Understanding the role of CHROMOMETHYLASES in response to cold in *Brachypodium distachyon*

Daniil Alexander Batanau

Department of Plant Science

McGill University, Montreal

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ABSTRACT

Spring and fall frost events will increase by the mid-21st century due to climate change, negatively impacting cereal production. Plants differentially express genes in response to abiotic stress, allowing adaptation to the environment. Evidence suggests adaptation to low temperature is partially regulated by the epigenetic mechanisms of histone modifications and DNA methylation, but their impact on cold response remains undefined. CHROMOMETHYLASE (CMTs) proteins are a conserved group of proteins that are responsible for the maintenance of DNA methylation marks in plants. This research hypothesizes that the modulation of DNA methylation through mutation in CHROMOMETHYLASE family proteins will alter cold acclimation, freezing tolerance, and vernalization in the model grass *Brachypodium distachyon (B. distachyon).* The objectives of this research project were four-fold; identify CMTs in *B. distachyon* (BdCMT) through *in silico* analysis, evaluate transcript accumulation profiles of *BdCMTs* and cold responsive genes in short- and long-term cold exposure, identify and validate mutant *B. distachyon* lines showing high to moderate impact mutations in *BdCMT* genes, and finally phenotype and characterize those mutants during cold exposure. The results herein identify 4 *CMT* genes in *B. distachyon*, sharing a high degree of homology to the conserved domains of other grasses. Additionally, gene expression analysis revealed that short- and long-term exposure to cold significantly alters the transcript accumulation profiles of *BdCMT* genes relative to ambient conditions. Sodium azide-induced mutant lines of *BdCMT* genes were validated through sequencing, identifying mutations in critical domains affecting protein function. Phenotypic analysis of these mutants under cold acclimation conditions showed altered transcriptional profiles of cold-responsive genes *BdCOR410* and *BdVRN1* relative to wild type, suggesting a regulatory role of *CMT* genes

in cold acclimation and vernalization. This study underscores the importance of *CMT* genes in the regulation of the cold stress response of *B. distachyon*, providing insights into the molecular mechanisms underlying plant adaptation to low temperatures. These findings reinforce the importance of epigenetic regulation in grasses in response to abiotic stress and highlights potential targets for epigenetic and genetic engineering for future crop development.

RÉSUMÉ

Les événements de gel printanier et automnal augmenteront d'ici le milieu du 21ème siècle en raison aux changements climatiques, impactant négativement la production céréalière. Les plantes expriment différemment les gènes de réponse aux stress abiotiques, permettant l'adaptation à leurs environnements. Les données recueillis jusqu'à présent suggèrent que l'adaptation aux basses températures est partiellement régulée par des mécanismes épigénétiques tels que la modification des histones et la méthylation de l'ADN, mais leurs impacts sur la réponse au froid restent flous. Les protéines CHROMOMETHYLASE (CMT) sont un groupe conservé de protéines responsables du maintien des marques de méthylation de l'ADN chez les plantes. Ma recherche émet l'hypothèse que la modulation de la méthylation de l'ADN par mutation dans les protéines de la famille CHROMOMETHYLASE modifiera l'acclimatation au froid, la tolérance au gel et la vernalisation chez la plante modèle *Brachypodium distachyon* (*B. distachyon*). Les objectifs de mon projet de recherche étaient: 1) Identifier les CMT chez *B. distachyon* (BdCMT) par analyse *in silico*; 2) Évaluer les profils d'accumulation des transcrits des BdCMT et des gènes régulés par le froid lors d'une exposition à court et à long terme; 3) Identifier et valider des lignées mutantes de *B. distachyon* montrant des mutations à impact modéré à élevé dans les gènes BdCMT; 4) Phénotyper et caractériser ces mutants pendant l'exposition au froid. Les résultats présentés ici identifient quatre gènes CMT chez *B. distachyon*, partageant un haut degré d'homologie avec les domaines conservés chez des CMT d'autres graminées. De plus, l'analyse de l'expression génique a révélé que l'exposition au froid à court et à long terme modifie de manière significative les profils d'accumulation des transcrits des gènes BdCMT. Les mutations des gènes BdCMT induites par l'azide de sodium ont été validées par séquençage, identifiant des

mutations dans des domaines critiques affectant la fonction des protéines. L'analyse phénotypique de ces lignées mutantes sous conditions d'acclimatation au froid a montré des profils transcriptionnels modifiés des gènes connus comme étant régulés par le froid, BdCOR410 et BdVRN1, par rapport aux plantes de type sauvage, suggérant un rôle régulateur des gènes CMT dans l'acclimatation au froid et la vernalisation. Cette étude souligne l'importance des gènes CMT dans la régulation de la réponse au stress froid de *B. distachyon*, fournissant des indices sur les mécanismes moléculaires sous-jacents à l'adaptation des plantes aux basses températures. Ces découvertes renforcent l'importance de la régulation épigénétique chez les graminées en réponse au stress abiotique et mettent en évidence des cibles potentielles pour l'ingénierie épigénétique et génétique pour le développement futur des cultures.

Table of Contents

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

ABA: Abscisic acid

AGO: ARGONAUTE

BAH: BROMO ADJACENT HOMOLOGY

Bp: Nucleic acid base pairs

CA: Cold acclimation

CA2+: Calcium ions

CAMTA: CALMODULIN-BINDING TRANSCRIPTION FACTORS

CBF: C-repeat binding Factor

CG methylation: 5mC DNA methylation in the CG nucleotide sequence context

CHG methylation 5mC DNA methylation in the CHG nucleotide sequence context, where H= A, C, or T nucleotides

CHH Methylation: 5mC DNA methylation in the CHH sequence context

CHROMO: CHRomatin Organization MOdifier

CMT: CHROMOMETHYLASE

COLD1: CHILLING TOLERANCE DIGERENCE

CS: Chilling stress

DCL: DICER-LIKE

DGAT1: DIACYLGLYCEROL ACETYLTRANSFERASE 1

DME: DEMETER

DML: DEMETER-LIKE

DNA: Deoxyribonucleic acid

DNMT: DNA METHYLTRANSFERASE

DRBE: DEHYDRATION RESPONSIVE BINDING ELEMENT

DRM2: DOMAINS REARRANGED METHYLASE 2

FLC: FLOWERING LOCUS C

FPKM: Fragments Per Kilobase of transcript per Million mapped reads

FT: Freezing tolerance

FS: Freezing stress

GbM: Gene body Methylation

H3K4me3: Histone H3 lysine residue 4 methylation

H3K9me: Histone H3 lysine residue 9 methylation

H3K27me3: Histone H3 lysine residue 27 methylation

HAT: HISTONE ACETYLTRANSFERASE

HII: Hexagonal II phase

HMT: HISTONE METHYLTRANSFERASE

HOS1: OSMOTICALLY RESPONSIVE GENE 1

ICE1: INDUCER OF CBF EXPRESSION 1

IRI: ICE RECRYSTALIZATION INHIBITOR

JmJC: Jumanji domain

KRY: KRYPTONITE

LB: Luria-Bertani growth media

LEA: LATE EMBRYOGENESICE ABUNDANCE

LSD1: LYSINE SPECIFIC DEMETHYLASE

LT: Low temperature

MET1: METHYLTRANSFERASE 1

MS MEDIA: Murashige and Skoog media

NaN: Sodium azide

NLS: Nuclear localization signal

NPR1: NON-EXPRESSER OF PATHOGENETISIS-RELATED GENE 1

OST1: OPEN STOMATA 1

PCR: Polymerase chain reaction

RDR2: RNA-DEPENDENT RNA POLYMERASE 2

RGA1: G-PROTEIN SUBUNIT 1

RNA: Ribonucleic acid

RNA Pol II: RNA polymerase II

RNA Pol IV: RNA polymerase IV

RNA-seq: Ribonucleic acid sequencing

ROS: Reactive Oxygen Species

ROS1: REPRESSOR OF SILENCING 1

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

RVR1: REPRESSOR OF VERNALIZATION 1

SHH1: SWADEE HOMEODOMAIN HOMOLOGUE 1

SNP: Single Nucleotide Polymorphism

SOC1: Suppressor of Overexpression of Constans 1

SUVH4: SUPRESSOR OF VERIEGATION

TE: transposable element

TSS: Transcription start site

5mC: 5-methylcytosine

6mA: n6-methyladenine

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Preface

This thesis is composed of original work in its entirety. It is organized in the traditional monographic style. The contributions of co-authors to this work are detailed bellow

Contributions of co-authors:

Chapters I, II, III and IV were written by Daniil Batanau with supervision from Jean-Benoit Charron. Experiments in Chapter III were designed by Daniil Batanau and Jean-Benoit Charron. qPCR data generation, plant phenotyping, phylogenetic analysis, and mutant line verification was completed by Daniil Batanau. SNP lines were generated and provided by the Vogel Lab in the joint genome institute. Data analysis was performed by Daniil Batanau.

CHAPTER I: INTRODUCTION

1.1 Introduction

Abiotic stressors are a primary cause of crop yield losses in Canada, particularly affecting regions like the Canadian prairies where temperature and moisture stress are prevalent (Bueckert & Clarke, 2013). With an ever-expanding demand and limited opportunities to increase land use, agricultural productivity could be increased by minimizing crop loss caused by abiotic stress. Cold and frost stress impact a plant at a physiological and molecular level, reducing growth and yield (Bhattacharya, 2022; Steponkus & Webb, 1992; Yadav, 2010). To compensate, plants alter their morphology and gene expression to adapt to these local environmental changes (Colton-Gagnon et al., 2014; Ding et al., 2020; Juurakko et al., 2022; Mayer et al., 2020). Much effort has been spent in understanding how plants adapt to cold in the interest of generating cold acclimated crop lines, but research is limited due to the trade-off of stress tolerance and overall growth and development, potentially reducing yield (Karasov et al., 2017). Key regulators including the C-repeat binding factor family (CBF), flowering regulator VERNALIZATION 1 (VRN1), and their accompanying downstream targets have provided insight regarding plant strategy in response to cold and frost stress (Dubcovsky et al., 2006; Medina et al., 1999; Stockinger et al., 1997). Plants optimize their expression profiles to adapt to their environment through transcriptional regulation, but the complete mechanism is not fully elucidated. Difficulty in uncovering the complete mechanisms derives from variability in epigenetic regulation as speciation occurs, with greater divergence in patterning and function as evolutionary distance increases (Takuno & Gaut, 2013). Evidence suggests that epigenetic regulators influence transcriptional control and

gene regulation in response to cold stress (Gutschker et al., 2022; Lämke & Bäurle, 2017; Mayer & Charron, 2021).

DNA methylation is an epigenetic mechanism that has been shown to play a role in abiotic stress and plant development. Prolonged exposure to low temperature induces significant changes in DNA methylation patterns, which in turn affect gene expression related to its stress response, suggesting an active regulatory response (Bhattacharya, 2022; Gutschker et al., 2022; J. Liu & He, 2020).

The maintenance and removal of DNA methylation marks involve various enzymes, including DNA methyltransferases and demethylases. Direct alterations in methylation patterning, as opposed to passive demethylation are conducted by the RNA directed DNA methylation pathway (RdDM), establishing *de novo* DNA methylation in response to environmental cues (H. Zhag et al., 2018). Maintenance of such marks is conducted by the CHROMOMETHYLASE (CMT) family proteins. Commonly consisting of three distinct isoforms in plants, this highly conserved protein family facilitates the deposition and maintenance of non-CG methylation pattern marks. CMT proteins share an interplay with histone modifications associated with transcriptional silencing, suggesting a shared function in gene regulation (Du et al., 2012). While there is evidence to suggest their involvement in abiotic stress tolerance, their function and local mechanisms of activity remain elusive.

Brachypodium distachyon (B. distachyon) is an undomesticated dicot grass native to the Mediterranean, sharing high synteny with commercial crops including Wheat and Barley. *B. distachyon* has been shown to be cold responsive, increasing its tolerance to cold through transcriptional changes collectively termed cold acclimation. Evidence

suggests epigenetic regulatory mechanisms are involved, presenting an opportunity to elucidate its role in grasses. Previous work established that while *B. distachyon* relies on the same cold response machinery as temperate cereal crops, the perception and interpretation of seasonal low-temperature signals appear to be specific to the species, which could represent an undomesticated characteristic of the model and an opportunity for domesticated crop improvement (Mayer *et al.,* 2020;2021). In addition, several reports indicate that the DNA methylation machinery may be responsible for establishing patterns of DNA methylation at stress-responsive loci in plants (Papareddy et al., 2021). This project thus aims at understanding the contribution of the CMT family of DNA methyltransferases to the cold responses of *B. distachyon*.

1.2 Research hypothesis

Disrupting DNA methylation through mutation of CHROMOMETHLASE genes will lead to alterations in the cold stress responses of *B. distachyon.*

1.3 Objectives

1. *In silico* characterisation of the *Brachypodium distachyon* CHROMOMETHYLASES
2. Determine transcript accumulation profiles of *CHROMOMETHYLASE* genes in responses to cold acclimation in *Brachypodium distachyon*
3. Identification and validation of *Brachypodium distachyon* sodium azide treated lines showing high and moderate impact mutations in Bd*CHROMOMETHYLASE* genes
4. Phenotypic and molecular characterization of the selected sodium azide lines under control conditions and during cold exposure

CHAPTER II: LITERATURE REVIEW

2.1 Plant Responses to Cold

2.1.1 The effects of cold exposure to plant physiology

Throughout their lifecycle, plants are exposed to a spectrum of temperatures. Being sessile organisms, they are especially sensitive and at risk of exposure to temperatures outside of their natural range. Cold stress occurs when environmental temperatures deviate below this natural range. The range that a plant is capable of tolerating and growing within varies for each plant species, being dependent on the climates they naturally acclimate to. For example, tropical plants are highly sensitive to cold, showing symptoms of injury after exposure to temperatures below 10°C (Scholthof et al., 2018). On the other extreme, woody plants found in the boreal forests of the arctic are capable of surviving in temperatures below -40°C (Strimbeck et al., 2008). Plants which naturally grow in colder climates such as temperate region grasses are capable of minimal loss of function in lower temperatures and can continue growing in colder temperatures following a period of adjustment (Colton-Gagnon et al., 2014). While plants are exposed to deviations from their natural temperature ranges, with appropriate physiology, plants can survive some natural temperature variations.

Low temperature (LT) stress can be broadly categorized into two groups, chilling stress (CS); stress which may occur at temperatures between 10°C and 0°C, and freezing stress (FS); which occurs at temperatures below 0°C. Both stresses share similar responses but pose unique sets of challenges for a plant to adapt to. Plants are capable of developing tolerance to reduced temperatures through a process termed cold acclimation (CA) in which a plant changes its physiological and molecular profile to adapt

to LT. The changes in a plants physiology and molecular profile through CA additionally increase a plant freezing tolerance (FT); the capacity to survive in temperatures below 0°C.

Regardless of each plant species ability to adapt to the cold, the strain on a plant as a result of CS remains consistent between species, impacting physiology, cell integrity, and molecular kinetics. CS derives from a reduction in temperature, generating a thermodynamically unfavourable local environment, resulting in abiotic strain on the plant. The severity of damage caused by CS is dependent on a variety of factors including the severity, duration, exposure number, and whether additional stresses are simultaneously occurring (Shinozaki, 2015, *p. 1055*). Exposure to CS impact a plants membrane structure and overall physiology (Bhattacharya, 2022)**.** During CS, the available transpirable water in both the cell and soil decreases due to a reduction in waters viscosity, resulting in cellular dehydration (Steponkus & Webb, 1992; Yadav, 2010). The decrease in water availability and molecular kinetics resulting from decreased temperature slow molecular machinery and lead to reduced enzymatic efficiency or complete loss of function, slowing down the metabolism of the organism and reducing respiration capacity (Levitt, 1980; Yamori & Caemmerer, 2009).

In addition to the physiological impacts associated with CS, prolonged or intense exposure impacts photosynthetic systems and phenological milestones. Exposure to CS impacts photosynthetic systems in plants due to the reduction in kinetics and membrane fluidity. Photosynthetic rates, chlorophyll composition, stomatal conductance and transpiration rate are all impacted by sub 15°C temperatures, and in turn growth and photosynthesis (Wei et al., 2022.; Zhang & Scheller, 2004). The reduction in temperatures

additionally impact plants phonologically, impacting growth rate and delaying developmental milestones (Kudo et al., 2019; Levitt, 1980; J. Li et al., 2024). Dependent on the plant, specific strategies may be adopted to delay or reduce growth rates until local conditions improve, including delaying the transition to flowering (Amasino, 2004; Park et al., 2018; Ream et al., 2014; Woods, Bednarek, et al., 2017). Unseasonably cold temperatures have detrimental impacts on flowering. Sudden reductions in temperature during a plants reproductive period can result in damage to fruit, uneven ripening, seed sterility, booting, panicle exertion, and increased spikelet sterility (Thakur et al., 2010). Altogether, reduced temperatures impact the vegetative, photosynthetic, and reproductive systems of plants.

While CS causes damage through prolonged exposure to cold, FS, that which is below 0°C, results in severe damage to the organism even if the exposure is brief. Damage from FS is most apparent at a cellular level. At sub-zero temperatures, the plasma membrane begins to lose fluidity and becomes ridged (Yadav, 2010). The membrane composition may rearrange into a structurally unsound hexagonal II (HII) phase. Upon thawing, this phase transition can lead to a disorganized plasma membrane, disrupting its integrity and making it more prone to rupture (Webb et al., 1993). The rigidity of the membrane makes it prone to cracking, resulting in electrolytic leakage of cytoplasmic fluid and metabolites. Ice crystals begin to form in the apoplastic regions of the cell, causing a loss of cellular compartmentalization and further exacerbating cellular dehydration (Steponkus & Webb, 1992; Thomashow, 1999). This adverse exposure to freezing stress manifests itself on a tissue level through surface lesions, desiccation and

shrivelling of leaves, discolouration, accelerated senescence wilting and decay, cell death and necrosis (Steponkus & Webb, 1992).

2.1.2 Plant adaptations and adjustments to low temperatures

While seasonal fluctuations and sudden deviations in temperature perturb plant growth, plants which have evolved to grow in colder climates adjust their phenotype in response to LT based the duration of exposure time and other environmental factors, increasing their chances of survival and reproduction. These adaptation patterns are categorized into four groups, those being chilling tolerance (CT), CA, FT and vernalization (Amasino, 2004). In short term exposure to stress, plants adapt to survive lower temperatures (15-0°C) without injury or damage through CT (Levitt, 1980). Through this short-term adaptation, plants alter their metabolism to compensate for the drop in temperature, altering enzymatic function and photosynthetic rates, compensating for reduced metabolic function and mitigating further damage (Juurakko et al., 2022). Plants with high tolerance to cold can survive short and long term cold exposure as well as some mild freezing stress and include temperate grasses such as Wheat and *B. distachyon* (Amasino, 2004; Colton-Gagnon et al., 2014; Ding et al., 2020; Juurakko et al., 2022; Mayer & Charron, 2021). This short-term response does not allocate significant resources to adapting to cold, as these changes do not significantly alter the physiological characteristics of the plant and allow for a rapid return to baseline function following the short exposure to cold.

Under prolonged exposure to cold, CT plants trigger CA. Cold acclimation permits the plant to survive initial cold exposure, increase FT, and prepare for recurrent cold stress

(Ding et al., 2020; Thomashow, 1999). Through changes in physiological, biochemical, and morphological profile, Cold acclimated plants can survive prolonged cold exposure and exposure to freezing temperatures. This reversable long-term acclimation causes complete restructuring of the metabolic, physiological, morphological, and biochemical profile through alterations in transcriptomic structure, allowing for long term survival until conditions improve (Ding et al., 2012; Mayer et al., 2020). While CA significantly increases FT, this phenotypic change comes at the expense of growth and temporary reproductive success (Karasov et al., 2017; Kudo et al., 2019).

