CHROMATIN DYNAMICS OF COLD-STRESS RESPONSES IN *CANNABIS SATIVA* L.

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"...the historically important uses of Cannabis have been largely forgotten, lost behind a smoke screen of unresolved controversy" Robert C. Clarke

ABSTRACT

Although *Cannabis sativa* L. has been grown for millennia as a multipurpose crop, little is known about its freezing tolerance and capacity to cold acclimate. Plants native to temperate regions are able to increase their cold hardiness when exposed to low temperatures through a process called cold acclimation. These organisms developed different strategies to deal with cold, and may involve alterations of the photosynthesis apparatus, the accumulation of carbohydrates and the production of protective molecules. Such strategies often start with the induction of cold-regulated genes (*COR* genes) notably via changes in chromatin structure. This thesis focuses on the cold acclimation capacity and freezing tolerance of hemp and uncovers underlying epigenetic and molecular mechanisms. The first objective consisted in ranking nine *Cannabis sativa* L. varieties approved for production in Canada according to their cold acclimation capacity and freezing tolerance. A whole-plant freezing test revealed a range of phenotypic variations for these traits, which enabled us to classify nine *Cannabis sativa* L. varieties into three groups. The first group holds varieties that acclimate the most efficiently, the second group includes varieties with modest cold acclimation capacities while the third group comprises sensitive varieties that do not acclimate. However, further analysis indicated that all varieties displayed similar cold-response strategies regarding photosynthesis and the accumulation of soluble sugars. In the second objective, we identified *COR* genes in the genome of *Cannabis sativa* L. and by monitoring their expression levels, we uncovered that all varieties could induce the expression of *COR* genes in response to cold. However, the varieties that could cold acclimate the most efficiently accumulated *COR* transcripts to higher levels. In the third objective, we observed that all varieties modified their epigenome in response to cold with

however a distinction that the most successful varieties displayed more pronounced global DNA methylation dynamics and a wider range of variations of cytosine methylation and histone modifications at *COR* gene loci. Altogether, this study is the first to report the cold acclimation and freezing tolerance capacities of *Cannabis sativa* L. characterised at the physiological, metabolic and molecular levels. Findings from this study may serve as stepping-stones for the sustainable development of *Cannabis* culture in Canada and other northern countries.

RÉSUMÉ

Bien que le *Cannabis* soit cultivé à travers le monde depuis des millénaires, très peu d'informations sont disponibles au sujet de sa tolérance au gel et de sa capacité à s'acclimater au froid. En fait, les plantes indigènes de régions tempérées sont capables d'augmenter leur tolérance au gel en démarrant un processus d'acclimatation lorsqu'elles sont exposées à des basses températures. Les plantes ont développé des stratégies différentes pour s'acclimater et lutter contre le froid, tels que des ajustements au niveau de l'appareil photosynthétique, l'accumulation des sucres et la production de molécules protectrices. Ces stratégies sont toutes coordonnées par l'expression de gènes régulés par le froid (gènes *COR*) à travers l'action de certains facteurs de transcription et via des changements de structure de la chromatine. Ce document vise à examiner la capacité du chanvre à s'acclimater au froid et de tolérer le gel, et à identifier les mécanismes épigénétiques associés à ces deux phénomènes. Le premier objectif de cette étude consistait à classifier neuf variétés de *Cannabis sativa* L. approuvées pour la production au Canada par rapport à leur capacité à s'acclimater au froid et à tolérer le gel. Un test de congélation révéla que les variétés étudiées ne sont pas toutes capables de s'acclimater au froid avec le même succès, ce qui nous a permis de les séparer en trois catégories. Une première catégorie regroupa les variétés qui ont la capacité de s'acclimater au froid, une seconde celles qui s'acclimatent de façon modérée et une troisième celles qui sont incapables de s'acclimater et d'augmenter leur tolérance au gel. En approfondissant l'analyse, nous avons observé que les variétés ne se démarquent ni au niveau de leur stratégie photosynthétique et ni au niveau de l'accumulation des sucres. Au cours du deuxième objectif, nous avons identifié des gènes *COR* dans le génome du *Cannabis* et, en mesurant le niveau relatif d'ARNm de ces gènes, nous avons

observé que toutes les variétés sont capables d'enclencher l'expression de gènes *COR* en réponse au froid. Cependant, les variétés qui ont montré les plus fortes capacités d'acclimatation accumulent des transcrits à des niveaux plus élevés que les autres variétés. En répondant au troisième objectif, nous rapportons, qu'elles soient capables ou pas de s'acclimater, les variétés de chanvre modifient leur épigénome en réponse au froid. Néanmoins, les résultats démontrent une distinction: les variétés qui sont capables de s'acclimater montrent des mécanismes de réponse de plus grande ampleur, tant au niveau global (méthylation de l'ADN), que spécifique (modifications d'histones et méthylation de l'ADN sur les gènes *COR*). En conclusion, cette étude est la première à rapporter des analyses physiologique, métabolique et moléculaire de l'acclimatation au froid chez le chanvre. Les variations de capacité d'acclimatation au froid et de tolérance au gel que nous rapportons pourront servir de base au développement d'une culture durable du *Cannabis* au Canada et dans les pays nordiques.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

A	Adenine
ABA	Abscisic acid
ADA2	Transcriptional adaptor 2
AGO4	Agonaute 4
AP2	APETALA 2
ATGOLS3	*Arabidopsis thaliana* galactinol synthase 3
ATP	Adenosine triphosphate
AZA	5-azacytidine
bp	base-pairs
C	Cytosine
CA	Cold-acclimated/Cold acclimation
CA14	Cold-acclimated 14 days
CA7	Cold-acclimated 7 days
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBF	C-repeat binding factor
CBF1	C-repeat binding factor 1
cDNA	Complementary DNA
CMT3	Chromomethylase 3
COR	Cold-responsive genes
COR15A	Cold-regulated protein 15A

COR413pm	Cold-regulated 413 plasma membrane
COR413tm	Cold-regulated 413 thylakoid membrane
ChIP	Chromatin immunoprecipitation
ChIP-qPCR	Chromatin immunoprecipitation followed by qPCR analysis
DA	Deacclimated
DCL3	Dicler-like 3
DDR	Protein complex with DRD1, RDM1 and DMS3
DMS3	Defective in meristem silencing 3
DNA	Deoxyribonucleic acid
DRD1	Defective in RNA-directed DNA methylation 1
DREB	Drought-responsive element binding
DRM2	Domains-rearranged methyltransferase 2
dsRNA	double-stranded RNA
EL	Electrolyte leakage
FT	Freezing tolerance
G	Guanine
GCN5	General control non-repressible 5
H in CHH	A or T or C
H1/H5	Linker histone 1/5
H2A	Core histone 2A
H2A.Z	Core histone variant of H2A
H2B	Core histone 2B
H3	Core histone 3

H3K27me3	Histone 3 lysine 27 tri-methylation
H3K9ac	Histone 3 lysine 9 acetylation
H4	Core histone 4
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEN1	HUA enhancer 1
LT	Low temperature
MeC	Methyl-cytosine
MeDIP	Methylated-DNA immunoprecipitation
MeDIP-qPCR	Methylated-DNA immunoprecipitation followed by qPCR analysis
MET1	Methyltransferase 1
mRNA	Messenger RNA
NA	Non-acclimated controls
PGIP/AFP	polygalacturonase-inhibitor/anti-freeze protein
Pol IV	RNA Polymerase IV
Pol V	RNA Polymerase V
PRC2	Polycomb Repressive Complex 2
qPCR	quantitative real-time polymerase chain reaction
RdDM	RNA-directed DNA methylation
RDM1	RNA-directed DNA methylation 1
RDR2	RNA-dependant RNA polymerase 2
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RT-qPCR	Reverse transcription polymerase chain reaction
siRNA	Short-interfering RNA
SPAD	Soil and plant analyser development
ssRNA	single-stranded RNA
T	Thymine
THC	Δ^9-tetrahydrocannabinol
THCA	Δ^9-tetrahydrocannabinolic acid
TIL	Temperature-induced lipocalin
VRN1	Vernalization 1
WPFT	Whole plant freezing test
WSS	Water-soluble sugar

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PREFACE

All component of this thesis are original work. Chapter III has been formatted as a manuscript destined for publication in which the candidate is the primary author. The contribution of co-authors to this manuscript can be found below.

Contribution of co-authors

All experiments were designed by Jean-Benoit Charron and Boris F. Mayer. The experiments were carried out and the data analysed by Boris F. Mayer, except for the water-soluble sugar quantification that was done by Annick Bertrand's team (Agriculture and Agri-Food Canada). Mohamed Ali-Benali contributed with preliminary work. Boris F. Mayer, Jordan Demone and Jean-Benoit Charron wrote the manuscript.

CHAPTER I: GENERAL INTRODUCTION

1.1 Introduction

Abiotic stresses represent a major challenge for the agriculture industry. In fact, environmental factors are responsible for 50% of yield loss on a worldwide average (Ciarmiello *et al.*, 2011). In northernmost agricultural regions of the globe, the omnipresence of frost can greatly affect the cultivation of crops. Interestingly, plants native to temperate regions have developed the ability to acclimate to cold by altering their metabolism and cellular structures. These sessile organisms sense low temperatures in the fall and deploy cold tolerance mechanisms in an attempt to survive the freezing temperatures of an upcoming winter. For instance, plants can trigger the expression of specific genes, and thereby induce the production of protective molecules that inhibit the formation of ice crystals. The regulation of such genes is part of a tightly regulated network that can initiate a systemic response. The cold-response network most probably involves a specific epigenetic program. Indeed, through epigenetics organisms can rapidly modulate their genome.

The genus *Cannabis* is a fascinating group of plants that has been spread worldwide and grown by mankind at least since the beginning of written history. A *Cannabis* plant is truly multipurpose as it offers an interesting chemistry, solid fibres and nutritious seeds. *Cannabis sativa* L. (hemp), is grow for its fibres and seeds and was re-discovered as a sustainable multipurpose crop for its many attributes.

