosynthetic process	38	1085	6.10E-07	2.30E-05	GO:0051252	P	regulation of RNA metabolic process	29	1194	1.10E-05	0.00044
	GO:0031323	P	regulation of cellular metabolic process	38	1110	1.00E-06	2.70E-05	GO:0006355	P	regulation of transcription, DNA-templated	29	1192	1.10E-05	0.00044
	GO:0050794	P	regulation of cellular process	47	1437	1.50E-07	2.30E-05	GO:0032774	P	RNA biosynthetic process	29	1395	0.00016	0.003
	GO:0010468	P	regulation of gene expression	38	1101	8.70E-07	2.50E-05	GO:0016070	P	RNA metabolic process	30	1656	0.0011	0.016
	GO:0010556	P	regulation of macromolecule biosynthetic process	38	1085	6.10E-07	2.30E-05	GO:0006351	P	transcription, DNA-templated	29	1389	0.00015	0.0029
	GO:0060255	P	regulation of macromolecule metabolic process	38	1130	1.60E-06	3.80E-05							
	GO:0019222	P	regulation of metabolic process	38	1136	1.80E-06	4.10E-05							
	GO:0051171	P	regulation of nitrogen compound metabolic process	38	1092	7.10E-07	2.40E-05							
	GO:1903506	P	regulation of nucleic acid-templated transcription	38	1058	3.30E-07	2.30E-05							
	GO:0019219	P	regulation of nucleobase-containing compound metabolic process	38	1072	4.60E-07	2.30E-05							
	GO:0080090	P	regulation of primary metabolic process	38	1110	1.00E-06	2.70E-05							
	GO:2001141	P	regulation of RNA biosynthetic process	38	1058	3.30E-07	2.30E-05							
	GO:0051252	P	regulation of RNA metabolic process	38	1059	3.40E-07	2.30E-05							
	GO:0006355	P	regulation of transcription, DNA-templated	38	1058	3.30E-07	2.30E-05							
	GO:0032774	P	RNA biosynthetic process	39	1198	2.50E-06	4.80E-05							
	GO:0016070	P	RNA metabolic process	40	1495	0.00015	0.0023							
	GO:0003700	F	transcription factor activity, sequence-specific DNA binding	26	478	1.70E-08	2.30E-06							
	GO:0006351	P	transcription, DNA-templated	39	1195	2.30E-06	4.70E-05							

Appendix 3 Table 1: Gene ontology analysis of cold-responsive genes divided intro core memory, core no memory, naïve-specific (memory) and primed-specific (acquired), and comparison between *B. distachyon* and *B. hybridum*.

Primer	Primer sequence	Reference
VRN1_F	GCTCTGCAGAAGGAACTTGTGG	Ream et al. 2014
VRN1_R	CTAGTTTGCGGGTGTGTTTGCTC	
FT_F	TTCGGGAACAAGGAACGTGTCCAAC	Ream et al. 2014
FT_R	AGCATCTGGGTCTACCATCACGAG	
CBF1_F	ACCCGTACTACGAGATGGGC	Ryu et al. 2014
CBF1_R	ATCGGAGGAGGGTCAATGAG	
CBF2_F	GTGGCGCAGTCGTCTTCTT	Ryu et al. 2014
CBF2_R	GCTGGTCCTGCPAGTCACAC	
CBF3_F	TCGTCCTCCCTCACTGACAA	Ryu et al. 2014
CBF3_R	GCGTAGTAGAGGTCCCAGCC	
IR_F	TCTGGGACCTACCATGTCGT	Mayer et al. 2020
IRI_R	CGGACATGAGCTTCGTCAGT	
COR410_F	AGCAAAAGCCACAAGCCAAG	Mayer et al. 2020
COR410_R	GTCAAAGAGGCCCCTATCCG	
COR413_F	AGGTTGGTTGCTGGATTGCGTTC	Colton-Gagnon et al. 2014
COR413_R	TCCAGCCAATCAGGAAAGTGGCG	
UBC18_F	GTCACCCGCAATGTCTGTAAGTTC	Ream et al. 2014
UBC18_R	TTGTCTTGCGGACGTTGCTTTG	

Appendix 3 Table 2: Primers used in this study

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