Using the seasonal cues provided by temperature, some plants, notably temperate grasses, increase their reproductive success by timing the transition from vegetative to reproductive growth to occur following exposure to prolonged cold temperatures (Amasino, 2004; Woods, Bednarek, et al., 2017). Vernalization is the activation of a plants flowering response following prolonged exposure to LT. Vernalization is used in variable strategies in different plant species. Some organisms use it to time flowering in the late fall and winter, permitting their seeds to overwinter and germinate in the early spring. Alternatively, a plant may exist in its vegetative state in the winter and transition to flowering in the early spring. Overall, the use of temperature as a seasonal cue prevents premature flowering in the fall and late winter and permits rapid flowering in the early spring (Park et al., 2018). In addition to cold exposure, an inductive photoperiod typical of spring is required to induce flowering (Lang, 1965). The activation of vernalization in plants is tightly controlled through the regulation of the transcriptional system discussed later in this review.

2.1.3 The molecular response to cold in plants

Control of the LT responses in plants including CT, FT, CA, and vernalization are all tightly regulated by signaling pathways as a response to abiotic stressors. Common abiotic stressors including drought, heat, salinity, cold, and freezing all share similar signaling components between each respective transduction pathway (Lamers et al., 2020). While abiotic stress stimuli all share similar signaling components, each stress requires and generates a unique response to compensate for the respective abiotic stresses. The signaling pathways involved in abiotic stress begin with the detection of said stress, an initial signal transduction, activation of key regulator genes which in turn activate genes which code for proteins and macromolecules which permit the plant to acclimate and tolerate the abiotic stress it is exposed to and return to a local homeostasis.

2.1.4 Detection of reduced environmental temperatures

Response to cold begins with the detection of changes in the surrounding environment, generating a biological signal that is transduced through the organism, producing a cellular response (Quint et al., 2016). Significant effort has been aimed at understanding how plants are capable of directly detecting cold, specifically through the search for a “master regulator” or receptor. This search has pointed in some directions, such as membrane fluidity alterations caused by LT and carbon allocation, but no plant ligand receptor or function can be directly attributed as being the master regulator of temperature detection and downstream signaling (Chen et al., 2021). In the absence of a master regulator, multiple systems and candidates have been uncovered to be responsive to fluctuations in temperature. The collective system of independent temperature

detectors has been dubbed the integrated model, in which simultaneous sensors detect and respond to temperature shifts (Kerbler & Wigge, 2023). This concept permits multiple components and detection mechanisms in the cell to detect temperature change, allowing for the fine tuning of temperature responses over an immediate and longer-term temporal time scale. For example, responses to temperature have been studied and identified in light sensing phytochromes, suggesting an interplay between temperature and circadian rhythm (Jung et al., 2016). The explicit mechanisms of cold detection in plants remains elusive but the presence of multiple systems suggest a multi-actor model.

There are multiple transduction systems that have been studied and found to generate a signal in response to LT, some of which were found on the plasma membrane. The plasma membrane is particularly sensitive to fluctuations in temperature, as changes in local kinetics alters its fluidity (Kerbler & Wigge, 2023; Lamers et al., 2020; Steponkus & Webb, 1992). In reduced temperatures the cellular membrane becomes stiff and ridged. This shift in rigidity has been suggested to be a system of detection and a signal transducer for LT. In a study by Ma and colleagues looking at signaling in Japonica Rice, alterations in membrane fluidity were revealed to induce the function of the membrane bound protein CHILLING TOLERANCE DIVERGENCE 1 (COLD1). In response to LT, membrane bound COLD1 interacts with G-PROTEIN SUBUNIT (RGA1; Ma et al., 2015). Phosphorylation of COLD1 by RGA1 activates COLD1 leading to an influx in cytosolic Ca2+, a response correlated with CT and CA (Ma et al., 2015). While calcium influx is a common occurrence in response to abiotic stress, changes in the amplitude, frequency, and timing of Ca2+ cytosolic influx form patterns which may signal to the plant the specific abiotic stress it must respond to (Whalley & Knight, 2013). Reactive oxygen species

(ROS) are a by-product of LT cold exposure, in which their generation results in damage to lipids, proteins, and DNA through the introduction of free radicles in the cell. ROS additionally acts as a signaling molecule, activating antioxidant defenses, preventing further damage by free radicles. The presence of ROS in the cell additionally appears to deactivate calcium channels, suggesting an interplay between ROS and Ca2+ as a means of fine-tuning cold response (Choi et al., 2017). The signal detection by the plasma membrane activates a series of signal transducing molecules to induce transcriptional and physiological changes.

2.1.5 Effectors involved in signal transduction

The detection of reduced environmental temperature leads to the transduction of effectors throughout the cell. These effectors notably consist of transcription factors which alter the phenotype of the responding plant through the upregulation of cold responsive genes. One of the best studied regulatory networks in relation to cold stress is the C-repeat/DREB1 (CBF) pathway. At the center of this pathway are three redundant AP2 family proteins; the CBFs (CBF1, CBF2 and CBF3). Over-expression of all *CBF* genes in *Arabidopsis* leads to a heightened cold response, while knockout of all three *CBF* genes leads to substantial sensitivity to cold exposure (Pearce et al., 2013; Stockinger et al., 1997; Thomashow, 1999). CBFs function by binding to Dehydration Responsive Binding Element (DRBE) promoters, upregulating cold regulated (COR) genes leading to a response and phenotypic change (Colton-Gagnon et al., 2014; Stockinger et al., 1997). The expression of *CBF* genes in response to LT is rapid, peaking 1-2 hours following

cold exposure, leading to a rapid transcriptional response to LT in the short term, and CA in the long term (Y. S. Kim et al., 2015).

The importance of *CBF* genes in response to LT, their downstream induction of cold activated genes and the overall cold response makes them prone to tight regulation by a series of proteins, enzymes, transcription factors, and post-translational modifications to precisely control *CBF* expression, localization, and stability. In response to cold, positive induction of *CBF* genes may be mediated by a series of transcription factors and effectors. One of the most studied *CBF* regulators is the INDUCER OF CBF EXPRESSION (ICE1). Discovered in *A. thaliana*, this transcription factor binds upstream of *CBF* genes and promotes *CBF* expression (Zarka et al., 2003). Post translational modifications to ICE1 influence its ability to regulate CBFs, and in turn, the COR pathway. For example, ubiquitination of ICE1 by the RING finger protein HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE 1 (HOS1) promotes the degradation of ICE1, while phosphorylation of OST1 or sumoylation by E3 SUMO protein ligase (SIZI1) upregulate ICE1 and in turn promote FT (Dong et al., 2006; Miura et al., 2007). CALMODULIN-BINDING TRANSCRITION FACTORS (CAMTAs) have also been shown to upregulate the expression of *CBF*s, promoting the expression at the 2-hour peak following cold exposure (Ding et al., 2018). Both ICE1 and the CAMTA family transcription factors have been shown to be regulated by calcium signaling, reinforcing its importance in LT signaling (Kidokoro et al., 2022). Beyond expression, CBFs are controlled in a post-transcriptional modification manner, impacting their ability to promote the expression of *COR*s. BASIC TRANSCRIPTION FACTOR 3 (BFT3) is a protein that when

phosphorylated by OST1, interacts with CBFs and stabilizes them, improving their affinity to COR promotors and strengthening the cold response (Ding et al., 2018).

While *CBF* genes have been heavily studied, CBF controlled cold response only accounts for a fraction of expression in exposure to LT. Studies suggest that the CBF pathway only accounts for approximately 10-20% of transcriptional changes in response to cold stress (Jia et al., 2016). In transgenic apple plants, An et al., found that the MdHY5 upregulated cold responsive genes *MdPYL6*, *MdWRKY6*, and *MdSOC1* in a CBF independent manner (An et al., 2017). Additionally through salicylic acid mediated signaling in response to cold stress, NON-EXPRESSER OF PATHOGENESIS-RELATED GENE 1 (NPR1) oligomers localized to the nucleus activating NSFA1 and induced HSFA1 targeted *COR* genes (Olate et al., 2018). Transcription factors have been revealed to regulate cold expression independent of CBF, and hormones such as ABA, auxins, cytokinin's, salicylic acid and other hormones trigger transcriptional and physiological changes in response to cold stress (Ding et al., 2020; Lamers et al., 2020).

2.1.6 Molecular actors in response to cold stress

In the final stage of cold response, transcription factors bind to promoter regions of *COR* genes, recruiting transcriptional machinery and initiating a transcriptional response. The genes expressed and subsequent proteins restructure the phenotype of the cell to compensate for decreased temperature (Mayer et al., 2020; Thomashow, 1999). Membrane fluidity is increased through upregulation of desaturase enzymes, introducing double bonds, generating unsaturated fatty acid chains (Dar et al., 2017; Welti et al., 2002). Additionally, membrane fluidity is increased through the deposition of

triglycerides on the cell membrane through the upregulation of triglycerol catalyzing gene *DIACYLGLYCEROL ACETYLTRANSFERASE 1* (*DGAT1*) in response to freezing stress (Arisz et al., 2018; Lamers et al., 2020). To prevent the formation of ice crystals and subsequent cellular dehydration, plants upregulate genes that produce cryoprotectants, including glycerol, sucrose, mannitol, and proline (Tao & Li, 1986). Additionally, the upregulation of ICE RECRYSTALIZATION INHIBITOR (IRI) proteins occurs (Mayer et al., 2020).These cryoprotectants and proteins confer freezing tolerance by protecting cells from ice crystal formation, lowering the freezing point within the cell, maintaining turgor pressure, and stabilizing proteins under stress conditions (Bredow et al., 2016; Raymond & DeVries, 1977 Shinozaki, 2015, p.1055).

Protection against freezing induced dehydration is further conferred by (COR) genes, including LATE EMBRYOGENESIS ABUNDANCE (LEA) dehydrin proteins such as COR410 (Jin et al., 2019; Juurakko et al., 2022). LEA proteins are additionally responsive to drought stress, salinity, and application of exogenous ABA (Shinozaki, 2015, p. 1056). These genes appear to localize within plants and are suggested to be involved in the stabilization of macromolecules and proteins in response to abiotic stress (Y. Liu et al., 2017).

Recent studies have shown transcriptional changes in response to cold exposure by plants are mediated and influenced by epigenetic modifications (Ding et al., 2012; Mayer & Charron, 2021; Yang et al., 2022). This has most notably been studied through the process of vernalization, in which plants become competent for flowering following long term cold exposure and an inductive photoperiod. Vernalization in grasses, particularly *B. distachyon* is controlled by three main regulators, *BdVRN1*, *VRN2, and*

VRN3 (otherwise known as FT) (Woods, Bednarek, et al., 2017). Upon cold exposure, repressive histone H3K27me marks within the *VRN1* gene locus are depleted, and the same marks are deposited on *REPRESSOR OF VERNALIZATION 1* (RVR1; Woods et al., 2017). Following cold exposure, *BdVRN1* remains on, triggering the expression of *BdVRN3*. *BdVRN3* then accumulates in the flowers and buds of *B. distachyon*, and with the presence of a long photoperiod, induces flowering (Bäurle & Trindade, 2020). Overall, the response to cold stress is a tightly controlled consortium of regulatory mechanisms and proteins meant to alleviate and acclimate plants to cold stress.

2.2: Epigenetic control of cold responses

2.2.1 Epigenetics

The transcription of genes is tightly controlled in eukaryotic organisms. Based on environmental stimuli, plants orchestrate a precise response to achieve homeostasis to the environment. An epigenetic modification and in turn epigenetics may be considered a change in a genes expression profile without alteration of the genetic code (Sung & Amasino, 2004). These changes are often reversable and can be passed on to the next generation (J.-M. Kim et al., 2015). Epigenetic modifications allow for a rapid and sustained response to stimuli without the need for mutational genomic changes that would take eons of evolutionary time (Amasino, 2004). This capability expands the phenotypic plasticity of eukaryotic organisms, allowing sessile organism like plants to survive in variable environmental conditions (Mayer & Charron, 2021). Epigenetic changes come in four flavours; nucleosome positioning, in which the location of the nucleosome influences transcriptional capability, Small RNAs involved in RNA silencing. The other two forms of

epigenetic modification regulate gene expression through the deposition of marks directly on genetic information and its storage to influence its affinity to transcriptional machinery, those being modifications to the chromosome packing protein histones, and direct DNA modification through the insertion of epigenetic marks.

2.2.2 Histone modification

Histones are a group of proteins which compose the basic unit of chromatin condensation. An octamer of two proteins of each histone H2A, H2B, H3, and H4 create a complex that wraps 146 base pairs of DNA to condense it within the nucleus (Lämke & Bäurle, 2017). Wound DNA around a histone octamer is called a nucleosome, which further wraps into chromatin fibers. Tightly packed nucleosomes prevent replication machinery from associating and binding to DNA, preventing its transcription. Specific post translational modification of histones influence the affinity of DNA to histone octamers, altering the spatial organization of DNA in nucleosomes, and their interactions with transcriptional machinery. The modification of histones and its influence on transcriptional control adds a deeper layer of gene regulation known as the histone code.

There are over 26 forms of post translational modifications; covalent linkages of specific marks on regions of histone proteins including the terminal tails and globular regions alter the interaction of histones with DNA resulting in either an increase or decrease in their association (Guarino et al., 2022; J.-M. Kim et al., 2015). The type of mark and the histone residue which is it imprinted on determines its effect on chromosome condensation (K. Cheng et al., 2019). The best understood and commonly studied forms

of histone modification are methylation, acetylation, ubiquitination, phosphorylation, and sumoylation (J.-M. Kim et al., 2015; Zhao & Garcia, 2015).

There are three classes of proteins which interact and enforce the histone code: Readers, Writers, and Erasers. Readers are proteins capable of binding with specific histone patterns dictated by specific protein domains (Nicholson et al., 2015. p32.). Following the association to a gene, readers recruit proteins to carry out a designated response, that either impact the transcription of the gene, facilitate chromatin relaxation, or condensation (K. Cheng et al., 2019). Readers often consist of transcription factors whose interaction with histones can include the maintenance of present marks or the removal of marks if a cellular change occurs (Hyun et al., 2017). Readers often consist of transcription factors whose interaction with histones can include the maintenance of present marks or the removal of marks if a cellular change occurs (K. Cheng et al., 2019). The proteins which deposit marks on histones are known as writers. These proteins may contain a reader domain or are recruited by readers to deposit marks on histones (Nicholson et al., 2015, p. 34). Writers are classified based on the molecule they deposit on the histone or directly on DNA, those being histone methyltransferases (HMTs), histone acetyltransferases (HATs), DNA methyltransferases (DNMTs), and histone Kinases. The final actor within the histone code are erasers, proteins which are capable of actively removing histone modifications to alter histone-DNA interaction. The form of mark and the histone residue which is it imprinted on determines its effect on chromosome condensation (Hyun et al., 2017).

Histone markings found in specific residues of the histone tails and globular domains are associated with either the repression or promotion of DNA transcription. The

two most studied forms of histone modification being histone acetylation, and histone methylation. Histone acetylation enrichment of the 9^{th}, 14^{th}, and 27^{th} lysine residues of the H3 N-terminal tail have been shown to associate with gene upregulation upon stress exposure, while the removal of acetylated histones correlates with gene silencing or repression (Park et al., 2018). The effect of histone methylation is variable relative to acetylation, as the location and number of methylation marks as well its location on a gene dictates its effect on chromosome condensation and gene expression (Cao et al., 2002). The deposition of methylated marks is mediated by histone lysine methyltransferases and histone demethylases. Histone lysine writers are characterized by the presence of SET domains, while lysine specific demethylases contain either a lysine specific demethylase (LSD1) or a JmJC Jumanji domain (Bannister & Kouzarides, 2011). Histone modifications through deposition of methyl marks show variable effects on histone condensation. For example, tri-methylation of the fourth lysine of H3 (H3K4me3) promotes chromatin relaxation and is commonly found at the transcription start site of genes, while trimethylation of the 27^{th} lysine (H3K27me3) promotes chromatin condensation (Bannister & Kouzarides, 2011). The deposition of methyl and acetyl marks on the globular domains of histones are an important factor of gene regulation, controlling and tuning the response of plants to stimuli, including abiotic stress.

The regulation of transcriptional availability of DNA is not only regulated by deposition of histone markings, but the histone structure itself. Histone variants are histones with partially varying amino acid composition relative to canonical histone subunits that have different conformations and associations with DNA, impacting its interaction with and transcription of DNA. Each histone subunit has a set of its own unique

variants which have been found to associate with a specific functions and localizations in Eukarya. For example, the H2A.Z histone variant of histone subunit H2A can be found in histones wrapped around the coding region of a gene, supressing its expression through physical obstruction (Osakabe et al., 2018). The post translational modification, and the type of histone subunit associating with transcribable DNA influences its expression and response to plant stimuli.

2.2.3 DNA methylation

Along with the post-translational modifications of histones, epigenetic modification through the deposition of methyl groups directly on nucleic acid bases has been shown to alter gene transcription and gene topography. Termed DNA methylation, it classically consists of the direct deposition of a methyl group on the 5^{th} carbon of the carbon ring in cytosine generating a 5-methylcytosine (5mC) and is commonly associated with gene repression (J. Liu & He, 2020; H. Zhang et al., 2018). Recent research has revealed a secondary form of DNA methylation, in which a methyl group is deposited on the nitrogen group of the 6^{th} carbon of its adenine base termed N6-methyladenine (6mA) (Heyn & Esteller, 2015; Z. Liang et al., 2018). This newly identified form of methylation is present in low quantities in plant species and is believed to promote gene expression (H. Zhang et al., 2018; Q. Zhang et al., 2018; Zheng et al., 2020). DNA methylation marks are heritable between generations and can be dynamic and altered within a single lifespan. Due to the nature of this thesis, most of the DNA methylation discussion will be focused on cytosine methylation.

DNA methylation is distributed across the genome, with various patterns and localizations influencing its impact on gene expression. In plants, methylation marks are categorized by three patterns, these being CG, CHG, and CHH (H=A, T, C); their impact on expression is determined by its pattern and localization within the genome (J. Liu & He, 2020; H. Zhang et al., 2018). In *A. thaliana*, methylated cytosines are present in all sequence context in the genome, with 24%, 6.7%, and 1.7% of cytosines being methylated in CG, CHG, and CHH contexts respectively (Cokus et al., 2008). Repressive methylation marks are typically found in the transcription start site of genes, while methylation of the gene body is found on conserved, constitutively expressed genes (J. Liu & He, 2020). Methylation is key to genomic stability, in which repressive 5mC marks can be found in heterochromatin, pericentric regions, and around transposable elements in order to silence their actively (Hollister & Gaut, 2009; H. Zhang et al., 2018).

2.2.4 Deposition and removal of DNA 5mC marks

Methylation of genomic regions is a result of the interactions between and the regulatory dynamics of the establishment, maintenance, and removal of methylation marks in genetic regions. *De novo* DNA methylation is established through the RNA directed DNA methylation pathway (RdDM). This pathway can be directed by two types of RNA polymerases. In the canonical pathway RdDM is guided by RNA POLYMERASE IV (POL IV), while the non-canonical pathway is guided by RNA POLYMERASE II (Pol II; (Haag & Pikaard, 2011) Canonical *de novo* DNA methylation begins through the recruitment of RNA POL IV by SWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), a protein that binds to repressive H3K9me2 marks on histones (Law & Jacobsen, 2010) .