The culture of *Cannabis* has been criminalised for decades in most countries due to the presence of Δ^9-Tetrahydrocannabinol (THC), a prised psychoactive compound found in high levels in marijuana (Hartley-Whitaker *et al.*, 2001). In Canada, hemp is federally regulated by Health Canada's Office of Controlled Substances. In 1994, Health Canada allowed researchers to grow hemp in very tightly regulated conditions. In 1998, the creation of *Industrial Hemp Regulations* under the *Controlled Drugs and Substances Act* legally permitted commercial production of the crop with the acquisition of a license. The production of industrial hemp has since been expanding in Canada.

Although *Cannabis sativa* L. prefers temperate regions, these plants can thrive in almost any climate. Hardy, the genus probably developed efficient response mechanisms to respond to environmental changes. The intense breeding hemp has been submitted to may have isolated varieties that can efficiently cold acclimate or on the contrary varieties that lost the capacity to adapt to cold temperatures. A multitude of varieties exist, possibly with different abilities to cope with stress. This thesis aims at investigating the cold acclimation and freezing tolerance capacities of several varieties of hemp and uncovers the underlying epigenetic and molecular mechanisms responsible for these important traits.

1.2 Research hypotheses

1- *Cannabis sativa* L. varieties have different cold acclimation abilities and freezing tolerances.

2- Cold acclimation in hemp relies on the regulated expression of cold-responsive genes.

3- Chromatin dynamics are involved in cold response in hemp.

4- Hemp varieties with different cold acclimation abilities display distinctive cold-responsive gene expression and chromatin dynamics responses during cold acclimation.

1.3 Objectives

1 - Rank *Cannabis sativa* L. varieties approved for production in Canada according to their cold acclimation capacity and freezing tolerance.

2 - Identify cold responsive genes in the genome of *Cannabis sativa* L. and monitor their relative transcript accumulation during cold acclimation.

3 - Identify changes in chromatin modifications of *Cannabis sativa* L. during cold acclimation.

CHAPTER II: LITERATURE REVIEW

2.1 Hemp: a rediscovered crop

Cannabis (marijuana, hemp) is an herbaceous plant that has been cultivated for centuries as a source of food, fibre, seed oil, medicine and recreational use. Indigenous to Central Asia, *Cannabis* has been spread worldwide from subarctic to tropical regions (Bóksa & Karus, 1998; Boyce, 1900). Prior to 1000 B.C. up to the late 1800's, *Cannabis* was one of the most cultivated plant genus because of its many economically valuable attributes (Bóksa & Karus, 1998; Ranalli, 1999). Indeed, a multitude of products and necessities such as medicinal products (marijuana), food, lamp oil, clothing and cordage (hemp) can derive from this plant (Ranalli, 1999). For example, the versatile hemp (*Cannabis sativa* L.) fibres can be used to manufacture fine clothing as well as rope, twine and paper (Boyce, 1900).

The industrial cultivation and production of hemp products greatly declined during the 20th century. Until the popularisation of the mechanical cotton gin, hemp fibre was the fibre of choice for textile (Bóksa & Karus, 1998). Petroleum-derived products (fibres and kerosene) as well as acid-processed pulp paper have concealed important uses of industrial *Cannabis* (Ranalli, 1999). Moreover, the prohibition of marijuana by the United States Congress in 1937 has had a negative impact on the cultivation of hemp that however remained cultivated in the USA until the late 1950s (Roulac, 1997). Although the legislators of anti-marijuana laws never intended to affect hemp production, the blurry distinction between hemp and marijuana led to a

criminalisation of the industry that quickly declined (Roulac, 1997). Although morphologically different, a clearer distinction between marijuana and hemp is made by measuring contents of Δ^9-tetrahydrocannabinolic acid (THCA), the precursor of the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC) and cannabidiolic acid (CBDA), the precursor of the non-psychoactive cannabidiol (CBD) (Devinsky *et al.*, 2014). The content of these two major phytocannabinoids can vary between *Cannabis* plants, the ratio THCA:CBDA being typically high in marijuana and low in hemp (van Bakel *et al.*, 2011). The tall hemp plants and the low growing marijuana have been bred for different characteristics, i.e. hemp for fibre and seed yield and marijuana for THC and considerable efforts have been made to breed THC-free hemp. Although unsuccessful, hemp however typically contains very low amounts of THC (0<0.3% of dry weight) as compared to marijuana (up to 22.6%) (Tsumura *et al.*, 2012; van Bakel *et al.*, 2011). The decline of this historically important crop was nevertheless followed by a rediscovery at the end of the 20th century.

In the early 1990's, hemp was rediscovered as a multipurpose, universally resourceful crop (Bóksa & Karus, 1998). Indeed, the culture of *Cannabis* offers many alternatives and is revived by environmental concerns, especially as the world is moving away from petroleum-based products. Over the last decade, numerous research projects and industrial enterprises have investigated cultivation and applications of hemp (Amaducci *et al.*, 2008). Indeed, hemp thrives in most climates from the equator to 60^o longitude (Boyce, 1900), is a fast-growing crop which also discourages the growth of weeds and tends to be resistant to disease and insect pests, reducing the need to use herbicides and pesticides (Roulac, 1997). Useful for phytoremediation, hemp can efficiently decontaminate soils, e.g. from heavy metals (Mihoc *et al.*, 2012; Shi *et al.*, 2012). Indeed, hemp is a highly adaptive and useful crop.

Cannabis sativa L. is a diploid plant, possesses 20 chromosomes with 9 pairs of autosomes and a pair of sex chromosomes (X and Y) (Sakamoto *et al.*, 1995). *Cannabis* is diecious, baring male and female flowers on separate plants. Although sexually unstable, monoecious varieties are not uncommon. The sex determination is based on a X-to-autosome equilibrium in diecious varieties (XX for female and XY for male plants) while monoecious varieties appear to be XX and may have developed a genetic basis for sex determination (Faux *et al.*, 2014). In 2011, the draft genome of *Cannabis sativa* L. was published (van Bakel *et al.*, 2011), providing an invaluable resource for future research on the biology of *Cannabis*. As a hardy plant, hemp can provide interesting insights on stress response mechanisms in plants. The wide applications and growing range of hemp make this crop a model of choice to study stress responses.

2.2 Abiotic stress: cold

2.2.1 Challenges of crop production: abiotic stress

Environmental conditions strongly influence plant growth and development. Plants need to adapt to extreme conditions such as intense light, extreme temperatures, osmotic stress associated with salinity and drought stress. On average worldwide, the most important crops have their yield reduced by 50% because of adverse environmental conditions (Ciarmiello *et al.*, 2011). Over the past years, many reports have indicated that extreme weather conditions will become more frequent, which will greatly affect crop production (Trindade *et al.*, 2011). For example, the water demand for agriculture will increase by more than 30% by 2030 solely

because of climate change (Foresight, 2011). Besides, there are already huge economic losses associated with water stress. For example, drought caused an economical loss of 30 billion US$ in 2007 (Syngenta, 2012).

The study of plant resistance to abiotic stress should provide more opportunities to improve crop production in increasingly harsher environments. Research focusing on plant acclimation would eventually lead to the development of more resistant crops, potentially crucial for future generations. In some areas of the world, crop production naturally faces environmental challenges. For instance, the omnipresence of frost in northernmost agricultural regions of the globe greatly affects the production of crops. Finding more resistant varieties would greatly increase the economic potential of crops in such areas and reduce the costs associated with productivity losses. Hardier plants would also require fewer resources to grow. For example, drought-resistant crops would require less water for growth and development and potentially grow on marginal lands deemed too dry.

Plants have adopted many strategies to cope with abiotic stress. In fact, plants can rapidly react to environmental stress either with adaptation mechanisms or specific growth responses to circumvent the stress (Ciarmiello *et al.*, 2011). Response strategies include changes in metabolism, growth and development through signal transduction pathways and gene expression (Ciarmiello *et al.*, 2011; Shinozaki & Yamaguchi-Shinozaki, 2007). Adaptation mechanisms rely heavily on transcriptional and posttranscriptional regulation of genes that alter cellular functions and allow the plant to cope with stress (Jiang & Deyholos, 2006; Wong *et al.*, 2006; Yan *et al.*, 2006). In particular, cold stress is known to cause physiological, cellular and metabolic stress to which plants developed different response mechanisms.

2.2.2 Cold acclimation

A fluctuation in temperature induces changes in metabolic homeostasis and initiates physiological and cellular stresses in plants (Ensminger *et al.*, 2006). In fact, plants have developed different mechanisms to deal with cold stress and some employ mechanisms inducing physiological, biochemical and molecular alterations while others cannot properly adapt. Indeed, plants differ in their capacity to withstand freezing temperatures and some can alter their physiology to survive winter. Plants native to temperate zones can significantly improve their tolerance to freezing when exposed to low non-freezing temperatures (Thomashow, 1999). This process, termed cold acclimation, prepares plants for an upcoming winter and only allocates their resources for freezing tolerance during the fall (Thomashow, 1999). Characterised in several plant species (Colton-Gagnon *et al.*, 2014; Gilmour *et al.*, 1988; Puhakainen *et al.*, 2004; Welling et al., 2002), cold acclimation is initiated by environmental cues of the fall, e.g. low non-freezing temperatures and shorter days (Gray *et al.*, 1997; Puhakainen *et al.*, 2004; Takahashi *et al.*, 2013; Thomashow, 1999; Welling *et al.*, 2002).

Cold acclimation can involve the accumulation of protective molecules (e.g. carbohydrates, chaperones) and osmoprotectants (e.g. amino-acids, glycine betaine), changes in the composition of membranes (plasma and thylakoid), an increase in abscissic acid (ABA) levels and changes in gene expression (Breton *et al.*, 2003; Thomashow, 1999; Uemura *et al.*, 2006). Firstly, the accumulation of soluble carbohydrates limits cellular dehydration, helps protecting macromolecules by lowering the freezing temperature of cells and inhibits the formation of ice crystals. Moreover, these sugars can constitute a nutritional source for the plant during and/or after the cold stress. Secondly, cold both affects and is perceived by photosynthesis, at a level ranging from the inhibition of the electron transport chain to the decreased metabolic sink

capacity for photosynthates that ultimately cause imbalance of energy flow in the cell (Ensminger *et al.*, 2006). Plants respond partly to this imbalance by relocating and altering chloroplasts membranes (Ogasawara *et al.*, 2013), increasing thylakoid membrane fluidity (Shinozaki & Yamaguchi-Shinozaki, 1996), and by adjusting the expression of enzymes involved in photosynthesis (Liu *et al.*, 2012). In fact, cold acclimation is a complex multigenic system that has been estimated to affect the expression of about 10% of all genes (Ouellet & Charron, 2013) collectively referred to as cold-responsive genes (*COR* genes) (Thomashow *et al.*, 1990; Thomashow, 1999).