Through the assembly of a complex POL IV generates single stranded RNA (ssRNA), which is then converted into P4 double stranded (RNA) dsRNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), making P4 RNA (Matzke & Mosher, 2014). DICER-LIKE proteins (DCL2, DCL3, DCL4) then convert P4 RNA into 34nt siRNA. The DCL generated siRNA is then loaded into ARGONAUTE (AGO4, AGO6) proteins, beginning the assembly of the Pol V complex that deposits *de novo* methylation marks (Matzke & Mosher, 2014). POV V is recruited by SUPRESSOR OF VARIEGATION (SUVH4) by the binding of methylated DNA. POV V and AGO proteins are directed to the appropriate location. Once the complex is assembled DOMAINS REARANGED METHYLASE 2 (DRM2) catalyzes deposition of methyl groups (J. Liu & He, 2020; Zhong et al., 2014) Canonical DNA methylation is a multistep process that requires the association of multiple proteins and actors.

Demethylation is the removal of 5mC groups from cytosines and is important in the regulation of gene expression. Demethylation may occur in either an active or a passive pathway. Passive DNA demethylation occurs overtime when methylation patterns are not symmetrically re-established on the daughter strand during mitotic division. Active DNA methylation is the physical excision of the methyl group from the base. Demethylation in plants is mediated by the DEMETER family, a group of DNA glycosylase that remove methylated cytosines via the base excision repair pathway (Le et al., 2014). In *A. thaliana* there are four members of the DEMETER family: DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2), and DEMETER-LIKE 3 (DML3). ROS1, RML2, and DML3 are all expressed in vegetative tissue, while DME can be found exclusively in companion cells of female and male gametes (H. Zhang et al., 2018).

Quadruple mutants of the DEMETER family in *Arabidopsis* revealed variations in hypermethylation, gene body methylation, a lack of tissue specific methylation, and premature flowering (Williams et al., 2022). Each member of the DME family tend to target and act in a specific region, for example, DME prefers euchromatic AT rich transposons. On the other hand, ROS1 targets transposons in regions that are enriched for acetylated H3K18 (Tang et al., 2016). Interplay between methylation and demethylation is suggested to occur through ROS1. For example, expression of ROS1 decreased in RdDM mutants, in which hypomethylation did not occur (Williams et al., 2015). The deactivation of demethylation in the absence of fully functional RdDM mediated *de novo* methylation suggests a communication and interplay between removal and deposition of DNA methylation. Active and passive DNA demethylation are an important component of in the stability of genomic regions in plants.

2.2.5 DNA Methylation Maintenance and CHROMOMETHYLASE Proteins

DNA methylation maintenance involves the transmission of methyl marks following mitosis, or gametogenesis. Maintenance of marks depends on sequence context, determining which DNA methyltransferase and pathway propagates the 5mC mark. CG context methylation is maintained by METHYLTRANSFERASE 1 and is capable of recognizing hemi-methylated CG-dinucleotides following replication (Finnegan & Dennis, 1993; Kankel et al., 2003; H. Zhang et al., 2018). CHG methylation is maintained by CHROMOMETHYLASE 3 (CMT3), with some minor activity by CHROMOMETHYLASE 2 (CMT2; Fang et al., 2022; Lindroth et al., 2001; Stroud et al., 2014). Maintenance by CMT3 and CMT2 is dependent on H3K9 histone methylation. CMT proteins bind to

methylated histone sites through their BAH domain, recognizing and depositing methyl marks through its catalytic domain. H3K9 methylation is deposited by KRYPTONITE (KRY) proteins (Fang et al., 2022; Jackson et al., 2002). Interestingly, loss of KRY or loss of CMT2 and CMT3 function result in a loss of H3K9 methylation (Du et al., 2014; Stroud et al., 2014). This suggests a requirement of deposition and interplay between the two epigenetic systems. Asymmetrical CHH marks are regulated by CMT2 and DRM2, with each protein targeting specific genomic regions (Jeddeloh et al., 1999; Zemach et al., 2013). Triple mutants of non-CG methylation proteins, including CMT2, CMT3, and DRM2 results in the absolute loss of non-CG methylation in *A. thaliana (He et al., 2022)*.

CHROMOMETHYLASE (CMT) family proteins are a group of highly conserved DNA methyltransferases involved in the deposition and maintenance of non-CG methylation marks. These proteins are characterized by the presence of three conserved domains, those being the Bromo Adjacent Homology (BAH) and the CHRomatin Organization MOdifier (CHROMO) domains, both of which are required for the binding to H3K9 methylation marks on histones (Du et al., 2012; Jackson et al., 2002). Loss of function of these domains obstructs binding to H3K9 histone marks and prevents cytosine methylation (Du et al., 2012). The final domain found in CMTs is the C5-Methyltransferase domain, involved in catalyzing 5mC methylation in plants. In most plant species, there are three CHROMOMETHYLASE proteins, two of which were previously described, CMT1, CMT2, and CMT3 (Bewick et al., 2017; H. Zhang et al., 2018). In *A. thaliana*, CMT1 is truncated, resulting in a non-functional mutant, suggesting it is not essential to non-CG methylation in *Arabidopsis* (Stroud et al., 2014). Meanwhile CMT2 and CMT3 are both involved in CHH and CHG methylation to varying degrees (Shen et al., 2014).

2.2.6 Impacts of DNA methylation on gene expression

DNA methylation is capable of regulating gene expression through deposition and removal of methyl marks, influencing the targets transcription. The impact of DNA methylation on gene expression is dependent on the location of the methyl mark, and the gene and genomic regions it is targeting. As previously mentioned, methylation of the promoter regions of genes is commonly associated with genetic silencing (Niederhuth & Schmitz, 2017; H. Zhang et al., 2018). Marks deposited on promoter regions inhibit the binding of transcriptional activators or promoting the binding of transcriptional repressors through silencing the gene (Domcke et al., 2015). The degree to which promotor repression is present is species-specific with plants that have more complex genomes containing greater promotor repression to maintain genomic stability (Niederhuth & Schmitz, 2017). Opposite to promoter repression, depression of methylation around transcriptional stop sites and enrichment along the protein coding gene body is named gene body methylation (GbM; X. Zhang et al., 2006). This form of methylation tends to be non-CG context methylation and mostly present on exons (Bewick & Schmitz, 2017). GbM is associated with constitutive expression of essential genes in plants (Bewick et al., 2017; Bewick & Schmitz, 2017) Methylation patterning is consistent between orthologs such as those of *Oryza sativa* and *B. distachyon*, but as evolutionary distance increases, the function and patterns associated with GbM become less clear (Takuno & Gaut, 2013). In *A. thaliana*, GbM can be found in one third of protein coding genes (X. Zhang et al., 2006). On the other hand, species such as *Eutrema salsugenium* have lost GbM, which is hypothesized to be a result of the loss of *EsCMT3 (Bewick et al., 2017; Muyle & Gaut, 2019).* Comparisons between orthologs which contain GbM in *A. thaliana* and *E.*

salsugenium differ in gene expression, reinforcing the importance of species-specific methylation patterning on gene regulation (Muyle & Gaut, 2019).

Importantly, DNA methylation and modification regulates the activity of transposons and retrotransposons through active silencing (Hirsch & Springer, 2017). Transposons, commonly referred to as jumping genes, are rogue genetic elements capable of moving or replicating into various genomic regions. The risk of transposable elements consists of the chance they jump into a protein coding genomic loci or a regulatory region, inhibiting or impeding the function of the loci (Hirsch & Springer, 2017). The suppression of transposons through DNA methylation prevents such replication and mobility through active silencing, maintaining genomic stability (Cokus et al., 2008; Law & Jacobsen, 2010). Sequence context of methylation is dependent on chromosomal location and methylation context. In the pericentromic regions of *A. thaliana*, suppression of transposons can be found with all sequence contexts (Cokus et al., 2008). Meanwhile in euchromatic regions, transposons are commonly supressed through CMT2 CHH methylation, and generate a methylation buffer between coding regions and transposons (Stroud et al., 2014). DNA methylation is a context dependent regulation format important for the regulation of genetic suppression, the maintenance of genomic stability, and transposon control.

2.2.7 Epigenetic impacts on the plant stress response

Over the past 20 years, significant effort has been dedicated to understanding the link between epigenetics; specifically, DNA methylation, and abiotic stress. This effort has led to a substantial output of research linking DNA methylation alterations in response to

abiotic stress, including heat, drought, salinity, cold, and others (G. Liu et al., 2018; T. Liu et al., 2017; W.-S. Wang et al., 2011; H. Zhang et al., 2018). The earliest studies involving DNA methylation were limited by the sequencing technologies of the time, resulting in research focusing primarily on methylation changes at specific loci, or trends in whole genome methylation and pattering (Jean Finnegan et al., 2005; X. Zhang et al., 2006). With the advent of next generation sequencing and the benefits of increased sequencing depth and resolution at reduced cost, studies have analyzed alterations of DNA methylation in response to stress at single base resolutions (D. Liang et al., 2014). As previously described, DNA methylation is commonly associated with gene suppression, while the relief of DNA methylation is generally associated with transcriptional upregulation. While this general assumption is typically sound, does not paint the full picture of the influence of methylation. Methylation profile changes and their impact on transcription are dependent on the location in which they occur. For example, methylation relief in the promoter region are typically associated with transcriptional promotion, while relief of methylation upstream of the promoter may have variable effects (W. Wang et al., 2016). Additionally, sequence contexts such as CG, CHH, and CHG methylation all have different patterns of change in response to abiotic stress, in conjunction with their targets, different impacts on abiotic response. There has been substantial difficulty linking and developing a common thread or pattern changes or a clear standardized lexicon of change in methylation, as patterns, impacts, and expression profiles are different between species and in some cases genotypes. While epigenetic patterning may share similarities between closely related species, these similarities are less recognizable as evolutionary distance increases (Takuno et al., 2016; Takuno & Gaut, 2013).

A major avenue of understanding of DNA methylation and abiotic stress derives from methylation profile comparisons between control conditions and stress treatments, as well as methylation comparisons between stress tolerant and stress sensitive genotypes of the same species. A study conducted between Gutschker and colleges used methylome and transcriptomic data sets to analyze the link between gene expression and methylation status in *Beta vulgarus* (Gutschker et al., 2022). The study revealed a general trend of reduced global DNA methylation in response to LT. Interestingly, when linking methylome changes and RNA-seq data, there was not a clear trend of direct increases of expression and changes in methylation. However, genes that were generally highly expressed did show a reduction in CHH context methylation just upstream of the transcription start site, while no clear trend was present in CHG contexts (Gutschker et al., 2022). This suggests that sequence context and location of methylation are more important to transcriptional control and changes rather than strictly alterations in global methylation. In a cold tolerant variety of rice, methylation patterns and transcriptional responses of seedlings in response to LT revealed a link between cold responsive transcription factors and DNA methylation. The study revealed the relief of promoter methylation of the gene *OsOST1*, a previously described regulator of LT response, its increase in transcription, and overall increase in cold tolerance relative to cold sensitive conditions (Guo et al., 2019). These studies reveal that sequence context, location, and species are all important considerations in understanding how DNA methylation influences gene expression.

While there is evidence of a link between stress and epigenetics, research has been conducted to evaluate which potential DNA methylation actors are involved in

altering epigenetic profiles in the interest of improving cold response. Exposure to cold in plants reduces global DNA methylation, suggesting an increase in global demethylation (Mayer & Charron, 2021; W. Wang et al., 2016; B. Zhang et al., 2016). Decreases in methylation should result from a reduction in RdDM, maintenance, and an increase in demethylation. Studies have revealed upregulation of demethylase encoding genes in response to LT and a downregulation of *CMT2*, the methylation maintenance actor responsible for CHH methylation (Gutschker et al., 2022). In addition, lines which contain mutations in DML and ROS1 demethylase family proteins present an increase susceptibility to fungal infection (Schumann et al., 2017). While there is some evidence to suggest some actors are involved in abiotic stress, the complete picture remains elusive, presenting a prime candidate for study.

2.3 Brachypodium distachyon as a model of cold acclimation.

To gain further insight into the molecular and epigenetic mechanisms which govern CT, CA, and vernalization, more research must be conducted through model plants which excel in CT and FT. *B. distachyon* is a monocot temperate grass which has recently been adopted as a model organism for cereals (Dalmais et al., 2013; Olsen et al., 2006; J. Vogel et al., 2010). The plant is native to the Mediterranean region, growing in high latitudes, where the climate is characterized as being cold and having a high amount of precipitation (Des Marais & Juenger, 2016). A member of the *Poacene* family, *B. distachyon* is related to major cereal commercial crops including Wheat, Barley, Maize, and Rice (Sancho et al., 2018). The plant itself is small in stature, has a seed-to-seed

lifecycle of eight to twelve weeks, and is capable of self-fertilization (C. Li et al., 2012; Scholthof et al., 2018b).

In 2010, the genome of inbred Bd21 *B. distachyon* accession was sequenced, characterizing the size of the genome to be 272 Mb (Vogel *et al.,* 2010). This genome is fairly small compared to other grasses, making it a suitable candidate for the genomic research of grasses. Using 54 accessions of *B. distachyon*, the pangenome of the population was sequenced (Gordon et al., 2017). This study identified 7135 new genes distributed across the lines, revealing the extensive genomic and phenotypic variation between different populations across varying growing regions. To assist in studying *B. distachyon*, multiple research groups have created online resources, including genomic annotations, protocols for agrobacterium transformation, TDNA insertion lines, protocols for CRISPR mediated modification, an RNA-seq in response to abiotic stress database ,and a collection generated through TILLING (Bragg et al., 2012; Dalmais et al., 2013; Hsia et al., 2017; Sreedasyam et al., 2023). Overall, due to its small size, fast generation time, online resources, and active academic research, *B. distachyon* is an excellent model organism to study the molecular biology of abiotic stress.

2.3.2 Brachypodium distachyon transcriptional response to cold exposure.

As a model for grasses undergoing CS, B. distachyon shows a capacity to tolerate low and freezing temperatures when exposed to specific photoperiods and temperatures. Research initially suggested that *B. distachyon* has a weaker response to cold exposure relative to other grasses such as wheat (Colton-Gagnon et al., 2014). However, more recent research has shown that under diurnal freezing, the Bd21-3 accession of *B.*

distachyon has strong response to cold, characterized by a reduced lethality, and greater upregulation of cold responsive genes relative to continual chilling treatments (Mayer et al., 2020). At a molecular level, proteomic characterization reveals differential expression profiles of 1359 proteins in the first two days of cold exposure, including an increase in glucose, dehydrin, metabolic, and signal transduction related proteins (Juurakko et al., 2021).

Transcriptomic responses, including CA and vernalization, are governed by extensive regulation through epigenetics. Vernalization is a requirement for flowering in *B. distachyon's* long day flowering accessions. Flowering and vernalization activation are repressed in non-vernalized plants through the deposition of H3K27me3 marks on *VRN1* by the *REPRESSOR OF VERNILIZATION 1* (RVR1; Woods, Ream, et al., 2017). Upon cold exposure, these repressive marks are lost, and deposition of H3K4me3 methylation on *VRN1* occurs, promoting its transcription and activation of the vernalization cascade. Unlike histone modifications, literature related to DNA methylation of genes involved in vernalization is limited but is an area of research focus.

Epigenetic control of transcriptomic alterations in response to cold acclimation by *B. distachyon* have been recently proven. In a study conducted by Woods et al., changes in temperature lead to the ubiquitination of histone deacetylases by HOS15, leading to the acetylation of cold responsive *COR* genes histones and the subsequent transcription of *COR* genes (Woods et al., 2017). The link between cold acclimation and epigenetics is further reinforced when *B. Distachyon* is placed in diurnal freezing conditions, where repressive DNA methylation and histone marks decrease in cold related genes Bd*CBF1*, Bd*COR410*, and Bd*IRI*, and upregulation subsequently occurred. (Mayer & Charron,

2021). In another study, the deposited transcription activating histone marks between initial and secondary chilling exposure were retained, and the transcription of cold genes in *B. distachyon* are upregulated as the plant acclimates to cold exposure (Mayer *et al.,* 2020). These changes and upregulation in transcriptomic profiles in preparation of subsequent cold treatments is known as transcriptomic memories, in which plants show an improved response to secondary abiotic stress exposure through epigenetic priming (Ding et al., 2012). This is exemplified in a study conducted by Mayer & Charron, transcript abundance of Bd*CBF1, BdCOR410, BdIRI* increased following secondary exposure relative to primary exposure (Mayer & Charron, 2021). In addition, under long term cold exposure, plants began to reduce expression of cold responsive genes, in the interest of continual growth (Mayer & Charron, 2021).

While there is concrete evidence of cold induced transcriptomic memories and epigenetic response through histone modification, the influence of DNA methylation and its modification in response to LT remains elusive. Methylome variation in *B. distachyon* lines revealed evidence of phenotypic variation, but research has not investigated its effects on cold response (Eichten et al., 2016). In *Arabidopsis*, treatment with methylation inhibitory reagent 5-azacytidine has shown to increase freezing and cold tolerance (Xie et al., 2018). Further insight and research into the relationship between DNA methylation, vernalization, and cold acclimation is necessary to understand their interplay in temperate grasses under cold conditions.

2.4: Reverse genetics as a tool for studying cold tolerance

Reverse genetic screening has become a powerful method used to research the relationship between gene and phenotype. Through impeding the activity of a gene, the phenotype of the plant in the absence of the genes function is analyzed, permitting the extrapolation of the genes role in the organism. The activity of a gene is often impeded through the generation of mutant lines, which may impact the transcription, translation, or expression of a gene of interest, or the activity of its translated protein. Common tools used for the generation of mutant plant lines include transposon insertions, RNA interference, and others (B. Liu & Zhao, 2023). These techniques are successful at fully knocking out a gene, but their function is limited as these techniques are often time consuming, containing a limited mutation rate, and operating best on small model organism genomes (Dalmais *et al.,* 2013). To alleviate the difficulty of mutating specific genes, research consortiums generate large mutant libraries, where mutations are randomly generated in the genome, identified, and cataloged. These mutant libraries are placed on public databases where individuals may order seeds which contain a mutation of interest. High throughput sequencing and mutations have allowed for the generation of these mutant libraries, improving the accessibility of reverse generics research. One of the techniques of generating mutant lines is known as TILLING (Targeted Induced Local Lesion IN Genomes), a technique with uses chemical mutagenesis and high throughput sequencing to identify and generate a library of point mutations across an organism's genome (McCallum et al., 2000)

TILLING begins with the treatment of seeds in a chemical mutagen, commonly Ethyl methanesulfonate (EMS) or sodium azide (NaN; Kurowska et al., 2011). Both EMS

and NaN cause point mutations, in which base pair transitions in the C/G and A/T context occur. While the transition mutations are the same, both mutagens have a different method of action. EMS functions as an alkylating agent by adding an ethyl group to a guanine base in DNA (Sega, 1984). This alkylation leads to the mispairing of guanine with thymine during DNA replication. On the other hand, NaN acts as a deaminator of adenine bases in DNA, converting them into hypoxanthine. During replication hypoxanthine pairs with cytosine rather than thymine, resulting in a SNP conversion of C to T (X. Cheng & Gao, 1988). Both mutagens can be used on plants, however NaN cannot be used as a mutagen in animals due to its toxicity. While both mutagens generate the same transition mutations, their method of action differ, making both suitable candidates for TILLING, and a matter of preference for selection of mutagen.