2.2.3 COR *genes and regulation*

During cold acclimation, plants reprogram their transcriptome, notably through the regulation of *COR* gene expression. In Arabidopsis, *COR* genes constitute 4 to 20% of the genome (Lee *et al.*, 2005). For example, *COR* genes can encode for heat-shock proteins, dehydrins and thermal hysteresis proteins that reduce cold-induced dehydration and inhibit the recrystallization of ice and can also act as chaperones (Barrett, 2001; Ouellet & Charron, 2013; Thomashow, 1999). Dehydrins are major enactors of protection against abiotic stresses; they play a role in both the cold and drought stress response pathways (Ouellet & Charron, 2013). First identified in drought-stressed barley and corn, these small hydrophilic proteins are found throughout the cell but tend to localize to the nucleus, cytoplasm and the chloroplasts (Ingram & Bartels, 1996). Proteins such as *AFP*s and *IRI*s (inhibitor of recrystallization of ice) inhibit ice crystal formation and growth in the cell (Griffith & Yaish, 2004; Zhang *et al.*, 2010). *AFP*s are thought to have convergent functions, and present no universal functional domain. In plants however, *AFP*s tend to derive from proteins involved in protective pathogenesis-related

pathways (Griffith *et al.*, 1992; Sicheri & Yang, 1995). As cold primarily induces a cellular dehydration that damages cell membranes (Thomashow, 1999), plants also modulate the fluidity of the plasma membrane to compensate for the rigidity induced by cold through the production of proteins like cold-responsive 413 plasma membrane (*COR413pm*) (Breton *et al.*, 2003; Colton-Gagnon *et al.*, 2014) and temperature-induced lipocalin (*TIL*) (Charron *et al.*, 2005; Kjellsen *et al.*, 2010; Uemura *et al.*, 2006). Although COR413 and TIL are plasma membranes, the precise mechanism through which they operate remains unknown. Both identified in *Arabidopsis* and wheat (*Triticum aestivum*), *COR413* is typically upregulated in response to temperature and drought stress while the expression of *TIL* appears to correlate with increased stress tolerance in wheat (Charron *et al.*, 2005).

The most studied regulation pathway of *COR* genes involve C-repeat binding factors/drought-responsive elements binding (*CBF*s/*DREB*s) (Ouellet & Charron, 2013). Independently discovered in cold acclimation (Thomashow *et al.*, 1997) and drought response (Yamaguchi-Shinozaki & Shinozaki, 1994), the overexpression of *CBF* genes improves cold hardiness in plants (Jaglo-Ottensen *et al.*, 1998; Ryu *et al.*, 2014). These transcription factors are member of the AP2 class and are rapidly induced in cold exposure (Barrett, 2001). They subsequently bind to a core element of consensus sequence (A/G)CCGAC (termed CRT, DRE or LTRE) that is located in the promoter region of *COR* genes (Ouellet & Charron, 2013; Thomashow, 1999). However, many studies established that binding elements such as the *CBF* transcription factors are not the only regulators of transcription (Thomas & Elgin, 1988). Another mechanism that can affect the regulation of transcription involves chromatin, the structure into which DNA is packaged in Eukaryotes. The study of the effect of chromatin structure modifications on gene expression is called epigenetics.

2.3. Epigenetics: chromatin structure and histones

2.3.1 Chromatin dynamics and gene regulation

Eukaryotes differ from prokaryotes in their ability to pack their DNA into a nucleoprotein complex known as chromatin. Composed of histone proteins and DNA, the fundamental unit of chromatin is the nucleosome, which comprises 146 nucleotide-long DNA molecule wrapped around an octameric histone core: chromatin is often referred to as "beads on a string". The DNA can be tightly or loosely bound to the histones, forming the two major configurations of chromatin: euchromatin (relaxed) and heterochromatin (closed).

The two configurations of chromatin play important roles in gene regulation (Thomas & Elgin, 1988). In euchromatin, the DNA is loosely bound to the histones; this conformation permits gene transcription. Conversely, heterochromatin is more compact than euchromatin and prevents gene transcription. Indeed, heterochromatic nucleosomes are characterised by the tight association of histones and DNA, which physically prevents the transcription complex from binding to DNA. Therefore, genes that are located in heterochromatic regions are silenced, or "closed" while genes located in euchromatin regions are "open" and transcriptionally active.

Some regions of the DNA can retain their chromatic structure throughout the life of the organism. For instance, centromeric and pericentromeric regions are tightly packed as they are not transcribed - they are referred to as constitutive heterochromatin. Constitutive

heterochromatic regions also confer tissue specificity and condition cells to retain their identity over the mitotic cycles. On the opposite, some regions of the genome can dynamically switch between euchromatin and heterochromatin conformations.

The regulation of chromatin structure is thought to be a level of transcriptional regulation that operates through the action of numerous regulators or chromatin modifiers. These proteins can modify the structure of chromatin by modulating the structure of the nucleosome, for instance through covalent modifications of histones.

2.3.2 Histones in nucleosome remodelling

Histone modifications can affect the structure of chromatin: chemical changes of the histone subunits can affect the structure of nucleosomes. There are five main families of histone proteins that, with DNA, constitute chromatin: four core histones associate to form an octameric core that builds the nucleosome (H2A, H2B, H3 and H4) and one linker histone H1/H5 that stabilises the DNA onto the octameric core. Histones can be covalently modified on multiple sites, and this with different types of modifications. Such modifications are thought to either activate or repress transcription (a) by chemically altering the binding between the histone and the DNA strand and/or (b) by interacting with chromatin modifiers and proteins that, depending on the modification, can positively or negatively affect transcription (Sterner *et al.*, 1999). One such well-studied modification is the acetylation of histones.

By default, histones are positively charged and deacetylated, and bind with higher affinity to the negatively charged DNA molecule. Acetylation neutralises this positive charge and relaxes the chromatin structure. Thus, the addition or removal of acetyl groups on histones induce a higher or lower accessibility of the DNA. In fact, histone acetylation positively

correlates with gene expression (Y. H. He *et al.*, 2003; Turner, 1991). *In vivo* assays have shown that histones in transcriptionally active nucleosomes are typically hyperacetylated, while heterochromatic regions tend to lack a similar degree of acetylation, or remain deacetylated (Turner & Oneill, 1995). Importantly, acetylated histones interact with various chromatin modifiers and transcriptional adaptors *in vitro* (Sterner *et al.*, 1999).

Histone acetylation appears to be involved in cold-response mechanisms. First, *COR* genes are thought to be directly activated in stress exposure through locus-specific acetylation. This process relies on the transcription factor CBF1, the HAT (histone acetyltransferase) GCN5 and adaptor protein ADA2 (Stockinger *et al.*, 2001). However, recent work suggested a more complex implication of GCN5 and ADA2 in cold acclimation (Pavangadkar *et al.*, , 2010) and histone deacetylation (Zhu *et al.*, 2008). In maize, cold stress induces the expression of HDAC (histone deacetylases) and treating the plants with a histone deacetylation inhibitor causes a downregulation of *COR* genes (Hu *et al.*, 2011). This finding implies that the expression of *COR* genes is induced by deacetylation - suggestively through the downregulation of a repressor.

The histone 3 lysine 27 (H3K27me3) tri-methylation is another well characterised histone modification. The addition of a tri-methyl group by the Polycomb Repressive Complex 2 (PRC2) on H3K27 is thought to repress gene expression (Derkacheva *et al.*, 2013). The levels of H3K27me3 of two Arabidopsis *COR* genes, *COR15A* and *ATGOLS3*, permanently change upon exposure to cold; these levels do not revert back to control levels, implicating that H3K27me3 is a probable stress memory marker (Kwon *et al.*, 2009).

A variety of histone changes exist. Other than acetylation and methylation, lysines can be modified by ubiquitination and sumoylation. Methylation can occur on arginine residues,

while serines and threonines amino-acids can be phosphorylated (Vaquero *et al.*, 2003). Not only modified, histones can be substituted with variants, which affects the structure of chromatin. These histone variants demonstrate specific expression, function and localisation. For example, nucleosomes containing a variant of H2A - H2A.Z - appear to decrease in occupancy in temperature-responsive genes (Boden *et al.*, 2013). H2A.Z are also required for the correct perception of ambient temperature in Arabidopsis (Kumar & Wigge, 2010).

In addition to histone modifications and variants, covalent modifications on the DNA strand can alter the structure of chromatin. In eukaryotes, cytosine methylation is an essential mechanism of the regulation of gene expressoion.

2.4 Mechanisms of DNA methylation

2.4.1 Cytosine methylation

In eukaryotes, DNA methylation is more precisely termed cytosine methylation because it only happens on cytosine residues. These can be modified by adding a methyl group (CH3) on the fifth ring position to become 5-methylcytosine (MeC) and this without affecting the base-pairing capacity to guanine. This mark is a characteristic of heterochromatic states: MeC rushes the DNA to bind more tightly to histones, and consequently prevents transcription complexes to effectively access the DNA. Cytosine methylation can also affect gene expression via (a) specific transcriptional repressors that bind MeC and prevent the binding of other

proteins, and (b) the sensitivity to MeC that some transcriptional factors possess - e.g. enhancers. Some studies suggest an interaction between histone modification and DNA methylation mechanisms (He *et al.*, 2011). For example, some proteins involved in DNA methylation may interact with modifications on H3 to enhance the switching from euchromatin to heterochromatin and vice-versa (He *et al.*, 2011).

DNA methylation is an essential epigenetic mark that is involved in the regulation of gene transcription. The mechanism of DNA methylation plays an major role in growth, development and genomic imprinting (He *et al.*, 2011). Fundamental to life and to the cellular memory concept, DNA methylation in both bacteria and multicellular organisms, maintains cellular identity over cell divisions. Methylation defects in mammals are lethal at the embryonic stage, and can lead to pleiotropic morphological defects in plants (Law & Jacobsen, 2010). Used as a defence mechanism, DNA methylation is also an effective means to prevent the expression of viral sequences and repetitive elements in plants (He *et al.*, 2011; Wassenegger *et al.*, 1994; Zhang *et al.*, 2006). There are different pathways of the regulation of DNA methylation: the maintenance and the *de novo* pathways.

2.4.2 Molecular processes of DNA methylation and demethylation

Over cell divisions, heterochromatin is maintained through the addition of methyl groups on hemi-methylated DNA. This maintenance of methylation is carried out by constitutive methyltransferases. They copy methylation patterns over cell divisions and sometimes from one generation to the next, making DNA methylation central to the maintenance of cellular identity and epigenetic inheritance.

Methylation patterns can only be maintained if they are present in the form of symmetric methylation sites: CG and CHG (H can be A, T or C). During DNA replication, enzymes called methyltransferases transfer methyl groups onto non-methylated cytosine residues on 'naked' newly synthesised DNA strands. Such methyltransferases include Methyltransferase 1 or MET1 (methylation at CG sites) and the plant-specific Chromomethylase 3 or CMT3 (at CHG). Constitutive heterochromatin typically contains many symmetric methylation sites that are maintained by MET1 and CMT3. However, asymmetric methylation CHH sites are not directly maintained by MET1 and CMT3, although these may play a role in their maintenance (Meyer, 2011). Hence, methylated DNA patterns on asymmetric methylation sites are gradually lost over cell divisions. Through this process, heterochromatin can passively and gradually become euchromatin. This non-maintenance or loss of methylation is called "passive DNA demethylation".

Generally speaking, passive demethylation prevents CHH sites to be part of constitutive heterochromatin. In other words, facultative heterochromatin methylated only at CHH sites cannot typically become constitutive. Interestingly, passive DNA demethylation can be triggered by methylation inhibitors, such as 5-azacytidine (AZA). This molecule is a cytosine analogue that lacks the characteristic site of methylation (i.e. the fifth carbon of the ring) (Zhou *et al.*, 2002). The integration of AZA in DNA precisely prevents the maintenance of DNA methylation and forces passive DNA demethylation at all sites.

Hypomethylated or unmethylated DNA can become hypermethylated as part of a response mechanism. This response is called *de novo* DNA methylation. Triggered by the environment, *de novo* DNA methylation establishes new methylation patterns at all sites (CG, CHG or CHH) (He *et al.*, 2011). Technically, newly methylated CHH are lost by passive

demethylation, while methylated CG and CHG remain. Therefore, a methylated cytosine in a CHH context is typical of *de novo* DNA methylation. The methyltransferase Domains-Rearranged Methyltransferase 2 or DRM2 is able to catalyse the addition of a methyl group on cytosines at all DNA sites making it particularly involved in *de novo* DNA methylation. Thus, DNA retains its heterochromatic structure through the action of maintenance methyltransferases or switches from euchromatin to heterochromatin through *de novo* DNA methylation. Antagonistic to *de novo* DNA methylation, active DNA demethylation can also rapidly change gene expression. This process is also involved in chromatin dynamics and is responsible for the change from heterochromatin to euchromatin. By demethylating cytosines, active DNA demethylation catalyses gene activation.

The discovery of DNA methylation and DNA demethylation led to extensive efforts in the identification of demethylases. Demethylases seem to ultimately replace 5meC with C in a replication-independent manner (Swisher *et al.*, 1998; Wu & Zhang, 2010). Many studies suggested various mechanisms of active DNA demethylation. These proposed: the enzymatic removal of the methyl group, the involvement of a base excision repair mechanism, the deamination of 5meC to T then followed by base excision repair, nucleotide excision repair, oxidative demethylation and radical deamination (Wu & Zhang, 2010). Unfortunately, the understanding of DNA demethylation is limited, and the molecular mechanisms of its occurrence are still being debated. The underlying pathway of gene-targeted demethylation is largely unknown, but studies tend to implicate RNAi (Penterman *et al.*, 2007).

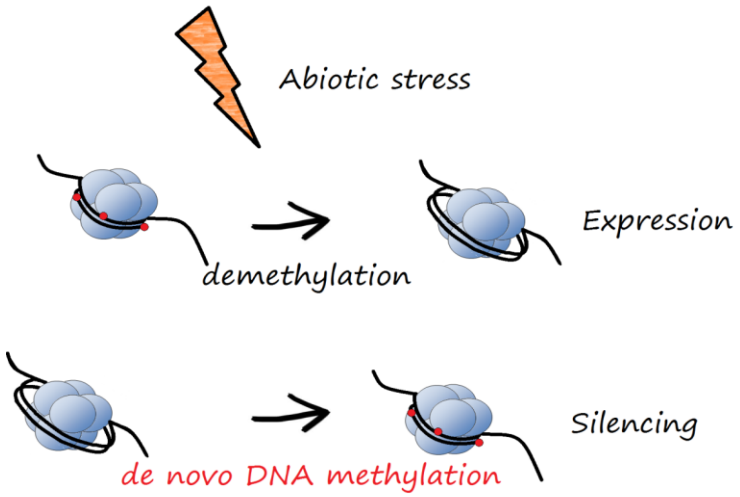

Figure 2.1: Nucleosome, DNA methylation and gene expression. Active demethylation of DNA leads to gene expression by switching a chromatin structure change from heterochromatin to euchromatin, or by increasing the affinity of transcription factors (or decrease the affinity of repressors). Antagonistic to demethylation, DNA methylation leads to gene silencing by the formation of heterochromatin.

Both active processes involved in chromatin dynamics, *de novo* DNA methylation and active DNA demethylation are most probably involved in plant response to abiotic stress. These two mechanisms would allow the plant to rapidly change the state of chromatin to either induce (demethylation) or repress gene expression (methylation). To establish whether DNA methylation is crucial in stress response, we need to understand what is behind the active methylation or demethylation of genes. However, the current knowledge of mechanisms of DNA demethylation are limited compared to what is known about *de novo* DNA methylation.

2.4.3 RNA-directed DNA methylation

The machinery behind gene specific *de novo* DNA methylation appears to be guided by RNA. This response pathway was termed RNA-directed DNA methylation or RdDM. One of the first clues of the existence RdDM was revealed in 1994 in potato. Viroid RNA triggered the methylation of inserted viroid DNA (Wassenegger *et al.*, 1994). Furthermore, RdDM has been shown to be involved in transposon suppression, gene imprinting, gene silencing and paramutation (He *et al.*, 2011).

RNA dependant DNA methylation begins with the transcription of target genes by the plant-specific DNA dependent RNA-polymerase IV (Pol IV) (He *et al.*, 2011). The single-stranded RNA molecule (ssRNA) generated by Pol IV is subsequently converted into double-stranded RNA (dsRNA) by a RNA-dependant RNA polymerase, known as RDR2 (Volpe *et al.*, 2002; Xie *et al.*, 2004). The dsRNA is cleaved into 24-nucleotide short interfering RNA (siRNA) fragments by DCL3, a multidomain RNaseIII-like dicer protein (Denli & Hannon, 2003; He *et al.*, 2011; Xie *et al.*, 2004). The siRNA is then methylated on its 2'-OH group on the 3' end by HEN1 (HUA enhancer), a S-adenosyl-L-methionine-dependant RNA methyltransferase (Yang *et al.*, 2006). This siRNA maturation step is followed by the loading of one of the strands of the siRNA onto Argonaute 4 (AGO4), or one of the closely related argonaute proteins, which are part of the RNA-induced silencing complex (RISC) (Wierzbicki *et al.*, 2012; Zhang *et al.*, 2007).

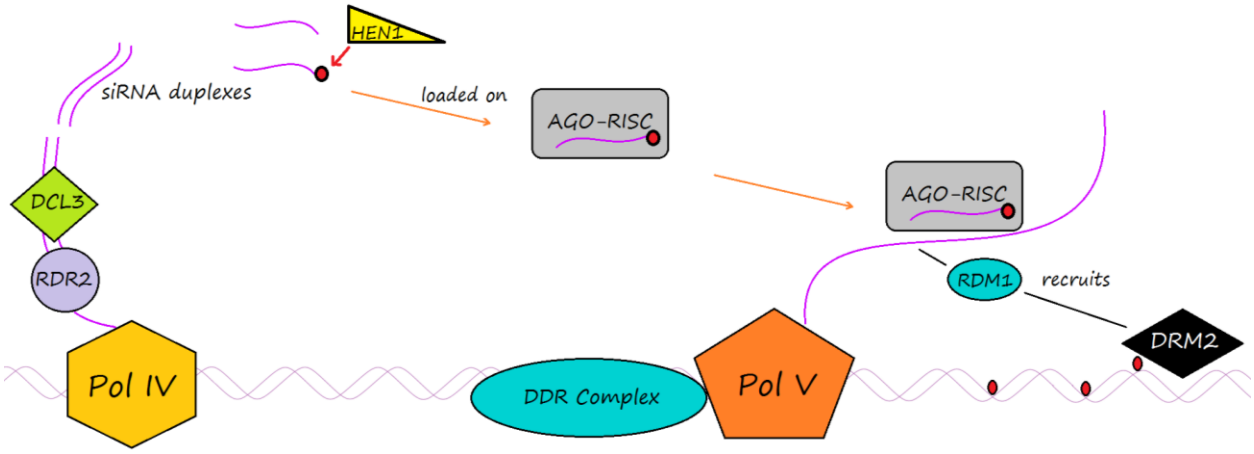

Figure 2.2: RNA-directed DNA methylation in *Arabidopsis*. The process of RdDM starts with the transcription of target genes by Pol IV. The produced ssRNA is converted to dsRNA by RDR2, which is subsequently cleaved into 24-nt siRNA fragments by DCL3. The siRNAs are then methylated by HEN1. This step is followed by the loading of one of the RNA strands onto AGO4 in RISC. Simultaneously, Pol V transcribes RNA target DNA being directed by the DDR complex. The ssRNAs produced by Pol V are used as scaffolds for the ssRNA-bound-AGO-RISC complex. Finally, the *de novo* methyltransferase DRM2 catalyses the cytosine methylation of the target gene.