In *Brachypodium*, NaN induced mutagenesis was used to generate TILLING lines due to the mutagens capability of generating a high quantity of mutants, while limiting chromosomal breaks common in mutagenesis (Dalmais *et al.,* 2013). Treatment using NaN mostly causes G/A and C/T base transitions (B. Thomas et al., 2016, p. 217). The initial mutant M1 population is self-fertilized, creating the M2 generation. Sequence data of the M2 population is collected and analyzed to identify mutations (Thomas *et al.,* 2016, p227). Commonly, heteroduplex mismatches indicative of point mutations are identified using heteroduplex specific endonucleases, which are then sequenced (Henikoff et al., 2004). Mismatches are then mapped to the known genome and a mutant library is generated. An SNP library of *B. distachyon* has been made, in which 5530 mutated lines with an average of 680 mutations per line (Dalmais *et al.,* 2013). Of the 680 mutations per line generated in the mutants, most mutations sequences synonymous, preserving

the amino acid sequence of the translated protein. Mutations that generate non-synonymous mutations are often present in a heterozygotic background, minimizing the damage the mutation generates.

TILLING has a series of benefits relative to other mutant generating techniques. Firstly, the chemical mutagen used has a high mutation rate, allowing for the generation of a higher density of mutation sites with a smaller sample size, reducing the amount of screening needed to generate a library (McCallum *et al.,* 2000). Secondly, mutations are generated consistently throughout the genome at a rate of one point mutation per 396 kB in *B. distachyon*, increasing the likelihood of obtaining mutants in smaller genes, a common issue in insertion based mutational techniques (Dalmais *et al.,* 2013). Thirdly, the random nature of SNPs allows for a gradient of mutations to occur, including missense, non-sense, and spice site interference (Kurowska *et al*., 2011). This variety of mutations generates a series of impaired function, as opposed to a full knockout, a favorable option when looking at genes where mutations generate a null mutant.

Disadvantages of using TILLING lines for research include the presence of background mutations which can impact the phenotype beyond the mutation of interest. To compensate for this, a study in which an SNP library was generated for *B. distachyon* used multiple lines in which the common denominator was a mutation in the gene of interest (Dalmais *et al.,* 2013). This combination generates a “guilt by association” conclusion, as similarities in phenotype between the lines are associated with the mutation of interest. As opposed to using tilling lines to conclusively link mutant to phenotype, selected lines may also be used as a source of preliminary data to evaluate if the mutated gene of interest plays a role in the phenomena of interest. If the phenotype

observed is of interest, knockout, CRISPR-cas9 directed mutagenesis, or backcrossed NaN lines may be generated to create a line in which the change of phenotype is conclusively linked to loss of function in the gene of interest. Mutated lines for the purpose of reverse genetics related studies provide an avenue of understanding phenotypic characteristics of plants.

2.5 Conclusion

To conclude, plants undergo significant transcriptional changes in response to cold stress, which affect their transcriptomes and morphology. Epigenetics, particularly DNA methylation, plays a crucial role in regulating gene expression in response to abiotic stress by altering DNA methylation profiles and the actions of DNA methyltransferases. Therefore, studying CHROMOMETHYLASE family genes in the cereal model *Brachypodium distachyon* can provide valuable insights into the role of DNA methylation dynamics and epigenetic modifications in the cold stress response of grasses.

CHAPTER III: RESEARCH FINDINGS

3.1 Materials and methods

3.1.1 Plant Materials and growth conditions

B. distachyon seeds from the *Bd*21-3 accession were used as the wild type for all experiments. Sodium azide treated lines studied in this work were created in the Bd21-3 background (J. Vogel & Hill, 2008). The seed sterilization procedure derives from a protocol formulated by Haas and Raissig (2020). Before planting, *B. distachyon* seeds were imbibed overnight in distilled water prior to surface sterilization. The lemma was

removed following imbibing, and the seeds were treated with a 1.5% sodium hypochlorite solution with agitation for 20 minutes. Following treatment, seeds were washed with sterile deionized water three times. Seeds were then cold stratified at 4°C in dark conditions in 2mL microcentrifuge tubes wrapped in foil for 5 days to promote uniform germination. Stratified seeds were sown in 2 x2 x3.5" 0.4L pots containing a 2:1 mixture of G6 Agromix™ (cat. no. AGRG6; Fafard, Sainte-Bonaventure, QC, Canada) and G10 Agromix™ (cat. no. AGRG10; Fafard, Sainte-Bonaventure, QC, Canada). Plants grown in control conditions were treated to a 16h:8h light:dark photoperiod at 22°C at a light intensity of 250 µmol $m^{-2}s^{-1}$. Plants were bottom watered twice a week and the trays were rotated weekly to minimize chamber effects. For cold kinetics experiments, plants were grown at 22°C in 16-hour photoperiods (16h light/8h dark; 150 µmol m^{-2} s^{-1}) for 14 days before being transferred to 4°C conditions under an 8-hour photoperiod (8h light:16h dark).

Plants grown for the short- and long-term cold acclimation experiments in objective for were prepared as previously described above (Haas & Raissig, 2020). 14 days following germination, three-leaf stage *B. distachyon* plants are either transferred into a 4°C growth chamber under an 8h photoperiod or remain under standard conditions four hours following the beginning of the photoperiod. Plant material is sampled at points 0, 1, 3, 6, 12, 24 hours post start of the experiment for the short-term kinetics experiment, and sampled 0, 7, 14, 21, and 28-days post start of the experiment for the long-term kinetics experiment. Following long term cold exposure, a final harvest was conducted following a 24-hour de-acclimation period. Three biological replicates are collected for analysis at each time point. Arial tissue was harvested for was sampled for each biological replicate

four hours-post the beginning of the photoperiod, and immediately flash-frozen in liquid nitrogen.

For phenotypic measurements of objective 4, Plants were prepared and planted following the previously described methods and grown in a 20h:8h light: dark photoperiod at 22 °C at a light intensity of 250 µmol $m^{-2}s^{-1}$. Plants were grown to the completion of their lifecycle, approximately 8 weeks.

3.1.2 In silico Characterization of BdCMTs

CMT genes in *B. distachyon* were identified using an InterPro (https://www.ebi.ac.uk/interpro) protein domain search for *B. distachyon* genes containing the chromo domain (PF00385). Gene information including accession number, sequences, and homologs of CMTs were retrieved through Phytozome (https://phytozome-next.jgi.doe.gov/). Information regarding domain composition and architecture of identified CMT proteins were analyzed using NCBI conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), ensuring that a BAH (PF01426) CHROMO (PF00385), and C-5 cytosine-specific DNA methylase (PF00145) domain were present. Nuclear localization signals of each *Bd*CMT protein were identified using NLSstradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/)

Data for the heatmap containing transcript accumulation data from *BdCMT* genes in response to CS over a time course of 49 days were obtained from the JGI gene atlas generated from Sreedasyam et al. (2023) hosted by the Joint Genome Institute (Berkely, California, United States). Expression data in Fragments Per Kilobase of transcript per Million Mapped reads (FPKM) for each data set was downloaded and visualized in R.

3.1.3 Multiple sequence alignment and phylogenetic analysis

To understand the relationship between CMT genes of *B. distachyon* and other grasses, a multiple sequence alignment and phylogenetic analysis were conducted between *B. distachyon* CMT genes and putative *CMT* genes in other grasses. Protein sequences of each grass CMT was obtained from Ensemblplants BLAST feature (https://plants.ensembl.org/index.html) using BdCMT (Taxid:15368) protein sequences as a reference to identify CMT protein sequences in Brachypodium stacei (Taxid: 1071399), *Zea Mays* (Taxid: 4577), Oryza sativa (Taxid:4530), *Orioetium thomaem* (Taxid: 1148796), *Panicum hallii* (Taxid:206008), *Panicum virgatum* (Taxid:38727), Hordeum vulgare (Taxid:112509), *Setaria Italica* (Taxid: 4555), *Setaria Virdis* (Taxid:4556), and Sorghum bicolour (taxid:4558) (**Table 3.1**). The presence of a BAH and CHROMO domain was verified in each protein sequence using the NCBI conserved domain search. Sequence alignment was completed using ClustalW and phylogenetic tree construction was done in MEGA11 software (Tamura et al., 2021)

3.1.4 Primer design

Primers for RT-qPCR and mutant line identification were generated using primer3 (https://primer3.ut.ee/). Primer pairs for RT-qPCR were generated with a strategy targeting an exon-exon junction to prevent genomic contamination. Primer pairs were generated for the genes Bd*CMT1, BdCMT2, BdCMT3a, and BdCMT3B,* and genes whose function is known to be involved in the monocots' cold response including *VERNILIZATION 1* (*VRN1*), AND *COLD REGULATED 410* (*COR410*; **Table 3.2**; (Colton-Gagnon et al., 2014; Mayer & Charron, 2021).

Primers for genotyping the sodium azide treated lines were generated using primer3 (https://bioinfo.ut.ee/primer3/). The region of interest was selected by identifying the location of the desired SNP and requesting the primers flank by 250bp on both ends. Annealing temperature for each primer pair was optimized using the NEB Tm calculator (https://tmcalculator.neb.com/#!/main) due to the high annealing temperature required by Q5 high-fidelity DNA Polymerase (**Table 3.2**).

3.1.5 RNA extraction and Transcript accumulation quantification in response to cold stress through Quantitative polymerase chain reaction (qPCR)

To determine transcript accumulation profiles of *BdCMT* genes in response to short term and prolonged CS, Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) was performed. *B. distachyon Bd21-3* seeds are sown, planted, and harvested as outlined in the previous section. RNA was extracted using the EZ-10 spin column plant RNA mini-prep kit (cat. no. BS82314; Bio Basic, New York, NY, USA) following the manufacture's protocol. Complementary DNA was synthesised from the RNA samples using XR-Advanced cDNA Synthesis Kit (cat. no. 801-100 XR; Wisent, Saint-Jean-Baptiste, QC, Canada) following the manufacture's protocol. RT-qPCR was performed using a CFX connect (BioRad) in the presence of Advanced qPCR MasterMix Lo-Rox (cat. no. 800-445 UL; Wisent, Sainte-Jean-Baptiste, QC, Canada). The optimization of the RT-qPCR reaction was performed according to the manufacturer's instructions for each primer pair, and differential expression was calculated using the ΔΔCT method using *UBC18* as the reference gene (Hong et al., 2008).

3.1.6 Statistical Analysis

All Statistical analysis was completed through JMP 17 (SAS Institute Inc., Cary, NC, USA). Experimental data for objectives 2 and 4 were first tested for normality using the Shapiro-Wilks test. Pairwise data was tested for significance using a one-way ANOVA and a Tukey's honesty significance difference test to compare expression between treated and control conditions and was used to compare significance between different mutant lines in objective 4. Phenotypic data that was found to be non-normally distributed was analyzed using a Dunn test all pairs for joint ranks test.

3.1.7 NaN line identification and genotyping

NaN lines were ordered from the Joint Genome Institute (Dalmaise et al., 2013). NaN lines containing mutations in the C5 methyltransferase, the CHROMO, or the BAH domains were selected that the desired mutation would result in a change in CHROMOMETHYLASE protein structure.

Verification of the presence of the mutation in each line of interest was done through sanger sequencing of the location of interest. Leaf tissue of each mutant line was harvested, and flash frozen in liquid nitrogen. Genomic DNA was extracted using the CTAB method following a modified protocol by *Aboul-Ftooh et al* (2019). Quality and purity of the extracted DNA was completed using a BioPhotometer with the uCuvette attachment (Eppendorf) set to 260/280 abs. The region containing the mutation of interest was amplified using specific primers and the Q5® High-Fidelity 2X Master Mix (cat. no. M0492SVIAL; New England Biolabs, Ipswich, MA, USA). The resulting amplicons were then run through a 1% agarose gel to ensure that non-specific bands were not present. If

non-specific bands were present, amplicons were run through a 2% agarose gel, and bands that were the expected product size were extracted using a scalpel and placed in a 2mL microcentrifuge tube. PCR cleanup was conducted using EZ-10 Spin column PCR products purification kit (cat. no. BS363; Bio Basic, New York, NY, USA) to remove PCR reaction reagents. Amplicons that contained only the target of interest were extracted using the PCR product purification protocol on page 11 of the handbook, while amplicons that that were excised from an agarose gel were extracted using the gel extraction protocol on page 13 of the instruction's handbook included in the kit. PCR products for each mutant line were ligated to the CloneJET PCR cloning kit (cat no. K1231, ThermoFisher, Whitby, ON, Canada) following manufactures protocols. The plasmid was the transformed into electrocompetent *E.coli* DH10B strains using the Multiporator (Eppendorf) Products were selected by spreading transformed product on LB plates containing ampicillin and incubated overnight. The presence of the plasmid of interest was verified through colony PCR using the sequencing primers included in the plasmid kit. Plasmids were extracted from colonies which contained the region of interest using EZ-10 Spin column plasmid DNA miniprep kit (cat. no. BS413; Bio Basic, New York, NY, USA) following manufactures protocols and the presence of the region of interest in the plasmid was further verified through a restriction digest of BglII. Plasmids were then sent out for sanger sequencing to Genome Quebec. Sequenced results were retrieved from NANUQ (https://ces.genomequebec.com/nanuqAdministration/) and compared to wild type sequences of the gene of interest using MUTALIGN (http://multalin.toulouse.inra.fr/multalin/) to validate the presence of the desired mutation.

3.1.8 Phenotypic measurements

Sodium azide treated *B. distachyon* lines were grown and compared to wild type to observe any deviations in phenotypic traits relative to wild type. Days to heading was measured starting from the period of planting to the first visible emergence of awns from the flag leaf sheath, otherwise known as Zadok 49 on the Zadok scale (Zadoks, Chang, & Konzak, 1974. Plant height was measured according to protocols outlined in Tyler et al. (2014).The number of tillers is counted according to a guide on YouTube (Randy Weisz, 2013) . To measure plant tissue weight, the shoot system is separated from the root system using scissors, cutting at the transition zone separating the two systems. Aerial tissue was then collected and measured using a scale.

Table 3.1 Species and sequences used for multiple sequence alignment

Species	Sequence Id	Protein
Brachypodium distachyon	Bradi1g68986	BdCMT1
Brachypodium distachyon	Bradi1g66167	BdCMT2
Brachypodium distachyon	Bradi3g21450	BdCMT3a
Brachypodium distachyon	Bradi3g39050	BdCMT3b
Setaria Italica	Seita.9G479200	SiCMT1
Setaria Italica	Seita.9G300300	SiCMT3
Setatia Italica	Seita.3G080000	SiCMT2
Brachypodium stacii	Brast03G093800	BsCMT1
Brachypodium stacii	Brast02G108300	BsCMT3
Oryza sativa	LOC_Os03g12570	OsCMT1
Oryza sativa	LOC_Os05g13780	OsCMT2
Zea mays	Zm00001d026291	ZmCMT1
Zea mays	Zm00001d002330	ZmCMT3
Panicum virgatum	Pavir.9NG370900	PvCMT3
Panicum virgatum	Pavir.3NG044900	PvCMT2
Panicum halii	Pahal.3G074800	PhCMT2
Panicum halii	Pahal.9G360100	PhCMT3
Hordeum vulgare	HORVU.MOREX.r3.6HG0628050	HvCMT2
Hordeum vulgare	HORVU.MOREX.r3.4HG0386550	HvCMT3
Setaria viridis	Sevir.9G306200	SvCMT3
Setaria viridis	Sevir.9G482900	SvCMT1
Setaria viridis	Sevir.3G081800	SvCMT2
Sorghum bicolour	Sobic.004G197400	SbCMT1
Sorghum bicolour	Sobic.006G214000	SbCMT2

Table 3.2 Primers Used in this study

Primer	Analysis	Primer Sequence	Reference
UBC18_F	RT-qPCR	GGTCATTTTCCTCAACCCGG	Ream et al. 2014
UBC18_R	RT-qPCR	GCGGCAGTTCCTAACATAGC	
CMT2_F	RT-qPCR	AGTGGGAAACGAGGAAAG	This paper
CMT2_R	RT-qPCR	GACCTCGCACTTCAGAAT	
CMT3a_F	RT-qPCR	AAGTACAACCACCCCGGAAC	This Paper
CMT3a_R	RT-qPCR	ATCCTCTGAGGGACTAGCCG	
CMT3b_F	RT-qPCR	AACCAATCTGCTCTCCTCCC	This Paper
CMT3b_R	RT-qPCR	GATCGGCCGTCTTCTTCTTC	
VRN1_F	RT-qPCR	GCTCTGCAGAAGGAACTTGTGG	Ream et al. 2014
VRN1_R	RT-qPCR	CTAGTTTGCGGGTGTGTTTGCTC	
CBF1_F	RT-qPCR	ACCCGTACTACGAGATGGGC	Ryu et al 2014
CBF1_R	RT-qPCR	ATCGGAGGAGGGTCAATGAG	
COR410_F	RT-qPCR	AGCAAAGCCACAAGCCAAG	Mayer et al. 2020
COR410_R	RT-qPCR	GTCAAAGAGGCCCTATCCG	
CMT1_NaN1080_F	Sequencing	GCTCTTGTCCTTGGAGATGC	This paper
CMT1_NaN1080_R	Sequencing	TGGCATAATTGAACACACCCA	
CMT1_NaN556_F	Sequencing	ACTTGGGATGATTGTTGCAGG	This paper
CMT1_NaN556_R	Sequencing	AGAAAACAGTACAGCGCGAC	
CMT1_NaN370_F	Sequencing	ACATGCCCTACTACACCGAC	This paper
CMT1_NaN370_R	Sequencing	GACGCCCTTGTAAACGAACC	
CMT1_NaN351_F	Sequencing	GATGGCCTTAGGTTGGTATGT	This paper
CMT1_NaN351_R	Sequencing	CACAAGCCGACTCAATGCAT	
CMT2_NaN208_F	Sequencing	TGTACTGTGGTTGTGGTGGA	This paper
CMT2_NaN208_R	Sequencing	CCGGACCTTTTAAAGCAGCA	
CMT2_NaN1609_F	Sequencing	GCCTTTTGCCACTTTGTAGAG	This paper
CMT2_NaN1609_R	Sequencing	TCACCTGGGATCGAATAAGAGG	
CMT2_NaN1732_F	Sequencing	TGTAGCGTAATTTGGTTGGGT	This paper
CMT2_NaN1732_R	Sequencing	TTTCCCTGAATTCTCCCAACA	
CMT3a_NaN207_F	Sequencing	CGCGACTTGGGATTATGGTC	This paper
CMT3a_NaN207_R	Sequencing	GCGAGCCAATTAAACACTTGC	
CMT3a_NaN326_F	Sequencing	TCTGCCGAAGTGATCCAACT	This paper
CMT3a_NaN326_R	Sequencing	GGGTGGTTGTACTTCAGGCT	
CMT3a_NaN0638_F	Sequencing	TCTTTCCATCCCCTCTCTCG	This paper
CMT3a_NaN0638_R	Sequencing	GACTCATCCTCCTCACCCAG	
CMT3b_NaN246_F	Sequencing	GAATGAACGGGCCCATCAAG	This paper
CMT3b_NaN246_R	Sequencing	TCATCTCCCCTCACCAATCC	
CMT3b_NaN2111_F	Sequencing	TGGATCGGAGGGAGTAACTT	This paper
CMT3b_NaN2111_R	Sequencing	GTTCAGGTCAACAGCCCATC	
CMT3b_NaN493_F	Sequencing	GCTGTTCCAATTCTCCAGACA	This paper
CMT3b_NaN493_R	Sequencing	CGGAAGTTGGCTCCCTAAAA	

3.2 Results

3.2.1 Brachypodium distachyon has 4 CHROMOMETHYLASE genes

To identify the number of CMT genes within *B. distachyon*, an *in silico* online search was conducted. Using a Pfam domain search through InterPro, with the query containing the requirements of the presence of a CHROMODOMAIN, a C5-methyltransferase, and a BAH domain, the search returned four protein sequences that fit the desired parameters of a CHROMOMETHLASE protein. Further information including the accession number, protein sequence, nucleotide sequence, and location obtained by searching for identical matches in EnsemblPlants, returning four genes, each categorized into one of the three types of *CHROMOMETHYLASE* classes. *BdCMT1*-predicted (BRADI_1g68985v3), *BdCMT2* predicted (BRADI_1g66167v3), and two *BdCMT3* predicted genes being *BdCMT3a* (BRADI_3g21450v3), and *BdCMT3b* (BRADI_3g39050v3). *BdCMT1* and *BdCMT2* were found on the first chromosome of *B. distachyon* located 68116732..68126086 forward, and 65658204..65671188 reverse respectively. The two *CMT3* class genes were found on the third chromosome at location Bd3:20856574..20864222 forward for *BdCMT3a*, and 41375766..41382138 reverse for *BdCMT3b*.