In parallel to the successive reactions catalysed by Pol IV, RDR2, DCL3 and HEN1, the other plant-specific DNA-dependant RNA-polymerase V (Pol V) produces RNA transcripts from "RdDM target" loci (Wierzbicki *et al.*, 2012), which can either be coding or non-coding (He *et al.*, 2011). The action of Pol V is directed by the DDR complex, that comprises DRD1 (a putative chromatin remodelling ATPase) (Kanno *et al.*, 2005; Wierzbicki *et al.*, 2008), DMS3 (related to cohesins' and condensins' hinge domain) (Kanno *et al.*, 2008; Wierzbicki *et al.*, 2009) and RDM1 (ssDNA binding, with preference to methylated DNA) (Gao *et al.*, 2010; Law

et al., 2010). The RNA transcripts produced by Pol V are used as scaffolds for the binding of AGO-RISC complex (Wierzbicki *et al.*, 2009). Subsequently, RDM1 (also part of the DDR complex) acts as an adapter between the AGO-siRNA-Pol V complex and the *de novo* methyltransferase DRM2 (Gao *et al.*, 2010; Wierzbicki *et al.*, 2012). Once recruited, DRM2 catalyses the methylation of the target DNA, which changes the chromatin structure from euchromatin to heterochromatin. The target DNA is consequently silenced.

Connecting statement

Hardy, hemp presents a real research potential in terms of stress-response strategies. Gathering information on how hemp responds to its environment can contribute to the rediscovery and recent interest this plant received. Grown in cold regions, hemp probably has the necessary molecular machinery to cold acclimate. By comparing varieties of this crop, we intend to expose specific and different cold acclimation strategies that most probably involve epigenetic modifications. We hypothesise that varieties that cold acclimate the most efficiently should develop a higher tolerance to freezing temperatures. Therefore, a freezing test can efficiently segregate the varieties. Although a 2004 study presented that hemp hypomethylates its genome in response to heavy metal stress (Aina *et al.*, 2004), no stress-responsive genes that are involved in response to heavy metal, freezing or any other stresses were identified in hemp. With the recently published draft genome, it is now possible identify stress-responsive genes and to perform mRNA quantification as well as techniques such as chromatin immunoprecipitation

(ChIP) or methylated DNA immunoprecipitation (MeDIP) on specific loci. Chapter III below is formatted as a manuscript that is destined for publication. The co-authors are listed as follows: Boris F. Mayer, Mohamed Ali-Benali, Jordan Demone, Annick Bertrand and Jean-Benoit Charron. A detailed description of each co-author's contribution can be found in the Preface.

CHAPTER III: *CANNABIS SATIVA* L. COLD ACCLIMATES THROUGH CHROMATIN CHANGES AND *COR* GENE EXPRESSION

3.1 Abstract

Plants native to temperate regions are often able to increase their cold hardiness when exposed to low temperatures (LT) by undergoing cold acclimation. Although *Cannabis sativa* L. has been grown from subarctic to tropical regions, little is known about its capacity to cold acclimate and develop freezing tolerance. This study investigates the cold acclimation capacity of nine *Cannabis sativa* L. varieties and the underlying genetic and epigenetic responses. The varieties were divided into three groups by comparing the survival of non-acclimated plants to the survival of cold-acclimated plants in a whole plant freezing tests. The first group holds varieties that acclimate the most efficiently, the second group includes varieties with modest cold acclimation capabilities and the third group comprises sensitive varieties that do not acclimate. All varieties showed an increase in total soluble sugar accumulation in response to LT although only the varieties that could acclimate maintained elevated soluble sugar content throughout the CA treatment. *Cannabis* varieties that acclimated the most efficiently accumulated higher levels of *COR* transcripts. Furthermore, all nine varieties responded to the cold acclimation treatment with an initial increase in global DNA methylation that was reverted during the treatment only in hardy varieties. Additionally, these varieties displayed significant increases in methylcytosine levels at *COR* gene loci when deacclimated, suggesting a role for locus-specific DNA

methylation in deacclimation. These results shed light on the molecular mechanisms underlying cold acclimation in *Cannabis sativa* L. and reveal higher levels of complexity regarding how genetic, epigenetic, and environmental factors intertwine.

3.2 Introduction

Cannabis (marijuana, hemp) is an herbaceous plant that has been cultivated for centuries as a source of food, fibre, seed oil and medicine. Hemp, *Cannabis sativa* L., comprises the varieties that produce the best fibre and seed oil yields with only traces of Δ^9-Tetrahydrocannabinol (THC), a prized psychoactive compound found at high levels in marijuana (van Bakel *et al.*, 2011). *Cannabis sativa* L. is diploid with 20 chromosomes, 9 pairs of autosomes and a pair of sex chromosomes (X and Y) and although not fully sequenced, a draft genome and transcriptome were published in 2011 (van Bakel *et al.*, 2011). The availability of this resource has been key in jumpstarting more in-depth research about hemp.

Stress response mechanisms have been scarcely elucidated in hemp. Nevertheless, numerous agronomic studies have been performed in regards to the effect of drought stress on yield and biomass, specifically reducing seed yield (Cosentino *et al.*, 2013; Lisson & Mendham, 1998). Biotic stressors that affect hemp growth and yield, such as insect and fungal pests, have been identified and documented (OMAFRA, 2013). Moreover, abscisic acid (ABA) causes a downregulation of secondary metabolites and photosynthetic components in hemp when applied exogenously (Mansouri & Asrar, 2011). Interestingly, hemp is also efficient in

phytoremediation and is considered relatively tolerant to heavy metals (Mihoc *et al.*, 2012). Proteomic analysis uncovered a group of protective peroxidases and formate dehydrogenases that functionally reduce the presence of damaging free radicals induced by heavy metal stress (Hartley-Whitaker *et al.*, 2001; Wintz & Vulpe, 2002). Moreover, copper stress can significantly alter root morphology and the development of aerial tissue (Liu *et al.*, 2012). Importantly, a study performed by Aina *et al.*, (2004) provided rare insights into molecular response mechanisms of hemp by showing that under heavy metal stress, hemp triggers a global DNA hypomethylation.

Very little is known in terms of the tolerance of hemp to frost and freezing. Low temperature (LT) and other abiotic stresses are among the most limiting factors to crop production worldwide (Xin & Browse, 2000). Indeed, LT limits crop production in temperate regions while freezing spells can be even more damaging and cause significant loss of yield. Primarily, cold induces cellular dehydration that ultimately causes mechanical damage to cell membranes (Thomashow, 1999). Moreover, cold can affect photosynthesis by increasing the rigidity of the thylakoid membrane (Shinozaki & Yamaguchi-Shinozaki, 2007), disrupting the function of the protein complexes involved in photophosphorylation and electron transport chain pathways (Wise & Ort, 1989).

Plants naturally vary in their ability to withstand freezing temperatures and most plants indigenous to temperate regions are able to increase their tolerance to freezing temperatures after an exposure to LT. This process has been termed cold acclimation (CA) and involves an array of physiological, biochemical and molecular changes. Cold acclimation has been well-characterized in several species, such as *Arabidopsis thaliana* (Gilmour *et al.*, 1988; Thomashow, 1999), *Triticum aestivum* (Christov *et al.*, 2007) and *Brachypodium distachyon* (Colton-Gagnon *et al.*, 2014; Li *et al.*, 2012). Plants respond to cold through both physiological and

metabolic changes. For instance, plants respond physiologically by relocating chloroplasts to low light-intensity areas of the cells, and metabolically by altering the expression of enzymes involved in photosynthesis (Liu *et al.*, 2012; Ogasawara *et al.*, 2013; Shinozaki & Yamaguchi-Shinozaki, 2007). Plants protect the photosystem II by producing specific isoforms of amylase - an enzyme involved in the production of maltose, a cryoprotective molecule (Kaplan & Guy, 2005; Kaplan *et al.*, 2007). In response to LT, tolerant plants accumulate osmoprotectants and soluble carbohydrates that limit cellular dehydration during freezing (Thomashow, 1999) and also increase the fluidity of both plasma and thylakoid membranes by altering their composition (Uemura *et al.*, 2006).

Cold acclimation is a complex multigenic process that can involve the expression of about 10% of all genes in a plant's genome (Hannah *et al.*, 2006; Ouellet & Charron, 2013; Thomashow, 1999). Cold acclimation triggers the expression of a wide array of genes, collectively referred to as cold-regulated genes (*COR* genes) (Hajela *et al.*, 1990; Thomashow *et al.*, 1990; Thomashow *et al.*, 1997). The promoter regions of *COR* genes often include short sequences known as a dehydration response element/C-repeat elements (DRE/CRT) containing the core *cis*-acting element (with the sequence CCAGC) that regulates cold and also dehydration stress responses (Baker *et al.*, 1994). Many *COR* genes are regulated at the transcriptional level by *trans*-acting regulators that include the extensively studied *CBF*s (C-repeat Binding Factors), a subset of the AP2/ERF class of transcription factors (Stockinger *et al.*, 1997). The overexpression of *CBF* genes improves cold hardiness in plants (Jaglo-Ottensen *et al.*, 1998; Ryu *et al.*, 2014).

Although only a few links have been established between stress response and chromatin dynamics in plants, transcriptional regulation of *COR* genes may also comprise

changes in chromatin structure (Pavangadkar *et al.*, 2010; Stockinger *et al.*, 2001). The structure of chromatin can be modified in various ways, notably through covalent modifications of histones and DNA methylation. For example, the acetylation/de-acetylation of H3K9 appears to play a major role in the transcriptional regulation of *COR* genes (Pavangadkar *et al.*, 2010; Stockinger *et al.*, 2001). Conversely, the tri-methylation of H3K27 may be involved in the repression of *COR* gene and in stress memory (Kwon *et al.*, 2009). In addition, *Arabidopsis* mutants for histone deacetylases develop a hypersensitivity to freezing temperatures (Zhu *et al.*, 2008). Furthermore, plants that were defective for subunits of histone acetyltransferase complexes induced *COR* genes to a lower level upon cold acclimation (Vlachonasios *et al.*, 2003). Vernalization, a process that is intertwined with the cold acclimation response in cereals, associates with histone acetylation changes (Amasino, 2004; Diallo *et al.*, 2012). In barley, the levels of H3 and H4 acetylation at the *Vernalization1* (*VRN1*) locus increase in response to LT (Oliver *et al.*, 2013). Moreover, treatment with the histone deacetylase inhibitor sodium butyrate promoted an increase in H3 and H4 acetylation and subsequently in *VRN1* transcript levels (Oliver *et al.*, 2013). The regulation of chromatin structure appears to be strongly linked to the regulation of stress-responsive genes. This study investigates the cold acclimation and freezing-tolerance capacities of nine *Cannabis sativa* L. varieties and focuses on the chromatin and molecular changes associated with these agronomically important traits.