To observe the configuration and if any additional domains exist in the proteins, a conserved domain search was completed. All BdCMT protein sequences contained the predicted CMT domains in the same order (**Figure 3.1**). The BAH domain and CHROMO domain involved in binding to methylated lysine sites, as well as facilitating protein-protein interaction, while the C5-DNA methyltransferase domain responsible for the deposition of the methyl mark on the cytosine of the DNA sequence were found. To verify that the CMT

proteins return to the nucleus following translation, nuclear localization signals (NLS) were identified using NLSradamus. Each CMT protein contained a N-terminal NLS at the beginning of the sequence. Nuclear localization signals for BdCMT1, BdCMT3a, and BdCMT3b were predicted to be within the first 50Bp of the start of their respective sequences. Interestingly, the predicted cite of the NLS for BdCMT2 was found deep within the protein sequences, starting 246bp past the start of the protein. The high probability of the presence of the three domains, as well as evidence of the nuclear localization signal, reinforce the idea that the four identified gene sequences are members of the CHROMOMETHYLASE family.

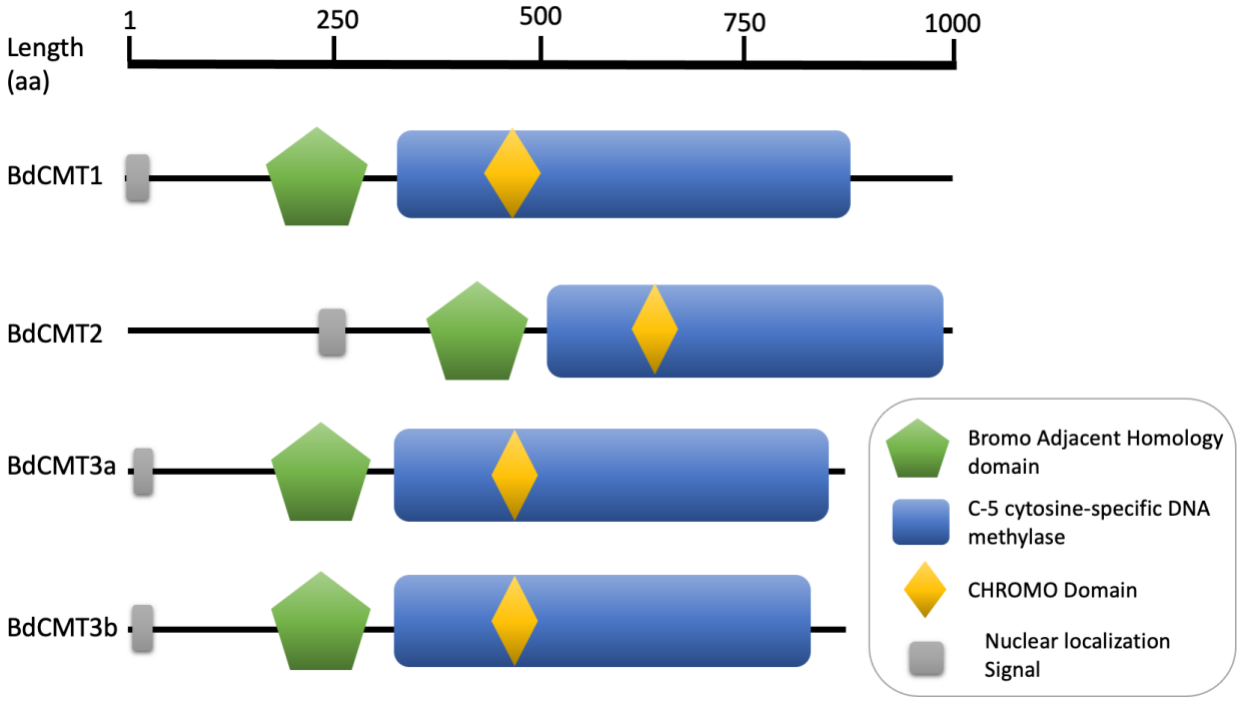


Figure 3.1 *Brachypodium distachyon* has four CHROMOMETHYLASES.
Predicted domain structure for four CMT proteins found in *B. distachyon*: BdCMT1, CMT2, CMT3a, and CMT3b. BdCMTs contain the BROMO adjacent homology domain, C5 DNA methylase, and CHROMO domains respectively responsible for protein-protein interactions, methylation of the C5-carbon of cytosine, and chromatin targeting through lysine binding and protein-protein interactions. Horizontal length of each colored shape represents length of each respective domain.

3.2.2 Brachypodium distachyon CMTs share conservation of structural motifs between monocots

Upon identification of the CHROMOMETHYLASE genes in *B. distachyon*, we wanted to understand the phylogenetic relationship of BdCMT proteins with CMT protein sequences in other commonly studied grass species. To do this, the CMT protein sequences of *B. distachyon* were used to identify CMT protein sequences in other grass species including, *Brachypodium stacei* (Bs), *Zea Mays* (Zm), *Oryza sativa* (Os),

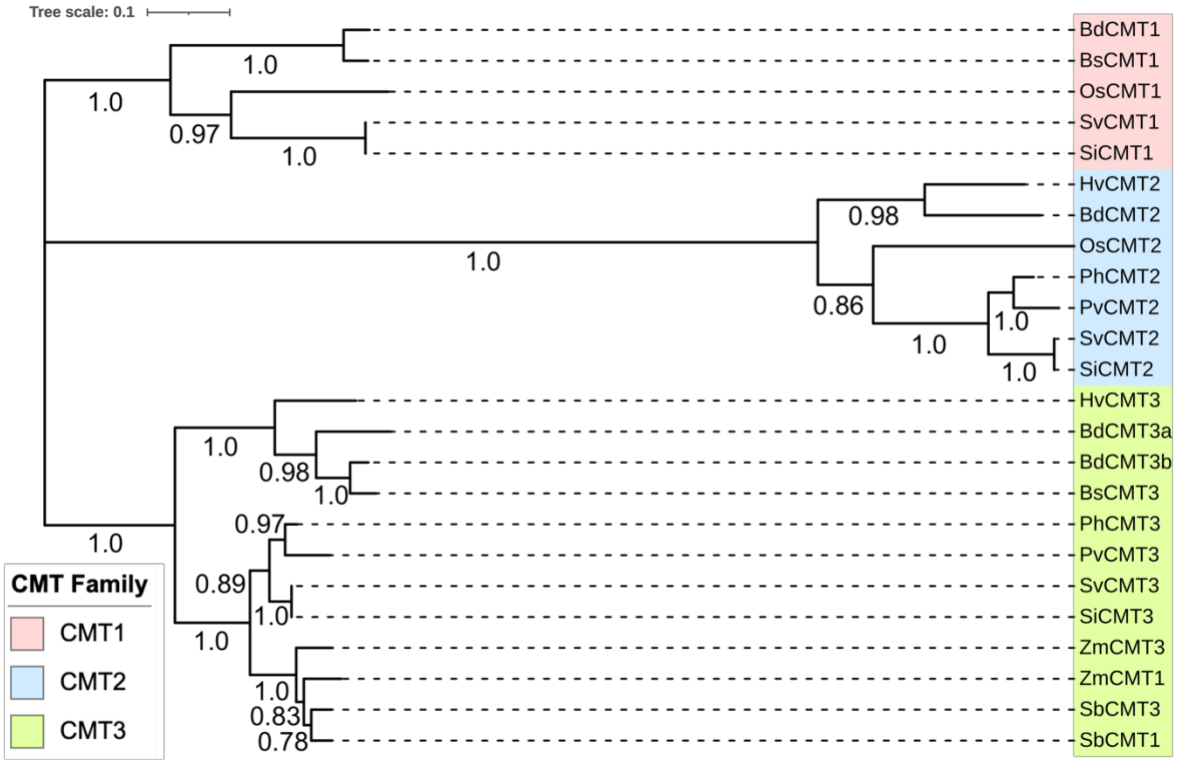


Figure 3.2 *Brachypodium distachyon* CMT amino acid sequences cluster with CHROMOMETHYLASE proteins of other monocots. Unrooted Maximum likelihood phylogenetic tree inferred from CHROMOMETHYLASE of selected monocot species. Proteins are clustered into three subgroups containing a total of 24 CHROMOMETHYLASE proteins sequences. Bootstrap support from the maximum likelihood analyses are indicated below branches

Orioetium thomaem (Ot), *Panicum halii* (Pi), *Panicum virgatum* (Pv), *Hordeum vulgare* (Hv), *Setaria Italica* (Si), *Setaria Virdis* (Vi), and *Sorghum bicolour* (Sb).

The query resulted in 42 sequences with an e-value under 1e-50. When sequences were retrieved, the presence of a CHROMO, BAH, and C5 DNA methylase domains were verified, refining the search to 24 protein sequences. Proteins were clustered using the CLUSTAL algorithm from MEGA11 and assembled using the maximum likelihood method with a replicate count of 1000 bootstraps. In the resulting unrooted phylogenetic tree, we observe that the sequences cluster into three distinct clades (**Figure 3.2**). Each of these clades associate and cluster with one distinct category of BdCMT gene. Each of the 10 species used in the phylogenetic analysis contained between two to four CMT proteins sequences. *Brachypodium distachyon* coded for the most CMT proteins with four, while *six* other species were revealed to only encode two CMT proteins. While *B. distachyon* sequences clustered with other CMT proteins in their respective clades, Zm and Sb CMT1 sequences preferentially clustered with CMT3 sequences from their own species within the CMT3 monophyletic group.

3.2.3 Virtual Expression analysis suggests BdCMT genes are cold responsive

Public RNA seq data was analyzed to observe if *BdCMT* genes were responsive to long term cold exposure. RNA seq data, as seen in a heatmap (**Figure 3.3**) shows changes in expression under long term 4°C cold exposure and a photoperiod of 10h light /14h dark conditions over a course of 49 days. In leaf tissue, Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values of *BdCMT1* are consistently low relative to the other four *BdCMT* genes. *BdCMT2* FPKM values increase over the duration of cold exposure, peaking at 29.215 FPKM at 28 days of cold exposure, relative to its initial FPKM of 18.577. *BdCMT3a* values are shown to remain

relatively consistent in virtual expression. However, the lowest value *BdCMT3a* exhibits during cold exposure is 2.877 relative to its 5.213 FPKM at time point zero, a nearly half reduction in *BdCMT3a* values of 55%. *BdCMT3b* virtual expression indicates a general trend of decreased transcripts as cold exposure progresses, with its lowest value exposure observed at 14 days of cold exposure at a value of 12.855 FPKM relative to its timepoint zero expression of 26.557. The RNA seq data obtained from the DOE Gene Atlas suggests that *BdCMT* genes change their transcriptional characteristics in response to long term cold exposure.

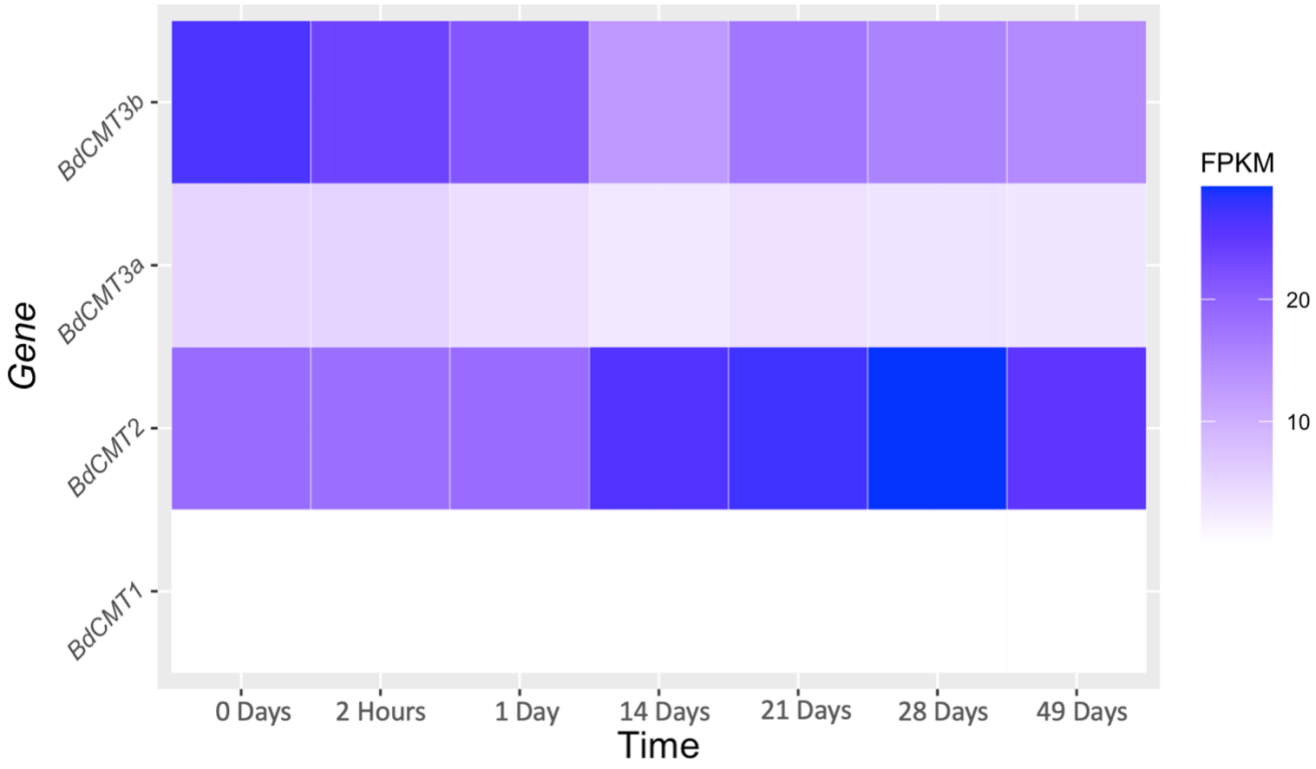


Figure 3.3 Virtual expression of *BdCMT* RNA transcripts suggests transcriptional changes in response to prolonged cold exposure. Heatmap of the FPKM values of *BdCMT* genes in response to 49 days of 4°C treatment. Raw expression data was obtained from Sreedasyam et al 2023

3.2.4 B. distachyon CMT2 is transcriptionally responsive to short term cold stress

To determine if *B. distachyon CMT* genes are transcriptionally responsive to short term cold exposure, RT-qPCR data of plants exposed to 4°C for 24 hours was generated (**Figure 3.4**). To verify that *B. distachyon* plants were indeed undergoing a response to cold exposure, transcript accumulation of the dehydrin *BdCOR410* was evaluated. The transcriptional response of *BdCOR410* relative to control conditions reveals a significant 109-fold increase in expression following 24 hours of cold exposure. Observation of *BdCMT2* expression relative to control conditions resulted in a significant 0.56-fold transcript level relative to control conditions.

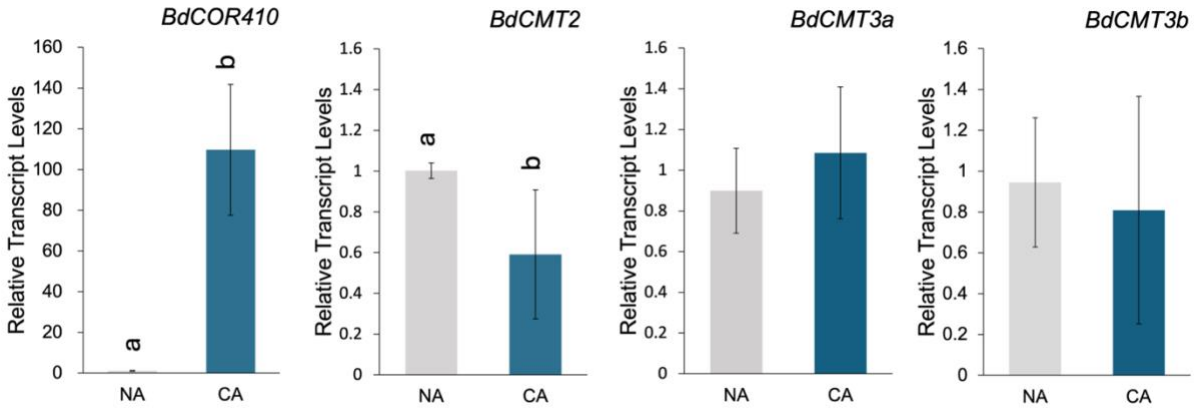


Figure 3.4 *BdCMT2* is transcriptionally responsive to short term cold exposure. RT-QPCR transcript accumulation profile of cold responsive COR410 and *B. distachyon CHROMOMETHYLASE* genes in response to 24 hours of cold exposure. Control group denotes 24 hours of long day conditions at 22°C, treated groups represent 24 hours of long day conditions at 4°C. Statistical significance is denoted with letters, representing an ANOVA and a Tukey's HSD test. Error bars denote standard deviation from the mean of 3 biological replicates.

While *B. distachyon* plants did indeed undergo a response to cold stress, BdCMT3 and BdCMT3b did not have a significant departure from control conditions following short term stress, with a relative transcript value of around 1, a level similar to control conditions. These results reveal that *BdCMT2*, but not *BdCMT3a* or BdCMT3b is

significantly transcriptionally responsive when exposed to cold stress over a course of 24 hours.

3.2.5 B. distachyon CMTs are transcriptionally responsive to long term cold acclimation

To determine if *B. distachyon CMT* genes are responsive to prolonged cold exposure, a transcript accumulation analysis of *BdCMT* genes and known long term cold responsive genes *BdCOR410* and *BdVRN1* was conducted. Plants were either left in control conditions (NA) for 0 and 7 days or cold acclimated (CA) in 4°C short day conditions for up to 28 days. Following the 28-day cold acclimation treatment, de-acclimation (DA) for 24 hours was conducted. *BdCOR410* showed a general trend of increasing in relative transcript levels as cold acclimation progressed (**Figure 3.5**). CA14 displayed a significant 15-fold increase in expression relative to NA7 and DA timepoints, while the highest expression level was seen upon 21 days of cold exposure, displaying 21-fold increase in expression relative to NA. The transcripts of the key vernalization regulator *BdVRN1* are known to accumulate following prolonged cold exposure (Colton-Gagnon et al., 2014). As expected, transcript accumulation of *BdVRN1* significantly increased during cold exposure. *BdVRN1* expression was significantly higher following CA14 relative to NA0 and NA7, with an expression peak of 333-fold at CA28. Following de-acclimation, transcript levels remain high at a level of 195-fold.

Knowing that our cold treatment successfully triggered *BdCOR410* and *BdVRN1* transcript accumulation, we aimed to observe the *BdCMT* genes transcriptional responses to long term cold acclimation (**Figure 3.5**). *BdCMT2* displayed a significant

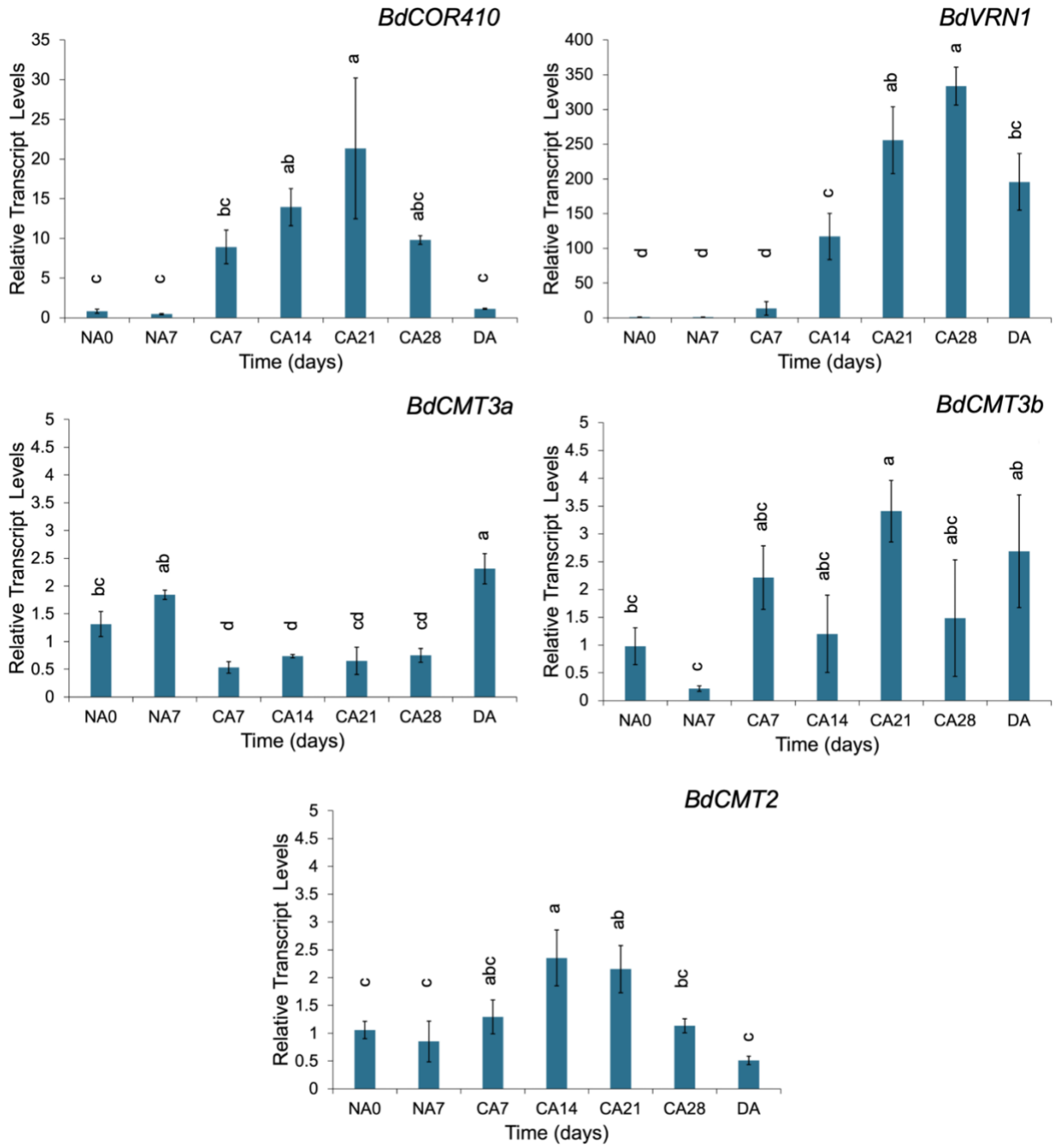


Figure 3.5 *BdCMT* genes are transcriptionally responsive to long term cold exposure. Transcriptional responses of *Brachypodium distachyon* genes exposed to non-acclimated (NA) condition for 0 or 7 days in long day conditions at 22°C , or *Bd* genes exposure to a 28 day cold acclimation (CA) treatment for 28 days followed by a 24 hour de-acclimation period (DA). Cold responsive genes *BdCOR410*, *VERNALIZATION 1* (*Bd*VRN1) and *Brachypodium distachyon* CHROMOMETHYLASE genes *BdCMT2*, *BdCMT3a*, and *BdCMT3b*. Statistical significance is denoted with letters, representing an ANOVA and a Tukey's HSD test. Error bars denote standard deviation from the mean of 3 biological replicates. Graphs denote levels of transcript levels relative to non-acclimated 22°C conditions.

increase in transcript levels relative to NA0, NA7. points following 14 and 21 days of cold exposure with a relative increase of transcript levels of 2.35 and 2.15 respectively. Following cold treatment, transcript accumulation levels during de-acclimation returned to expression levels similar to NA7. *BdCMT3a* experiences downregulation during cold exposure, reaching the lowest expression level (0.5-fold) at CA7. Following de-acclimation, *BdCMT3a* expression returns to levels similar to NA7. *BdCMT3b* did not show a clear trend of transcription upon exposure to cold stress, fluctuating in expression levels between 3.5 and 1-fold under prolonged exposure to cold. Significant differences in expression relative to NA0 were only observed at CA21, suggesting some response to cold stress.

3.2.6 Validation of Mutations in BdCMT NaN lines

Following the confirmation that *BdCMT* genes are transcriptionally responsive to CS, we wanted to functionally characterize *B. distachyon* plants with mutations in *BdCMT* genes. 15 Lines containing SNP mutations in *BdCMT* genes were selected from a NaN library. The open reading frames of *BdCMT* genes in these lines possessed either a missense mutation in the BAH, C5 domains, or CHROMO domain, or a mutation early in the open reading frame that would result in a premature stop codon. These mutations in the *BdCMT* genes would hypothetically disrupt the function of the respective domain, and in turn, the function of the translated protein. This led to the selection of 15 potential NaN lines (**Table 3.3**), Of the lines selected, 4 contained mutations in *BdCMT1*, 5 in *BdCMT2*, 3 for *BdCMT3a*, and four for *BdCMT3b*.

Table 3.3 15 *B. distachyon* sodium azide lines carrying mutations in functional domains of *BdCMT*s. The table indicates the position and impact of each SNP mutation on its respective targeted gene in the lines identified from Dalmais et al 2013.

Line	Gene	Mutation	Codon Change	Domain Targeted	Impact
370	*BdCMT1*	G to A	G11129E	Methylase	Missense
351	*BdCMT1*	G to A	G519D	Methylase	Missense
1080	*BdCMT1*	C to T	P678S	N/a	Missense
556	*BdCMT1*	C to T	P639S	N/a	Missense
367	*BdCMT2*	C to T	G316E	BAH	Missense
1644	*BdCMT2*	G to A	H368Y	BAH	Missense
1609	*BdCMT2*	C to T	W417*	BAH	Nonsense
208	*BdCMT2*	C to T	N/A	Methylase	Splice Site
1732	*BdCMT2*	G to T	N/A	Methylase	Splice Site
246	*BdCMT3a*	G to T	E/Stop	N/a	Nonsense
2111	*BdCMT3a*	G to A	G341E	Methylase	Missense
493	*BdCMT3a*	G to A	R707K	Methylase	Missense
0638	*BdCMT3b*	C to T	Q32*	N/a	Nonsense
326	*BdCMT3b*	C to T	L328F	Methylase	Missense
207	*BdCMT3b*	G to A	G614D	CHROMO	Missense

To verify the presence of the SNP mutations, amplicons of regions containing the predicted mutation of interest were generated and sent out for sequencing (**Figure 3.6**). Analysis through Mutaline revealed that only 11 of the 15 ordered lines contained their desired mutation, and subsequent experiments were conducted using only lines with mutationally verified lineages (**Table 3.4**) Of the four lines that did not contain the mutation of interest, 351 and 367 were homozygous for their predicted mutation while lines 1609 and line 207 were heterozygotic. Lines in which the presence of the desired mutation was ambiguous were re-sampled and sequenced again to ruling out the possibility that only one allele contained was amplified. Overall, 11 lines containing mutations in *BdCMT* genes were identified, and could be used in subsequent experiments.

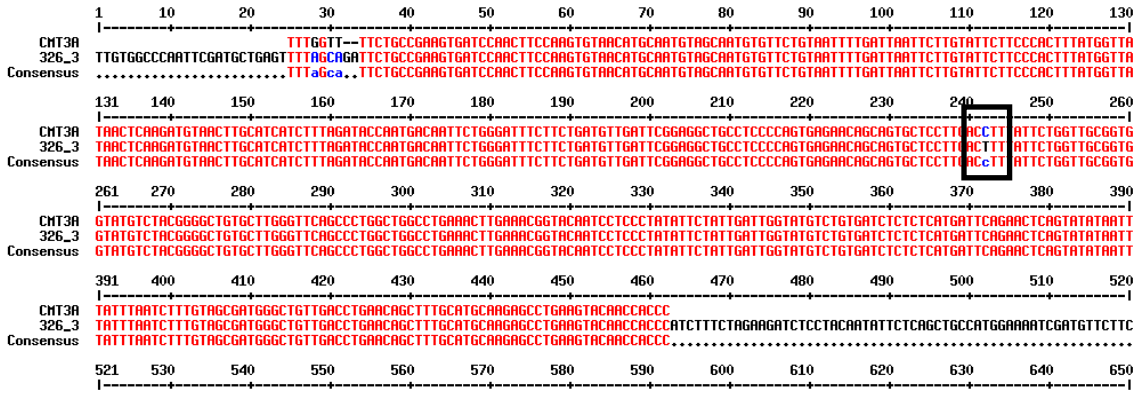


Figure 3.6 Selected line of *BdCMT* 326 contains the desired SNP mutation. Example of verification of the presence of mutations of interest in BdCMT genes using sanger sequencing and Mutalign. Location of mutation highlighted by black box.

3.2.7 Mutations in B. distachyon CMT genes alter transcript accumulation of cold responsive genes

Following the verification of mutational insertions in *BdCMT* genes, we wanted to observe if there would be changes in the transcriptional response of cold responsive genes *BdCOR410* and *BdVRN1*, as well as *B. distachyon* CMT genes *BdCMT3a* and *BdCMT3b* in verified mutant lines compared to wild type when exposed to prolonged cold exposure. Due to limited growth space constraints, only *BdCMT3a* and *BdCMT3b* mutant lines 493, 326, 2111, and 0638 were studied (**Table 3.4**). A long-term cold acclimation experiment setup identical to the cold treatment in objective 2 was conducted to assess transcriptional response. Analysis of the transcriptional accumulation of *BdCOR410* did not show a trend of significant changes in relative expression among all lines relative to wild type (**Figure 3.7A)**. However, at time point CA7, lines 493, 2111, and 0638 showed a significant difference in relative expression of COR410 compared to wild type. No statically significant differences were observed at CA14, CA21, CA28 and DA, indicating

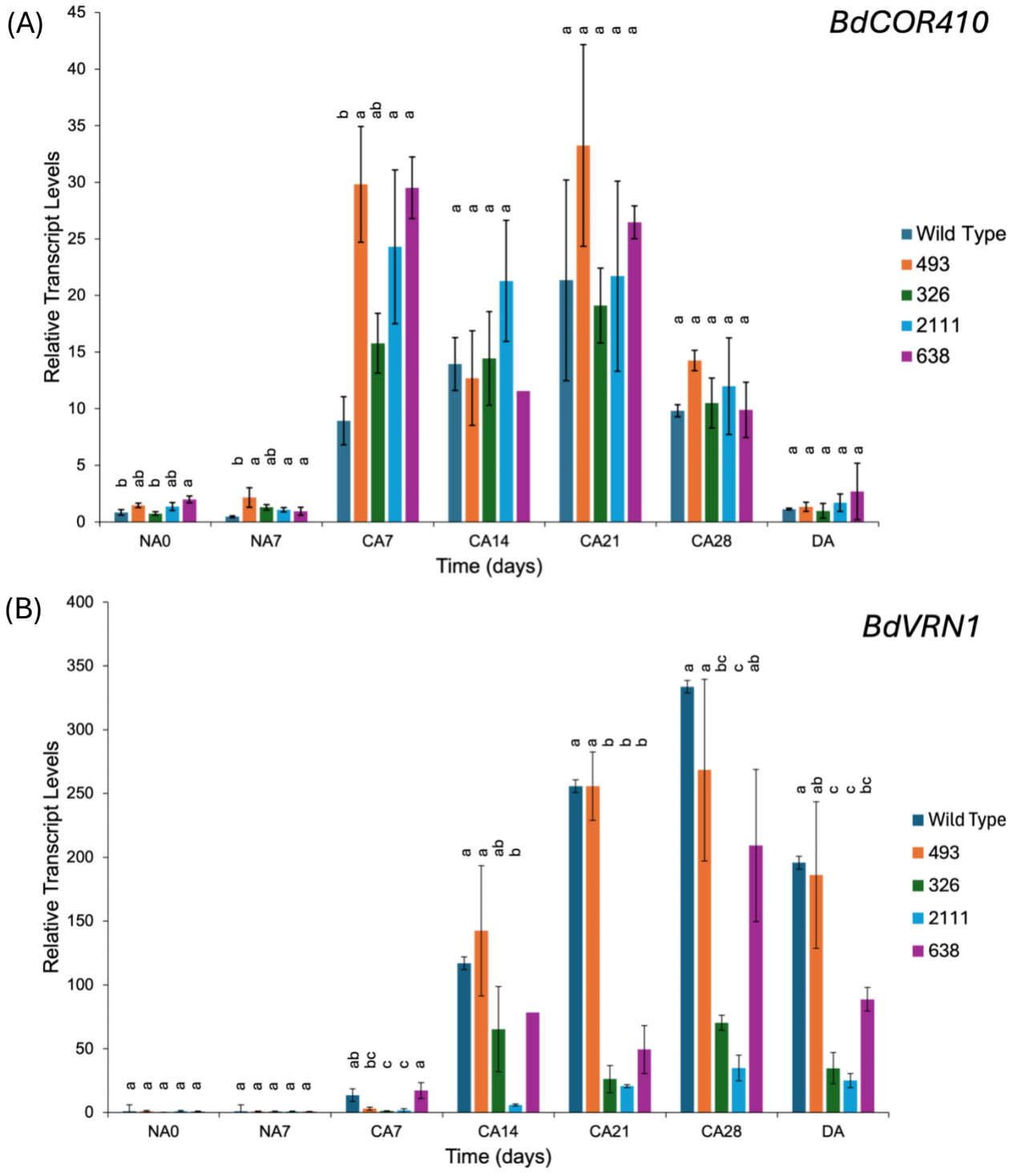
that the mutations in *BdCMT3* impact the accumulation of COR410 transcripts withing one week of cold acclimation.

Table 3.4 11 *B. distachyon* sodium azide lines with verified mutations in catalytic domains of BdCMTs. The table indicates the position and impact of each SNP mutation on its respective targeted gene in the lines identified from Dalmais et al 2013.

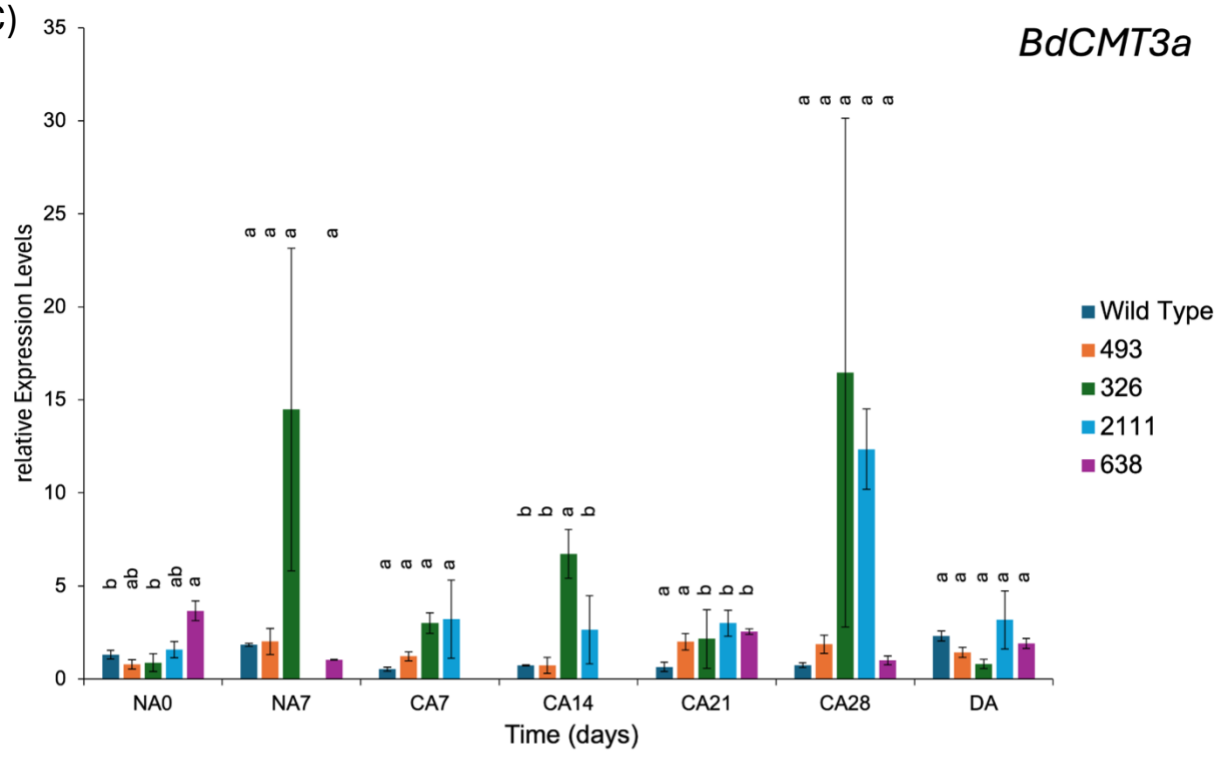
Line	Gene	Mutation	Codon Change	Domain Targeted	Impact
370	*BdCMT1*	G to A	G11129E	Methylase	Missense
1080	*BdCMT1*	C to T	P678S	N/a	Missense
556	*BdCMT1*	C to T	P639S	N/a	Missense
1644	*BdCMT2*	G to A	H368Y	BAH	Missense
1732	*BdCMT2*	G to T	N/A	Methylase	Splice Site
246	*BdCMT3a*	G to T	E/Stop	N/a	Nonsense
2111	*BdCMT3a*	G to A	G341E	Methylase	Missense
493	*BdCMT3a*	G to A	R707K	Methylase	Missense
0638	*BdCMT3b*	C to T	Q32*	N/a	Nonsense
326	*BdCMT3b*	C to T	L328F	Methylase	Missense
207	*BdCMT3b*	G to A	G614D	CHROMO	Missense

BdVRN1 showed significantly lower relative expression in three of the four mutant lines (**Figure 3.7B)**. Lines 326 and 2111 showed significantly lower relative expression at all cold time points and after de-acclimation. Line 493 shared a similar trend of expression to wild type, and revealed a similar level of expression at all timepoints. Line 0638 had a generally lower transcript accumulation at all cold exposure time points, but these changes are only statistically significant at timepoints CA21 and DA. Overall, changes in transcript accumulation of VRN1 is observed in 3 of the 4 lines containing mutations in *BdCMT3.* Transcript accumulation of BdCMT3a in the *BdCMT3* mutant lines showed relatively similar expression patterns over the time course of the cold acclimation experiment, with some significant changes in expression at specific time points (**Figure 3.7C)**.