3.3 Materials and Methods

3.3.1 Plant material

Nine industrial hemp (*Cannabis sativa* L.) varieties approved for production in Canada were studied: Alyssa, Anka, Canma, CFX-1, CFX-2, CRS-1, Finola, X-59 and Yvonne, according to the term specified in the licenses #12-C0142-R-01, 13-C0142-R-01, 14-C0142-R-01 delivered to Jean-Benoit Charron by Health Canada. For each cultivar, fifteen seeds were sown in three-inch pots containing Agromix PV20 (Fafard et Frères Ltd., Saint-Remi, QC, Canada) and grown in environmental growth chambers (Conviron, Winnipeg, MB, Canada) at 22°C/22°C (day/night) with a 16h photoperiod and a photosynthetically active radiation (PAR) intensity of 150 μmol m^{-2} s^{-1}. The plants were watered twice a week with a solution of a water-soluble fertilizer (Scotts Inc., Marysville, OH, USA). Three replicates were grown for each experiment. After 14 days of growth, plants were either left in the same conditions for another two days (non-acclimated, NA), or subjected to a LT treatment of 4°C ±1°C under an 8h photoperiod at a photon flux density of 150 μmol m^{-2} sec^{-1} for a duration of 7 (cold-acclimated, CA7) or 14 days (CA14). Deacclimated plants (DA) were placed back to control conditions for one day after 14 days of LT treatment.

3.3.2 Electrolyte leakage assay

A minimum of six seedlings per treatment (NA, CA7, CA14, CA21 and CA28) per assay were placed in 15 ml conical tubes containing 10 mL of distilled water and agitated at room temperature overnight. Following agitation the conductivity of the solution was measured using a conductivity meter (VWR International, Ville Mont-Royal, Canada). The solutions were then boiled for 20 min to lyse the cells. Once the samples returned to room temperature, the electrolyte conductivities were recorded as the absolute conductivity. The percentage of electrolyte leakage was calculated by dividing the initial conductivity by the absolute

conductivity. The experiment was repeated three times with independent biological replicates.

3.3.3 Whole plant freezing test

A whole plant freezing test (WPFT) was performed in a programmable LT-36VL low-temperature chamber (Percival Scientific, Perry, IA, USA) in order to determine the survival to freezing of nine varieties of industrial hemp with or without (NA) a prior cold acclimation period of 14 days (CA). Plants were subjected to a 2h equilibration period at -2°C and seeded with ice chips to initiate freezing after which the temperature was decreased by 1°C every hour (J. B. F. Charron, Ouellet, Houde, & Sarhan, 2008; Colton-Gagnon et al., 2014). After one hour at -3, -5, -7 and -9°C, a randomly selected pot of NA and CA were removed from the cold chamber for each cultivar. To minimise light stress effects after the test, plants were placed at 4°C for 24h in the dark before returning to control conditions. Survival counts were performed after two weeks of re-growth. The experiment was repeated three times with independent biological replicates.

3.3.4 SPAD assay

Ten successive readings were taken using a SPAD-502 chlorophyll meter (Minolta, Azuchi-Machi, Osaka, Japan) across five to six leaves per pot per treatment. The mean SPAD value was calculated from a minimum of 30 readings obtained from three independent biological replicates. All measurements were taken at 12 PM (4 hours into the illumination period).

3.3.5 Carbohydrates analysis

Whole aerial parts of the hemp plants were dried at 70°C for 30 minutes to inactivate degradative enzymes and further dried overnight at 37°C. The samples were ground into a fine powder using liquid nitrogen and sifted through a 50 μm sieve and stored at 4°C until further use. Soluble carbohydrates were extracted by adding 1 mL of deionised water to 200 mg of the ground sample. Extracts were heated at 80°C for 20 minutes and then incubated at 4°C for 24h. Extracts were centrifuged twice for 10 min at 3200 g and subsamples of supernatant were taken for soluble carbohydrate analysis. All extracts were stored at −20°C until analyses could be completed. Fructose, glucose, sucrose and raffinose levels were processed with the Waters ACQUITY ultra-performance liquid chromatography (UPLC) analytical system controlled by Empower II software (Waters, Milford, MA, USA). Sugars were separated on an ACQUITY UPLC BEH (ethylene bridged hybrid) Amide 1.7 μm (2.1 by 100 mm) column preceded by a VanGuard (2.1 by 5 mm) precolumn and detected on an electric light scattering detector set to a gas pressure of 310 kPa. Elution was performed at 35°C with a flow rate of 0.25 mL min^{-1} with the following gradient of eluents A (80% CH_3CN and 0.1% NH_4OH) and B (30% CH_3CN and 0.1% NH_4OH): 70% A and 30% B from 0 to 15 min and 30% A and 70% B from 15 to 17.5 minutes using curve 6, 70% A and 30% B from 17.5 to 17.51 min using curve 6, and 70% A and 30% B from 17.51 to 20 minutes. The drift tube was adjusted to a temperature of 50°C in the cooling mode. Samples were diluted 1:1 with CH_3CN and centrifuged for 3 minutes at 16,000 g and were then kept at 4°C in the sample manager throughout the analysis. Sugars were identified and quantified by reference to standards. The presented values are the added values of all quantified sugars for each variety at each time point.

3.3.6 Nucleic acids extraction

Aerial tissue from three biological replicates was sampled. For each replicate, >10 plants were flash frozen and kept at -80°C until use. The samples were then pooled and grounded in liquid nitrogen prior to nucleic acids extraction. Total RNA was extracted from ~200 mg of powdered tissue using TRI Reagent® (Sigma-Aldrich Corp., St. Louis, MO, USA) following the manufacturer's protocol. The extracted RNA samples were further purified using the EZ-10 Spin Column RNA Cleanup and Concentration Kit (Bio Basic, Markham, ON, Canada) and treated on-column with DNase 1 (Qiagen, Hilden, Germany). Genomic DNA was extracted from 200 mg of powdered tissue by phenol/chlorophorm/isoamylacohol.

3.3.7 Global DNA methylation

The global DNA methylation assay was performed using the Imprint® Methylated DNA Quantification Kit (Sigma-Aldrich Corp., St. Louis, MO, USA) according to the manufacturer's recommendations with 100 ng/μL of DNA per well. Each sample was measured in technical quadruplicate using a 680 Microplate reader (BioRad, Hercules, CA, USA).

3.3.8 Methylated-DNA Immunoprecipitation (MeDIP)

Prior to MeDIP the same genomic DNA was sonicated in a Bioruptor® UCD-200 (Diagenode, Seraing, Belgium) with 10 duty cycles ON/OFF for 30s/30s on the highest setting. The MeDIP was performed following the protocol described in (Mohn, Weber, Schübeler, & Roloff, 2009) except that immunoprecipitated DNA was cleaned using the MinElute Reaction Cleanup kit (Qiagen, Hilden, Germany). An antibody against methylated cytosines (the 5-mC Monoclonal Antibody (Diagenode, Seraing, Belgium) was used with the Protein G Magnetic Beads (Cell Signalling, Denvers, MA, USA) and the 6-Tube Magnetic Separation Rack (Cell

Signalling, Denvers, MA, USA). A subset of controls (input DNA and no antibody control) were also analysed by qPCR.

3.3.9 Chromatin Immunoprecipitation (ChIP)

Fresh plant tissues of NA, CA7, CA14 and DA from the different varieties were cross-linked and whole plant nuclei were extracted following the protocol described by (Bowler et al., 2004). Extracted nuclei were sonicated using the Bioruptor® UCD-200 (Diagenode, Seraing, Belgium) with 15 duty cycles ON/OFF for 30s/30s on the high setting. The sonicated nuclei were centrifuged for 5 min at 4°C to pellet the debris. The supernatant was used for ChIP following the Affymetrix® Chromatin Immunoprecipitation Assay Protocol (P/N 702238 Rev. 3) as described in (J. B. F. Charron, He, Elling, & Deng, 2009) and Protein G Magnetic Beads (Cell Signalling, Denvers, MA, USA). The antibodies used were Anti-Histone H3 (Abcam PLC, Cambridge, UK), Anti-trimethyl-Histone H3 (Lys27) and Anti-acetyl-Histone H3 (Lys9) (EMD Millipore, Billerica, MA, USA). A subset of controls (input reverse cross-linked chromatin and no antibody control) were also analysed by qPCR.

3.3.10 Target gene identification and primer design

The genes studied in expression analysis, ChIP and MeDIP were identified using *The Cannabis Genome Browser* (van Bakel *et al.*, 2011). Template amino acid sequences from *Arabidopsis thaliana* were used for all genes except for PGIP/AFP (*Daucus carota)* (see Table 3.1). Specific primers were designed using Primer-BLAST and ordered from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

3.3.11 Transcript accumulation analysis by quantitative real-time PCR

The cDNA was produced using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA, USA) as stated in the protocol. Quantitative real-time PCR was performed on a CFX Connect Real Time system (BioRad, Hercules, CA, USA) using SYBR green Supermix Sso-advanced (BioRad, Hercules, CA, USA). Three technical replicates for each sample were used and the experiment was repeated three times with different biological replicates. Controls without template were included for all primer pairs. The RT-qPCR data was analysed following the Livak method using 18S ribosomal RNA as the reference gene (Livak & Schmittgen, 2001).

3.3.12 ChIP-qPCR and MeDIP-qPCR analyses

The qPCR reaction was performed on ChIP and MeDIP samples following the same conditions described in the section *Transcript accumulation analysis by quantitative real-time PCR*. The results were analysed following the percent input method. In the presented results, the background signal (no antibody mock signal) was subtracted from the immunoprecipitated signal.

3.3.13 Statistical analysis

Statistical difference was analysed by performing an analysis of variance (ANOVA) test followed by a Tuckey's test using the software JMP®.

3.4 Results

3.4.1 CA induces electrolyte leakage in hemp

In order to rank the varieties according to their cold hardiness, we initially measured their electrolyte leakage (EL) after a cold acclimation (CA) treatment of 7, 14, 21 or 28 days. Interestingly, all varieties were significantly affected by LT at a different time (Fig. 3.1). The highest EL values were measured in two varieties at CA7, in four at both CA14 and CA21 and in eight at CA28. While most varieties displayed no increase in EL after 7 days, most exhibited significant leakage after 28 days of CA (Fig. 3.1). However at CA14 and CA21, the varieties displayed the largest response variability, suggesting that these two LT treatments had the potential to highlight differences in cold acclimation capacities. However, differences in plant stature and signs of leaf yellowing were visible in some varieties after 21 days of CA. Thus, we chose CA14 as the CA treatment to evaluate the cold-tolerance of morphologically similar *Cannabis sativa* L. plants (Fig. 3.2A).

3.4.2 The capacity to cold acclimate is variety-dependent

To investigate the freezing-tolerance of hemp, we performed a series of whole plant freezing tests (WPFT) (Fig. 3.2). The survival of non-acclimated (NA) plants was compared to the survival of plants exposed to the cold-acclimation treatment (CA plants) of 14 days at -3°C, -5°C, -7°C and -9°C. After a re-growth period of two weeks, most varieties had higher survival counts for CA plants compared to NA plants (Fig. 3.2B). Finola plants showed an increased survival of 25% at -3°C, of 60% at -5°C and of 35% at -7°C when cold-acclimated. However, neither NA nor CA Finola plants survived at -9°C (Fig. 3.2B). Further, 45% of CA Yvonne survived temperatures of -9°C while NA plants showed zero survival at that same temperature. Conversely, CA CRS-1 plants did not follow that trend and were more sensitive to

freezing than the NA CRS-1 plants, making this variety the most sensitive tested (Fig. 3.2B-C). Overall, the WPFT revealed three survival groups. The first group holds Finola and Yvonne - the two varieties that can cold-acclimate the most efficiently and develop freezing-tolerance. More precisely, Yvonne had the most robust FT with surviving plants showing mostly undamaged leaves when exposed to -9°C, whereas Finola showed the most pronounced survival gain at -3°C, -5°C and -7°C, even though the survivors were evidently damaged after the recovery period (Fig. 3.2C). The second group comprises varieties for which CA plants showed significantly higher survival than NA for only one of the freezing temperatures tested: CanMa, Anka, CFX-1, CFX-2 and X-59 (Fig. 3.2C). Lastly, the third group includes Alyssa and CRS-1, two sensitive varieties that show no ability to cold acclimate (Fig. 3.2C).

3.4.3 Cold acclimation treatment induces metabolic changes in Cannabis sativa L.

In an attempt to explain the cold acclimation capacity differences, we first investigated the impact of the CA treatment on photosynthesis NA, CA7, CA14 and deacclimation (DA) by indirectly measuring the chlorophyll content with a SPAD meter (Soil and Plant Analyser Development analysis). All varieties displayed significantly lower SPAD readings in response to LT (Fig 3.3A). Nevertheless, the readings were statistically similar amongst all nine varieties at a given time point, which indicated that none of these varieties had a clear photosynthetic advantage during the CA treatment. In addition, we quantified the total water soluble sugar (WSS) content per gram of dry weight (mg/gDW) including fructose, glucose, raffinose, stachyose and sucrose levels in the nine varieties at NA, CA7, CA14 and DA (Fig. 3.3B). As expected, all varieties showed a significant increase in the accumulation of total soluble sugars at CA7. However, CanMa, CFX-2 and Yvonne were the only varieties that

maintained elevated WSS content at CA14 (Fig 3.3B). Upon deacclimation, all varieties demonstrated WSS contents at, or below NA levels. These results indicate that LT clearly induces photosynthetic and sugar metabolism changes in *Cannabis sativa* L. but do not support a linear relationship between metabolic changes and freezing-tolerance level.

3.4.4 Cold acclimation efficiency positively correlates with COR gene transcript levels

We performed RT-qPCR to quantify relative mRNA abundance of four *COR* genes in the nine varieties following NA, CA7, CA14 and DA treatments: *Temperature induced lipocalin* (*TIL*), *Cold-regulated 413 plasma membrane* (*COR413pm*), *Cold-regulated 413 thylakoid membrane* (*COR413tm*) and a putative *polygalacturonase inhibitor/anti-freeze* (*PGIP/AFP*) that shares sequence homology with the *Daucus carota AFP* (Table 3.1). All nine varieties showed increased transcript accumulation of all 4 *COR* genes upon exposure to CA conditions. These levels generally returned to control values upon deacclimation. Unlike with *COR413pm*, *COR413tm* and *PGIP/AFP*, all varieties presented similar accumulation profiles of *TIL* transcripts when exposed to LT. Notably, the most variable mRNA accumulation profiles among the varieties were observed for *PGIP/AFP*. Alyssa, Anka, CFX-2 and X-59 showed little to no *PGIP/AFP* transcript accumulation, while CanMa, CFX-1, CRS-1 and Finola showed an induction of 2 to 4 fold. Yvonne stood out with a 59.8 fold induction of *PGIP/AFP* at CA14 while also exhibiting higher transcript levels of both *COR413pm* (12 fold) and *COR413tm* (5 fold). Unlike metabolic changes, the transcript accumulation of *COR* genes illustrated the differential cold acclimation capacity observed in the WPFT.

3.4.5 DNA methylation dynamics reflect cold acclimation capacities

We wanted to investigate the implication of DNA methylation in cold acclimation in hemp. We measured the global DNA methylation levels and the expression profiles of genes involved in the establishment and maintenance of DNA methylation in four selected varieties with contrasting freezing-tolerance capabilities (Fig. 3.5A). Based on the results from the WPFT and the *COR* gene expression data we selected CFX-2, CRS-1, Finola and Yvonne for the following experiments. These varieties represent the 3 groups of different cold acclimation capacities described earlier and also displayed different transcript accumulation profiles for the *COR* genes. Under control conditions CFX-2, CRS-1 and Finola had similar levels of global DNA methylation while Yvonne's NA level was lower by 75%. The varieties showed an increase in global DNA methylation upon exposure to the CA treatment (Fig. 3.5A). The stronger global DNA methylation shift was displayed by Yvonne, which increased by 5.2 fold at CA7. Similar trends were observed at a smaller scale in CFX-2 (2.5 fold) and in Finola (1.4 fold). Conversely, CRS-1 global DNA methylation levels remained stable at CA7. At CA14, elevated global DNA methylation levels were observed in CFX-2, CRS-1 and Yvonne but not in Finola. Importantly, the varieties that cold acclimate the most efficiently, Finola and Yvonne, both reduced their global DNA methylation levels at CA14 after an initial hypermethylation at CA7. Once deacclimated, the global DNA methylation levels in CRS-1 and Finola returned to control levels (Fig. 3.5A). However, this reduction in global DNA methylation was not observed in CFX-2, which maintained high levels of DNA methylation at DA. Interestingly, Yvonne displayed lower global DNA methylation at DA than at NA and was the only variety displaying significant differences in DNA methylation levels between every time point.

Changes in DNA methylation levels suggested the activity of DNA methyltransferases and other genes involved in its establishment and maintenance. We thus

monitored mRNA abundance of three DNA methyltransferases: *Methyltransferase* 1 (*MET1*), *Chromomethylase* 3 (*CMT3*) and *Domains-rearranged methyltransferase* 2 (*DRM2*) in response to the CA treatment (Fig. 3.5B). We also monitored the expression profiles of genes involved in *de novo* DNA methylation (Fig. 3.5C). No significant variation in the expression profile of *MET1* was observed in CFX-2, whereas modest transcript abundance decreases were observed in CRS-1 and Finola at CA and DA (Fig. 3.5B). Similar trends were observed for *CMT3* and *DRM2* in these three varieties with the exception of *DRM2* in Finola whose levels initially increased at CA7 and dropped below control levels at CA14 and DA. Conversely, Yvonne displayed clear expression profile variations for both *MET1* and *CMT3* transcripts with 3 to 5 fold increases at CA14 and DA as well as a 22 fold increase of *DRM2* transcripts. All methyltransferases' transcript levels decreased to control levels at DA (Fig. 3.5B). Modest differences in expression profiles were observed for *RDR2*, *DCL3*, *HEN1* and *AGO4* in all four varieties (Fig. 3.5C). CRS-1 showed a general downregulation upon CA and DA for all of the tested *de novo* DNA methylation genes. CFX-2, Finola and Yvonne displayed the highest transcript accumulation of these genes during the CA treatment. Yvonne showed a 20-fold increase in *AGO4* accumulation at CA14, which decreased to close-to-control levels at DA (Fig. 3.5C). Interestingly, *RDR2* and *DCL3* transcript accumulation patterns were alike and displayed very similar profiles in the four varieties in response to LT. *AGO4* and *DRM2* also showed a similar transcript accumulation pattern. Once again, Yvonne stood out with the most dynamic range of global DNA methylation and high accumulation of *MET1*, *CMT3*, *DRM2* and *AGO4* transcripts (Fig. 3.5C).

3.4.6 All varieties displayed differential locus-specific histone modifications and MeC

To investigate whether epigenetic landscapes at specific loci are modulated in response to LT, we monitored changes in three well-characterised epigenetic modifications: acetylation of histone 3 lysine 9 (H3K9ac), tri-methylation of histone 3 lysine 27 (H3K27me3) and methylation of cytosine (MeC) at two *COR* gene loci (Fig. 3.6). Specific primer sets were designed to amplify regions where the presence of these two modifications normally correlates with gene expression (He *et al.*, 2003) (Fig. 3.6A). Accordingly, we found higher levels of H3K9ac at the N-terminal region of the *COR413pm* gene than at the C-terminal region in response to CA treatment (Fig. 3.6B). This difference was not observed for H3K27me3. No significant differences in the deposition of H3K9ac at the *TIL* locus was observed for CFX-2 and Finola. However, both CRS-1 and Yvonne showed a clear reduction of H3K9ac on *TIL*. Nevertheless, both varieties were able to reset their *TIL* H3K9ac signal to NA levels following deacclimation (Fig. 3.6B). All varieties showed similar decreases in H3K9ac signal on*COR413pm* at DA with the exception CFX-2. Interestingly, in Yvonne and Finola, the varieties with strong cold acclimation responses, H3K9ac on *COR413pm* returned to control levels at DA while in the more sensitive varieties CFX-2 and CRS-1 it did not (Fig. 3.6B).