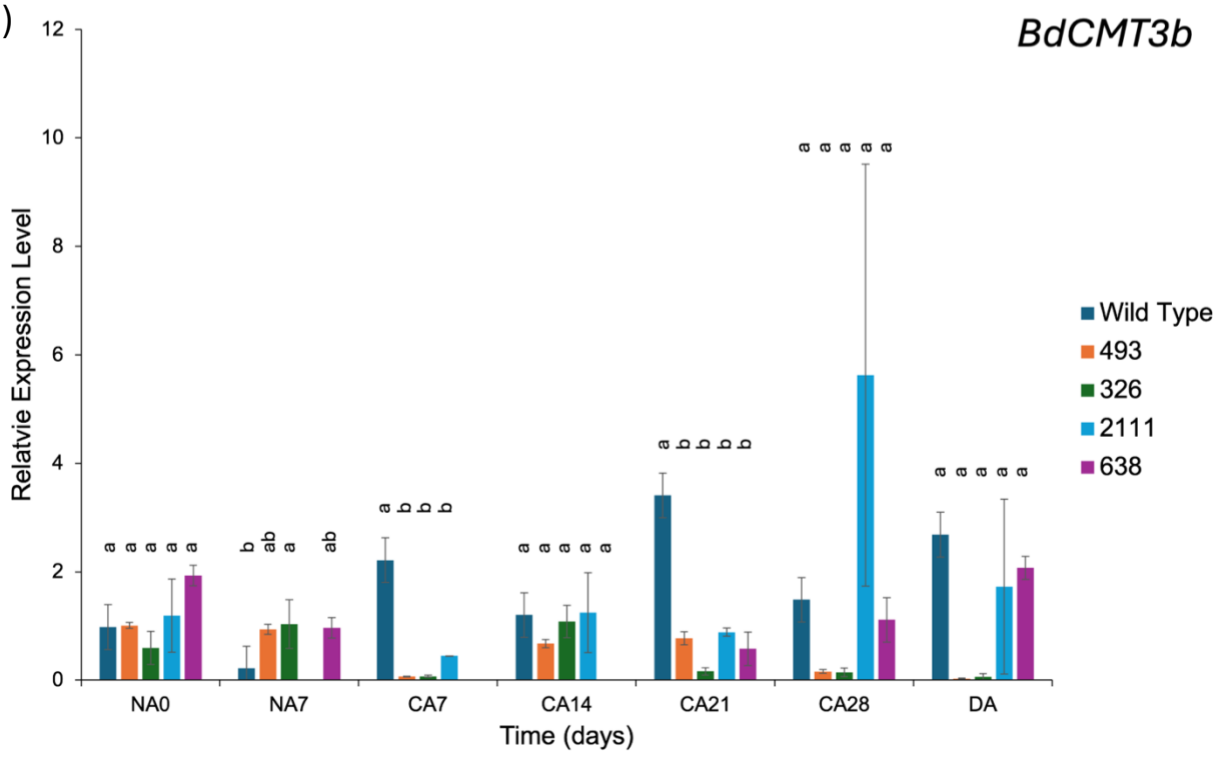
Figure 3.7 Mutations in *BdCMT* genes change the transcription of cold-responsive genes under long term stress. Transcriptional responses of cold activated and *CHROMOMETHYLASE* genes in response to non-acclimated (Na), cold acclimated (CA) or de-acclimated (DA) conditions in Bd21-3 NaN lines and Bd21-3 plants. Plants are exposed to either a seven day non acclimated NA treatment or a 28 day cold acclimation treatment (CA) are 4c for 28 days followed by a 24 hour de-acclimation period at 22c. (A-B) Cold responsive genes *BdCOR410* and *BdVRN1*. (C-E) *B. distachyon CHROMOMETHYLASE* genes *CMT2*, *CMT3a*, and *CMT3b*. Statistical significance is denoted with letters (P value < .05) through an ANOVA and a Tukey's HSD test. Error bars denote standard deviation from the mean of 3 biological replicates. Graphs denote levels of transcript levels relative to non-acclimated 22°C conditions.


(A)
BdCOR410
Relative Transcript Levels
Time (days)
NA0
NA7
CA7
CA14
CA21
CA28
DA
Wild Type
493
326
2111
638
(B)
BdVRN1
Relative Transcript Levels
Time (days)
NA0
NA7
CA7
CA14
CA21
CA28
DA
Wild Type
493
326
2111
638

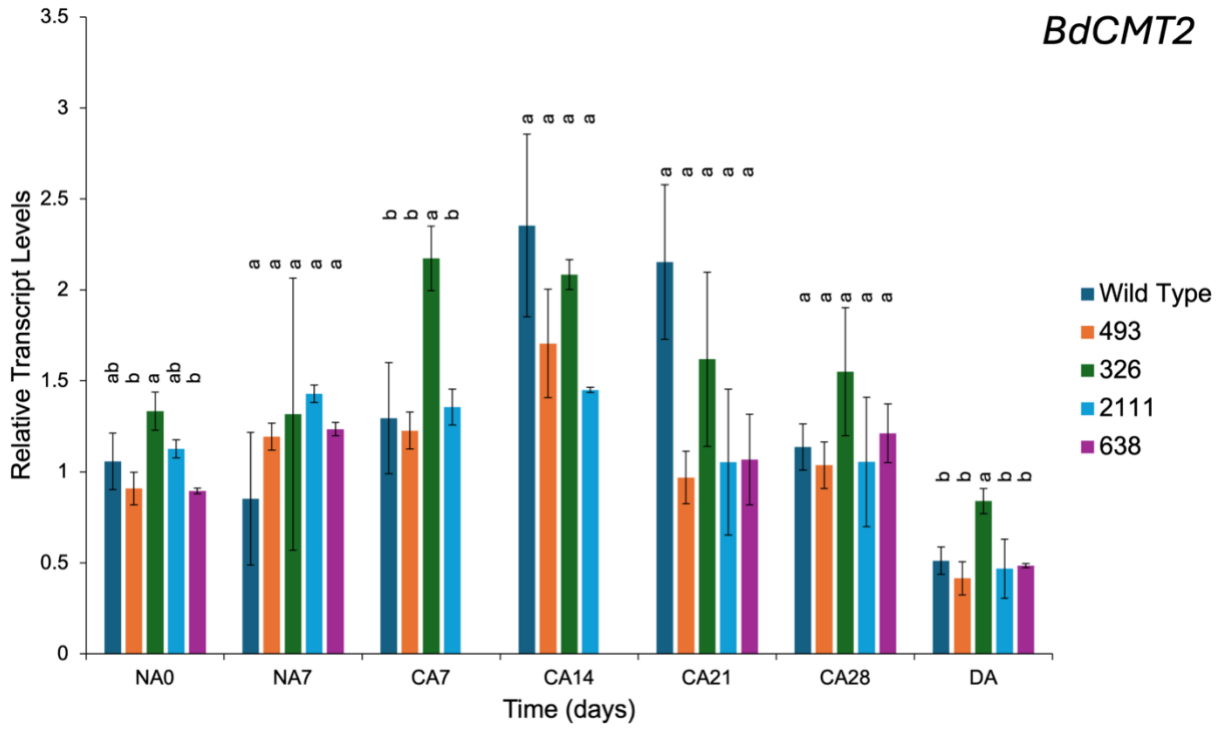
(C)


BdCMT3a
relative Expression Levels
35
30
25
20
15
10
5
0
NA0
NA7
CA7
CA14
CA21
CA28
DA
Time (days)
Wild Type
493
326
2111
638

(D)


BdCMT3b
Relatvie Expression Level
12
10
8
6
4
2
0
NA0
NA7
CA7
CA14
CA21
CA28
DA
Time (days)
Wild Type
493
326
2111
638

(E)


BdCMT2
Relative Transcript Levels
3.5
3
2.5
2
1.5
1
0.5
0
ab b a ab b
a a a a a
b b a b
a a a a
a a a a a
a a a a a
b b a b b
NA0
NA7
CA7
CA14
CA21
CA28
DA
Time (days)
Wild Type
493
326
2111
638

Basal level expression of line 0638 at NA0 were significantly higher relative to wildtype, while lines 493, 326, and 2111 shared slightly higher or similar transcript levels compared to wild type. Following 14 days of cold exposure, line 326 had significantly higher transcript levels relative to wild type, while all other lines shared similar transcript levels compared to wild type. At timepoint CA21, lines 0638, and 2111 had higher expression compared to wild type. Timepoints CA28 and DA shared variable expression patterns in all lines but was not statistically significant.

Expression trends in *BdCMT3b* transcript accumulation in CMT mutant lines were variable, but similar expression behaviors were seen at specific timepoints **(Figure 3.7D).** All lines shared statistically similar expression patterns at timepoints NA0 and NA7, while at timepoints CA7 and CA21, all lines showed significantly significant lower transcript levels relative to wild type. Analysis of expression of *BdCMT2* genes revealed similar expression patters between mutant lines, with the only significant differences in expression coming from line 326 at timepoints CA7 and de-acclimation (**Figure 3.7E)**. Overall, some differences in mutant lines were observed across *BdCMT* genes in response to prolonged cold exposure.

3.2.8 Brachypodium distachyon lines containing mutations in BdCMT3 genes share similar vegetative and flowering growth features

Following transcriptomic analysis, we wanted to determine if modulation of the function of *BdCMT3* genes manifest in the plant's phenotype. We followed a "guilt-by-association" strategy, in which if all lines share a similar difference in expression relative to WT, we can assume that this change in phenotype is a result of the mutation in *BdCMT*

genes. To do this, mutant lines and wild type plants were grown at 22°C under flowering inducing 20 hour of light conditions, and phenotypic traits related to flowering and morphology were collected. Recorded phenotypic traits of plant height, number of spikes, and days to heading were statistically insignificant of the four lines relative to wild type (**Figure 3.8A, C, E**). Tiller count, a trait used to predict the total number of seeds, as well as seed count itself were significantly lower in line 0638 relative to wild type with an average tiller number and seed count of 1.85 and 25 respectively (**Figure 3.8B, D**).

Traits associated with size were analyzed to determine if mutations in BdCMT3 genes would alter morphological features of *B. distachyon* plants relative to wildtype. Plant heights of lines 326 and 0638 were significantly greater than the heights of lines 493 and 2111, while the wild type lines shared similar heights to all four mutant lines (**Figure 3.9A-B**). Overall dry weight did not significantly differ between wild type and mutant lines. While individual plant presented some significant phenotypic differences in flowering and growth, these differences were not present across all mutant lines suggesting mutations in BdCMT3 genes did not influence traits associated with flowering and morphology.

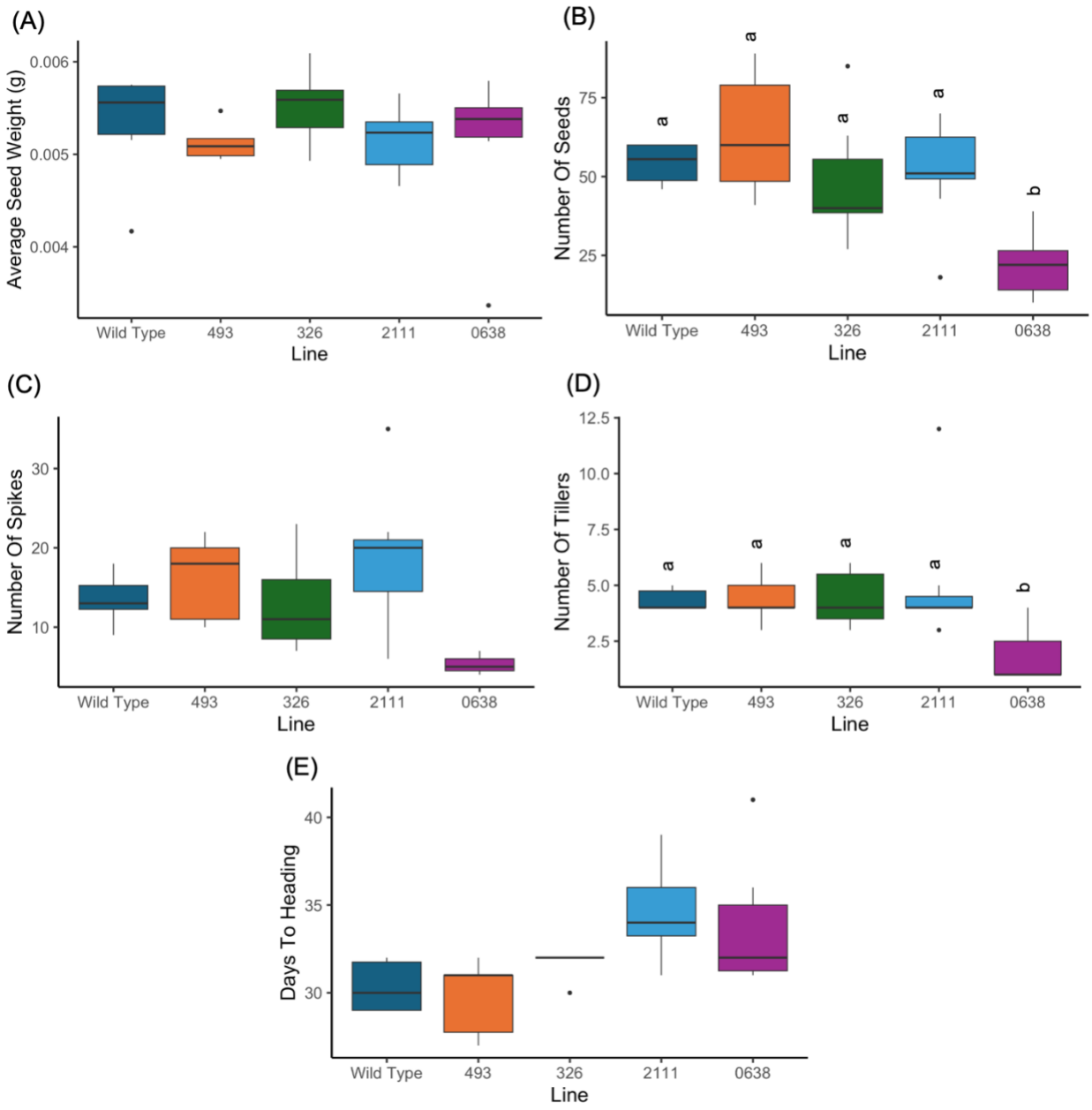


Figure 3.8 Mutations in *BdCMT* genes do not influence phenotypic traits associated with flowering. Phenotypic traits associated with flowering as denoted in box plots. Median represented through the line within the box. Upper and lower hinges correspond to first and third quartiles, while whiskers represent greatest and lowest values within 1.5 standard deviations of the interquartile range. **(A-D)** Phenotypic traits of wild type and mutant lines were grown in long day conditions (20 hours light: 4 hours dark) conditions at 22C. 6,9,11,7, and 7 biological replicates are used for wild type, line 493, line 2111, line 0638, and line 326 respectively. **(A)** Average seed weight of Bd21-3 Wild Type and *Bdcmt* NaN mutant lines. **(B)** Number of seeds in Bd21-3 Wild Type and *Brachypodium* lines with mutations in *CMT3* genes. The data was determined to be non-uniformly distributed through a Shapiro-Wilks test (p = .502). letters indicate a statistical significance (p <.05) and determined though an ANOVA and a Tukey-Kramer HSD. **(C)** Number of spikes in Bd21-3 wild type and BdCMT3 mutant lines. **(D)** Number of tillers in Wild Type and Lines with Mutations in *BdCMT3* genes. The data was determined to be non-uniformly distributed through a Shapiro-Wilks test (p = <.0001). Dunns method of ranking was used to compare

Figure 8 continued. means. letters indicate statistical significance (p <.05) and determined using a Dunn Method for joint ranking analysis. **(E)** Flowering, as represented through days to heading, is not delayed through mutations in *BdCMT3* genes. Plants are grown in long day conditions (20 hours light: 4 hours dark) conditions at 22C. 6,8,10,6, and 5 biological replicates are used for wild type, line 493, line 2111, line 0638, and line 326 respectively. The data was determined to be non-uniformly distributed through a Shapiro-Wilks test (p = <.0001). Dunns method of ranking was used to compare means.

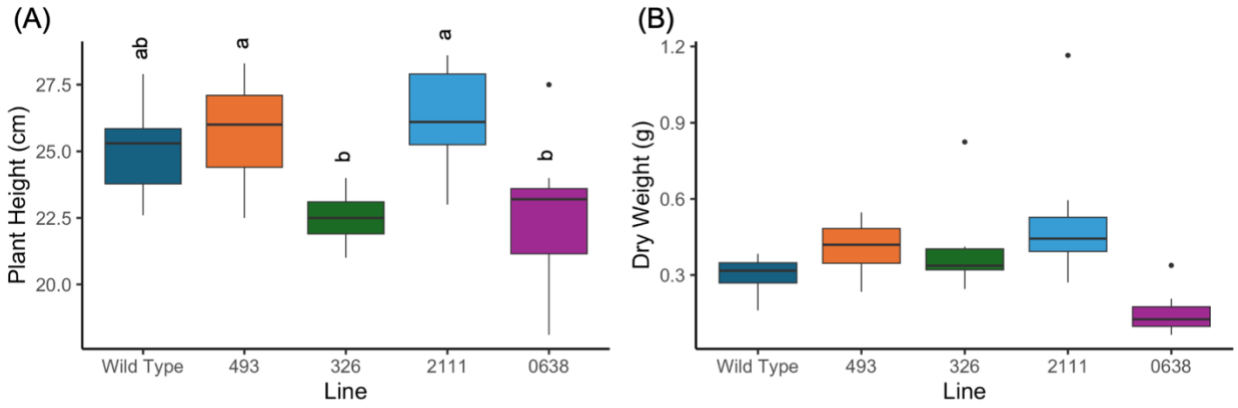


Figure 3.9 Mutations in *BdCMT* genes do not change phenotypic traits associated with size. Phenotypic traits associated plant size as denoted in box plots. Median represented through the line within the box. Upper and lower hinges correspond to first and third quartiles, while whiskers represent greatest and lowest values within 1.5 standard deviations of the interquartile range. **(a)** Plant height between Bd21-3 and *BdCMT3* mutant lines as represented in a box plot. Data determined to be uniform through a Shapiro wilks test. Plant Height Different letters indicate significant differences between *Brachypodium lines,* as determined using a Tukey-Kramer HSD (P>.05). **(B)** Dry Weight of Bd21-3 and BdCMT3 mutant lines as represented using a boxplot.

3.3 Discussion

DNA methylation deposition and maintenance is crucial for gene regulation and genomic integrity, however the role of specific methyltransferases in relation to abiotic stresses remains elusive. In this project, we aimed to observe if disrupting the function and activity of DNA methylation maintenance proteins of the CHROMOMETHYLASE family in *B. distachyon* would modulate the expression of known cold-responsive genes and the plant's physiological phenotype.

To begin our study, we wanted to identify the number of *CMT* genes in *B. distachyon.* We identified 4 genes predicted to contain the three protein domains that are

a prerequisite for CMT classification. Interestingly, we found two copies of *BdCMT3* genes in different locations of the chromosome, *BdCMT3a* and *BdCMT3b* (**Figure 3.1**). It is unclear if this duplication is evolutionarily new as its closest neighbor, *Brachypodium stacii*, only has one copy of *CMT3* (Gordon et al., 2020). On the other hand, the allotetraploid grass *Brachypodium hybridum* is suggested to contain 3 copies of *CMT3*, two from *B. distachyon* and one from *B. stacii* (Gordon et al., 2020). Both copies *BdCMT3* genes appear to express some degree of transcriptional response to cold stress through our virtual expression analysis, suggesting both are transcriptionally active (**Figure 3.3**). These paralogs may be undergoing sub functionalization, suggesting that over evolutionary time, one of the *BdCMT3* copies will become either non-functional, develop a new function, or both genes may remain functional and act in a dose dependent manner (Birchler & Yang, 2022). The evolutionarily close species *Oryza sativa* contains two *CMT3* copies, in which *OsCMT3b* is suggested to have sub functionalized as a backup to Os*CMT3a*, which only functions when all other DNA methyltransferases are mutated (He et al., 2021) Studies in hexaploid Wheat analyzing the evolutionary fate of CG methylation gene *TaMET1* revealed that the *TaMET1-b* lineage undergoes purifying selection relative to other *TaMET1* paralogs, while genes such as *TaMET1-a* has already undergone pseudogenization (M. Thomas et al., 2014).

Brachypodium distachyon contains four isomers of the *CMT* family genes. These Isoforms share highly similar conserved domains but share variability in their overall amino acid sequences. Between all other 3 genes *BdCMT2* shares the lowest % identity with BdCMT1, BdCMT3a, and BdCMT3b, with approximately 46% between all three. On the other hand, paralogs BdCMT3a and BdCMT3b share an 86% identity between each

other. BdCMT1 shares a 60% identity with the CMT3 paralogs. This divergence in sequence is consistent with the common consensus that CMT1 and CMT3 isoforms are more closely related to CMT2 (Bewick et al., 2017).While each *CMT* gene shares some similarities in structure their functions in relation to epigenetic maintenance and their targets are different. This functional divergence is present in all plant species that contain CMT isoforms, suggesting their targets and function is evolutionarily conserved subfuctionalize to maintain specific sequence contexts at specific developmental intervals, such as CMT1 showing its highest expression during germination and seedling development. *B. distachyon* and its closest neighbour *Brachypodium stacii* share significant similarity between their respective orthologs, with CMT1, CMT2, and CMT3 sharing 94.75, 90.98, and 94.86 percent identity with each other. This high sequence similarity reinforces the idea that CMT protein function is conserved within plant species. While it is known that CMT genes act in specific patterns, future studies would benefit from elucidating each isoform is used in specific temporal and sequential contexts.

All 4 BdCMT peptide sequences contained a NLS, allowing for transport back into the nucleus. BdCMT1, BdCMT3a, and BdCMT3b all contained predicted NLS patterns near the beginning of the protein sequence, while BdCMT2 contains a predicted NLS 250bp following the translation start site. While nuclear localization signals are typically found in the N-terminus of the peptide sequence, there is no specified position or consensuses in the exact location of the NLS (Lu et al., 2021; Martoglio & Dobberstein, 1998). Future studies may verify the localization of BdCMT proteins to verify their migration to the nucleus and their function within it.

Validation of BdCMT through phylogenetic analysis clustered in groupings with putative CMT proteins in other monocots, reinforcing the idea that they are indeed members of the CHROMOMETHYLASE family (**Figure 3.2**). While searching for other CMT proteins in grass species, it is noted that some species were missing at least one of the CMT protein sequences. The incomplete collection may have come as a result of the initial query, in which sequences were discarded if they contained the presence of a domain outside of the 3 prerequisite CMT domains. Future studies may attempt to generate a larger tree outside of grasses and relax the strict parameters of this study. *Zea mays* and *sorghum biocolour* CMT1 protein sequences clustered in a species-specific manner, clustering with their CMT3 copy in the CMT3 monophyletic group. This preferential clustering could be a result of superfamily specific evolution caused by selective pressures. Both *Zea mays* and *sorghum bicolour* are evolutionarily similar as both are members of the Andropogoneae tribe (Soreng et al., 2017). It is possible that CHROMOMETHYLASE proteins between these two species have undergone non-synonymous substitutions in regions outside of the BAH, CHROMO and C5-Methyltransferase domains, resulting in greater divergence between species rather than genes. To accommodate for this, future studies may look at only domain regions of the gene and increase the number of CHROMOMETHYLASE protein sequences from various species in the monocot clade.

Virtual expression data from public databases suggests that over the course of 28 days, *BdCMT* genes *BdCMT3a* and Bd*CMT3b* undergo a decrease in transcript abundance while *BdCMT2* undergoes upregulation (**Figure 3.3**). *BdCMT1* expression was meager, barely being detected in leaf tissue. This low expression is consistent with

literature, as levels of *CMT1* activity are known to be low in leaf tissue. CMT1 is commonly associated with genomic imprinting and seedling development, in which its expression is highest in flowering tissue development (Klepikova et al., 2016; Henikoff & Comaii, 1997). Future studies may look at specifically floral tissue or seedlings *BdCMT1* expression in response to CS. Overall, virtual expression suggests that *BdCMT* genes are transcriptionally responsive, and that DNA methylation patterning through *CHROMOMETHYLASE* genes changes in response to cold. Future studies looking at virtual expression should analyze both *CHROMOMETHYLASE* family genes, other methyltransferases, as well as cold responsive genes to predict correlations between cold stress and other DNA methyltransferases factors such as MET1 and DRM2.

Our study aimed to determine if *B. distachyon CMT* genes are transcriptionally responsive to short- and long-term cold stress. As expected, transcript levels of the known cold-responsive gene *BdCOR410* significantly increased after 24 hours of cold treatment (**Figure 3.4**). Dehydrins such as *COR410* are known to be rapidly expressed in multiple grass species including Wheat and *B. distachyon* (Colton-Gagnon et al., 2014; Mayer & Charron, 2021; Y. Wang et al., 2014). The gene is additionally known to be epigenetically regulated through histone modification, making it a potential candidate for evaluation of DNA methylation changes in response to cold stress.

Among the CHROMOMETHYLASE genes, only *BdCMT2* showed significant differential expression in response to 24-hour cold exposure (**Figure 3.4**). This may be the first direct evidence suggesting CMT's responsiveness on such a short temporal scale, typically associated with short-term stress (Gutschker et al., 2022; Sreedasyam et al., 2023). Although the statistical analysis indicates significance, the error bars suggest

substantial variability in the data. This variability implies that the significance should be interpreted with caution, as it may affect the robustness of the findings. BdCMT2 expression results through qPCR conflict with virtual expression data, which suggests that BdCMT2 expression remains consistent over the 24 hours timescale. Transcript accumulation of Both *BdCMT3* remained consistent over 24 hours (**Figure 3.4**), aligning with the common consensus that methylation is a long-term response relative to 24 hours. *BdCMT3* expression reinforces virtual expression data which showed minimal change in *BdCMT3* FPKM values over 24 hours. Global DNA methylation has been shown to change in such a short temporal scale, but expression of specific methyltransferases was previously not analyzed. Future studies may analyze methylation patterns and BdCMT localization in loci around known cold responsive genes and transposons to confirm if *BdCMT* genes to directly influence methylation patterns of cold responsive genes.

Prolonged cold acclimation studies in grasses have elucidated the epigenetic profiles of both histone modification and DNA methylation of many cold responsive genes and epigenomes. Our study reinforces that *BdCOR410* and *BdVRN1* are transcriptionally upregulated in response to prolonged cold acclimation. *BdCOR410*, similarly to short term stress, is rapidly upregulated in response to CS (**Figure 3.5**). Global DNA methylation changes have been studied in response to LT in which differential methylation across the transcriptome has been uncovered, but little work has directly observed dehydrin methylation changes in response to CS. Epigenetic regulation of dehydrins such as COR410 may occur indirectly through epigenetic regulation of regulators in the CBF-DRBE pathway, pathway, a regulatory system known to undergo DNA demethylation in response to cold stress.

Our study reaffirms that *BdVRN1* transcription is significantly enriched following cold acclimation. *BdVRN1* is a tightly controlled gene in regard to flowering, as to correctly time flower development following spring. Significant upregulation of *BdVRN1* was observed following 14 days of cold stress, continuing to increase as exposure progressed and remained active following de-acclimation. The upregulation of *BdVRN1* during cold exposure in *B. distachyon* was also seen in previous studies in which plants were exposed to a cold period of 58 days (Colton-Gannon et al 2014). However, in that study *B. distachyon* plants only experienced a significant upregulation following 21 days rather than the 14 days found in this study. Being a facultative accession Bd21-3, does not necessarily require vernalization to flower. However, exposure to cold is known to accelerate flowering and allow for a faster maturation (Vogel et al. 2010). Overall, cold responsive genes *BdCOR410* and flowering activator *BdVRN1* are transcriptionally active in response to cold stress, protecting plants from cold stress and inducing flowering respectively.

A period of 28 days of exposure to CS resulted in significant differences in expression in all *BdCMT* genes relative to control conditions. Transcriptional levels of CHROMOMETHYLASE genes and DNA methylation levels tend to be positively correlated in response to abiotic stress (Gutschker et al., 2022). However, epigenetic changes in response to such stress tend to be species-specific, reducing the probability of reliably expecting specific methylation patterns to occur (Niederhuth & Schmitz, 2017). During cold exposure, we observed an upregulation of *BdCMT2*, peaking at 14 days. *BdCMT3a* underwent significant downregulation during cold treatment and returned to basal levels during de-acclimation. Conversely, *BdCMT3b* display a variable pattern

during cold treatment, showing an irregular and variable trend. CMT2 is known to be involved in the deposition of CHH methyl patterning in all plant species, implying an increased deposition of such marks in *Brachypodium* (Stroud et al., 2014). Interestingly, while up and downregulation of CMT2 marks correlate with the associated marks, species undergo variable changes in DNA methylation. For instance, prolonged cold exposure in sugar beet was associated with the downregulation of *CMT2* expression, as well a decrease in CHH methylation (Gutschker et al., 2022). Additionally in *Arabidopsis*, mutations in *AtCMT2* and lower global CHH methylation in *Arabidopsis* ecotypes were found to grow in colder climates of western Europe, suggesting lower CHH methylation improves cold acclimation (Shen et al., 2014). Conversely in Orchid Grass, following eight weeks of exposure to 4°C, CHH and CHG methylation upstream of the transcriptional start site and of protein coding genes and transposons increased as vernalization continued, suggesting hypermethylation improves the cold response of these grasses. (Yang et al., 2022). Regardless of methylation status patterning, the changes in methylation across these species resulted in improved cold acclimation and cold tolerance. Future studies investigating the link between cold acclimation and epigenetic modification profiles should compare methylation patterning upstream of *B. distachyon* cold responsive genes such as *BdVRN1* and *BdCOR410*.

Verification of *BdCMT* mutations only revealed 11 of the 15 candidates contained the desired mutations. The absence of mutations predicted by Dalmais and colleagues (2013) may be a result of a series of reasons. Missing mutations using the outlined verification are unlikely as multiple plants for each mutant line were sequenced multiple times, confirming the true absence of the mutations. Additionally, while *B. distachyon* is

capable of self-pollination, lines may have lost their mutations due to accidental cross pollination of lines during bulking. A final reason for the absence of the mutations may have been caused by mis sequencing of the lines, as these mutations were predicted to exist, but not directly verified individually until now.

Reverse genetics has been a powerful tool to elucidate the function of genes across organisms. Using multiple sodium azide mutant lines containing mutations in *BdCMT3* genes, transcriptional analysis of cold responsive genes *BdCOR410* and *BdVRN1* revealed significant differences in expression levels relative to wild type in *B. distachyon*. We observed significant changes in *BdCOR410* expression relative to wildtype early on in our cold treatment in lines 493, 2111, and 0638, showing a significant difference in relative expression at CA7. Variable expression between mutant lines was additionally observed in non-treated groups NA0 and NA7. Increased expression early on in treatments suggest that *BdCMT3* may play a partial role in the suppression of expression of *BdCOR410*, preventing leaky expression when *B. distachyon* is not exposed to LT. Little work has been done to evaluate DNA methylation profiles in Dehydrins. However, studies have shown significant enrichment of H3K4me3 and H3K27me3 histone marks following expression as well as an increase in global DNA methylation in *B. distachyon* following cold treatment, implying that the cold regulated genes are epigenetically controlled (Mayer & Charron, 2021).

VRN1, a gene known to be responsive and epigenetically regulated to induce flowering, showed significantly lower relative expression in three of the four mutant lines. Lines 326 and 2111 had consistently lower expression across all cold exposure timepoints and the de-acclimation period, and lines 0638 exhibited generally lower transcript

accumulation, with significant differences observed at CA21 and DA. Contrary to this, line 493's expression pattern was similar to wild type, showing no significant differences. These observations suggest that mutations in *BdCMT3* can affect *VRN1* expression, suggesting that BdCMT3 methylation may play a critical role in the epigenetic regulation of VRN1 during vernalization. A previous study revealed treatment of Orchid grass with methylation inhibitor zebularine resulted in a delayed flowering time and reduction of expression of vernalization genes, reinforcing the idea that deposition of methylation marks promotes vernalization in grasses (Yang et al., 2022). While we can observe that loss of CMT function through mutations in their functional domains results in a reduction in transcript abundance of *VRN1*, the direct impact of CMT gene mutation remains elusive. In Wheat, *BdVRN1* homolog *VRN-A1* does contain non-CG methylation in its first intron in a region containing transposable element fragments, suggesting some role of DNA methylation in regulation of the gene (Khan et al., 2013). Alternatively, research has shown that the disruption of CMT3s BAH domain completely arrests H3K9me2 methylation in Arabidopsis, a histone mark associated with transcriptional repression (Du et al., 2012). Considering this, it would be hypothesized that the disruption of CMT3 activity may result in a depletion of H3K9me2 deposition of upstream negative regulators of VRN1, reducing its expression and preventing a strong induction of flowering. Many other methyltransferase and demethylase genes are involved in the regulation of VRN1. Considering this, BdVRN1 expression may have been reduced in this study due to an adjustment of another methyltransferase due to the loss of BdCMTs, resulting in the reduced expression we observe in this study. Future studies should observe methylation patterning of known regulators of VRN1 expression in wild type *B. distachyon* and mutant

lines in the interest of observing any discernible differences in DNA methylation patterning.

Transcript accumulation of *BdCMT2*, *BdCMT3a*, and *BdCMT3* did not significantly deviate in our *BdCMT3* mutant lines relative to wild type (**Figure 3.7C-E**). While there were some changes in expression, such as expression in CA 14 in CMT3a and CA7 in *BdCMT3b*, significant differences in trends or expression were not visible. Being NaN lines, the genes were expected to still be expressed within the plants, but functions in either BAH domains or CHROMO domains modulate the function within the genes, and the epigenetic profiles of cold responsive genes, as seen in *BdVRN1*. Multiple studies have assessed the function of various methyltransferases across plant species. Loss of methyltransferases drm2/cmt2/cmt3a/and cmt3b resulted in complete loss of non-CG methylation in *Oryza sativa* (Hu et al., 2021). In the context of our study, modulation of BdCMT3 function may have only resulted in the partial loss of non-CG methylation, preventing the observation of a complete picture of the impacts methylation poses on the whole plant. Future studies may be interested in generating complete mutants by deleting BdCMT function through CRISPR-cas9 mutation.

Phenotypic analysis of mutant *B. distachyon* plants did not reveal significant differences in traits associated with flowering and growth (**Figure 3.8 and Figure 3.9)**. While line 0638 did reveal significant changes in seeds count, tiller count, and plant height other lines did not share these significances. These results conflict with the hypothesis and cannot be attributed to mutations in *CMT* genes due to our guilt by association approach, in which a majority if not all lines need to show variability relative to wild type to be clearly attributed to mutations in CMT genes. A lack of change may be attributed to

growth conditions and other mutations present in the lines that may be impacting growth. Statistical power was limited in the experiments, and a larger sample size may improve statistical robustness and introduce alternative techniques. While the experiments did not show a complete change across the board, line 0638, which contains a heterozygotic premature stop codon in *BdCMT3b*, could be a candidate for further studies. Alternatively, future studies could generate a mutant line of *BdCMT3s* to further reinforce the data generated.

Chapter IV: CONCLUSION

4.1 General Conclusion

We hypothesized that the disruption of DNA methylation through mutations in *CHROMOMETHYLASE* genes will lead to alterations in the abiotic stress response in *B. distachyon* including cold acclimation, cold tolerance, and vernalization and our findings support this. We identified four isoforms of *B. distachyon* CMT genes, with at least 1 identified sequences for the CMT1, CMT2, and CMT3 class. *B. distachyon CMT2* was found to be significantly transcriptionally responsive to short- and long-term stress, while *BdCMT3a* and *BdCMT3b* were found to only be transcriptionally responsive to long term stress. 5 lines containing mutations in *BdCMT3* genes were found to promote early activation of cold responsive gene *BdCOR410*, and weaken the vernalization response through *BdVRN1*, implying that CMT3 mediated DNA methylation plays a role in the function of vernalization. Mutants in *BdCMT3* did not however result in significant changes in phenotypic characteristics across all lines in non-vernalized *B. distachyon* mutant lines relative to wild type, as we did not see changes in features related to growth such as height and weight, or features associated with flowering such as days to heading, number of tillers, or number of seeds.

4.2 Contribution to Science

- Completion of Objective 1 identified 4 CHROMOMETHYLASE family genes in Brachypodium distachyon and verified their close homology to CMT protein

sequences in other grasses, presenting the potential for *B. distachyon* as a model organism for grasses.

- By completion of Objective 2, we have determined that 3 *CHROMOMETHYLASE* genes are transcriptionally active in response to cold stress in *B. distachyon*.
- Completion of objective 3 identified 11 lines containing mutations in *BdCMT* genes, which may act as candidates for future studies into epigenetic regulation in *B. distachyon* and other grasses.
- By completing objective 4, this study has determined the epigenetic contribution of CMT3 in the early transcription of *BdCOR410* and transcript accumulation of *BdVRN1* in response to cold stress in *B. distachyon,* presenting an avenue for epigenetic modifications to be used in agricultural practices.
- Objective 4 additionally revealed that partial loss of *BdVRN1* is not sufficient to stop flowering in *B. distachyon*.

4.3 Future directions

The research presented provides a foundation for future studies into CHROMOMETHYLASE proteins role and function in the abiotic stress response in *B. distachyon* and grasses. While this study does provide evidence of CHROMOMETHYLASES influence on the abiotic stress response, future studies are proposed to further elucidate CMTs direct action and influence on cold response.

Due to the limited space of the institution, biological replicate count was limited for the phenotypic analysis of *BdCMT* mutant lines. The experiment may be repeated under similar conditions, but the plants may be vernalized first to see if the mutations may

influence flowering and growth when in vernalization conditions under a mutant background. Finally, a whole plant freeze test should be conducted on the studied mutant lines to determine if mutations in *CMT* genes would influence the survivability of *B. distachyon* under freezing conditions.

While this study did observe changes in expression in *BdVRN1* and *BdCOR410* in a *B. distachyon* mutant background, the remainer of the transcriptome, including genes beyond BdVRN1 and BdCOR410 remains elusive. Future studies should look at global transcriptional changes and methylation changes through RNA-seq and bisulphite sequencing in mutant and wild type backgrounds. This information would provide insight into the degree to which our mutations of interest influence the background of methylation and transcription in both standard and cold acclimation conditions.

Finally, while our mutant lines did contain mutations in *BdCMT* genes, the presence of additional SNPs in the mutant background may act as confounding variables in the experiment. For this reason, future studies should generate a knockout or overexpression line of *BdCMT2* and *BdCMT3* in Brachypodium. This mutant line would allow is to clearly determine if the mutations in this study were a result of the modulation of *BdCMT* gene function and provide a line that may be used in other studies related to *CMT* genes in grasses.

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