In CFX-2, we observed no significant differences in the levels of H3K27me3 on both *TIL* and *COR413pm* during the CA treatment. However, CRS-1 and Finola displayed reductions in H3K27me3 levels at both loci in response to the CA treatment. Unlike CRS-1, Finola reverted its H3K27me3 levels to control levels on *TIL* and *COR413pm-2* at DA. Yvonne was the only variety to display an increase in H3K27me3 levels at both loci during CA.

The profiles of MeC at the two studied *COR* gene loci varied greatly among the four varieties. CFX-2 displayed elevated levels of MeC on *TIL* under NA conditions. Upon exposure to the CA treatment, the MeC signal progressively decreased below detection level and

no MeC signal was detected at DA. The latter shift was also observed on *COR413pm* at DA with the exception that no significant changes were observed between NA and CA for this variety. CRS-1 displayed only modest MeC level changes on both *TIL* and *COR413pm* while Finola and Yvonne actively modulated their MeC levels at these loci during CA. These two varieties displayed significant increases in MeC signals at DA, at both loci for Yvonne but only on *TIL* for Finola. Yvonne also progressively increased its levels of MeC during CA, which was not observed in Finola. These results indicate that in *Cannabis sativa* L., cold acclimation involves specific changes in chromatin.

3.5 Discussion

Interest in industrial hemp has been gaining momentum worldwide due to its robust and rapid growth habits and to an increasing demand for biodegradable and natural products. As a result, over thirty countries now grow industrial hemp, challenging this crop to grow in a wide range of environmental conditions. This situation creates the need but also provides opportunities to understand the adaptive mechanisms of hemp. With the recent release of a draft genome sequence and transcriptomic data in 2011, *Cannabis sativa* L. now falls into the non-traditional model plant category alongside other plant species such as *Thlaspi arvense* L., *Thellungiella parvula* and *Eucalyptus grandis* (Dorn *et al.*, 2013; Gion *et al.*, 2011; Oh *et al.*, 2010; van Bakel *et al.*, 2011).

Our study is the first to expose a cold acclimation response in hemp and to demonstrate that certain varieties are able to induce mechanisms to increase their freezing-

tolerance. The whole plant freezing-tolerance test performed in this study uncovered clear phenotypic differences in cold acclimation capacities in hemp. Overall, most hemp varieties tested demonstrated the capacity to acclimate to cold and were able to significantly increase their tolerance to freezing. On one hand, Yvonne and Finola showed the highest increase in survival to freezing following the CA treatment and thereby demonstrated a superior cold acclimation capacity. On the other hand, CRS-1 and Alyssa showed significantly lower survival in response to freezing temperatures after an exposure to LT. The CA treatment appears to be damaging to these two varieties, suggesting a lack of proper cold acclimation machinery.

Both acclimated and non-acclimated freezing-tolerance were examined in this study. Non-acclimated freezing-tolerance measures a plant's innate ability to withstand a sudden drop in temperature. Acclimated freezing-tolerance reflects a plant's acquired freezing-tolerance developed during an exposure to low, non-freezing temperatures through the process of cold acclimation (Thomashow, 1999). While both Yvonne and Finola could efficiently increase their freezing-tolerance through cold acclimation, they both displayed different non-acclimated freezing-tolerance. Finola showed the lowest level of acclimated freezing-tolerance amongst the nine varieties and yet showed the largest increase in freezing-tolerance. The mechanism behind non-acclimated freezing-tolerance is not as well-understood as cold acclimation, partly because it is governed by genetic loci independent of those involved in cold acclimation and acquired freezing-tolerance (Hannah *et al.*, 2006; Stone *et al.*, 1993; Teutonico & Osborn, 1995). Thus, the hemp model, due to this newly uncovered range of phenotypic variation in freezing-tolerance and cold acclimation capacity, can be a potentially valuable tool to uncover key determinants behind non-acclimated freezing-tolerance. Furthermore, the analysis of phenotypic variation for freezing-tolerance and other traits might be efficient in providing evidence towards genetic

relatedness among modern varieties, especially as biosystematic classifications and breeding history records are almost inexistent for many popular *Cannabis sativa* L. varieties. While only short seed and dual-purpose (seed and fibre) varieties were the object of this study, two varieties grown primarily for their high seed yields, CRS-1 and Finola, revealed contrasting cold acclimation responses. Both varieties were selected for early maturation and bred to develop short statures to maximise seed yield. However, Finola is specifically adapted for production north of the 48th parallel, having been developed in Finland. Similarly, Yvonne (the variety that displayed the strongest cold acclimation response) was developed for production in northern climates. It is also the tallest variety tested in this study and is highly regarded for the quality of its fibres. Therefore, the superior CA capacity that Finola and Yvonne display may have been unintentionally co-selected with the desirable agronomic traits.

Similar to the observations of Hannah *et al.* (2006), our results do not support a linear relationship between metabolite changes and acclimated freezing-tolerance, although cold acclimation in *Cannabis sativa* L. is clearly associated with metabolic changes. In some herbaceous plant species, sugar content changes are well correlated with freezing-tolerance levels, while others show little correlation (Sasaki *et al.*, 1996). In this study, the sugar accumulation profiles only partly correlate with sensitivity to cold. Indeed, all the tested *Cannabis sativa* L. varieties accumulated soluble sugars in response to the CA treatment, but in the most sensitive variety CRS-1 as well as in the varieties with modest CA responses Anka and X-59, the CA14 levels dropped below NA levels. This drop may indicate that these varieties are not able to sustain this type of metabolic adjustment under LT stress. Although the varieties able to cold acclimate did not show this decrease between CA7 and CA14, their profiles of sugar accumulation were not significantly different from one another. Together these data suggest that the monitoring of the

variations in soluble sugars content in hemp during a LT stress can potentially be an indication of sensitivity, but does not allow for an elaborate variety classification based on cold acclimation capacity.

Hannah *et al.* (2006) have shed light on a significant association between the complexity of the transcriptional response and the cold acclimation capacity in *Arabidopsis*. Comparing nine accessions, they observed that the amplitude of gene expression was greater in accessions with a higher acclimation capacity, which implied that more dynamic changes may be functionally relevant for successful acclimation. This observation has also been made at a smaller scale in wheat and other cereals where transcript abundance of genes such as *TIL*, *COR413pm*, *COR413tm* and antifreeze proteins correlates with freezing-tolerance levels (Breton *et al.*, 2003; Charron *et al.*, 2002; Tremblay *et al.*, 2005). In our case, the transcript accumulation profiles of *COR* genes *COR413pm*, *COR413tm*, *TIL* and *PGIP/AFP* evident that all varieties responded to the CA treatment by inducing the expression of *COR* genes. More specifically, the varieties able to cold acclimate tended to accumulate more *COR* gene transcripts than those who could not. The variety Yvonne stood out with significantly higher transcript accumulation levels at CA14 for *COR413pm*, *COR413tm* and *PGIP/AFP*. Similarly, Finola showed increased transcript accumulation for two of the *COR* genes tested (*COR413pm* and *PGIP/AFP*). Interestingly, *TIL* showed very similar trends across all nine varieties, which suggests its transcript abundance does not correlate with cold acclimation capacity and freezing-tolerance in hemp, despite the fact that it plays an important role in freezing-tolerance (Charron *et al.*, 2008; Takahashi *et al.*, 2013). Therefore, cold acclimation in hemp appears to heavily rely on the expression of some *COR* genes. In brief, our data indicate that differences in cold acclimation capacity and acclimated freezing-tolerance in *Cannabis sativa* L. relies on the expression of *COR* genes.

This study also reports global DNA methylation profiles of hemp varieties in response to a CA treatment. DNA methylation is an epigenetic mark that plays an important role in cell differentiation, genome imprinting, development, defence, chromatin activation and gene expression (Ibarra *et al.*, 2012; Zhang *et al.*, 2006). Recent papers suggest that DNA methylation is involved in mechanisms of stress response in plants (Aina *et al.*, 2004; Verhoeven *et al.*, 2010; Wang *et al.*, 2011). Moreover, DNA methylation imparts persistent control over some defence genes in *Arabidopsis* under control conditions and change dynamically to alter gene expression in response to environmental stimuli such as the pathogen *Pseudomonas syringae* (Dowen *et al.*, 2012).

Cannabis sativa L. responds to LT by modulating its global DNA methylation level. While all varieties showed a hypermethylation at some point during CA, the timing of that response varied. At CA14, the time point at which we performed the WPFT, varieties unable to acclimate kept elevated global DNA methylation whereas the varieties with strong cold acclimation responses reduced their global DNA methylation state down to NA levels after an initial hypermethylation event at CA7. Interestingly, it was suggested that a DNA hypermethylation may be viewed as a defense strategy that prevents genomic instability, allowing survival in extreme environment (Kovalchuk *et al.*, 2003). In addition, a DNA hypomethylation caused by cadmium exposure was observed in root tissues of a hemp variety known to be tolerant to heavy metal stress (Aina *et al.*, 2004). Thus, the decrease of global methylation during the CA treatment could indicate better adaptability and hence serve as an indicator of acclimated freezing tolerance.

All four varieties showed locus-specific changes in H3K9ac, H3K27me3 and MeC in response to the CA treatment and at DA. However, the profiles obtained for the two histone

marks conflict with previously reported observations in other plant species. For instance, global H3K9ac levels have been shown to decrease when maize plants are exposed to LT, but to increase at *COR* gene loci, supporting the gene activation role of H3K9ac (Hu *et al.*, 2011). In the present study, we show that H3K9ac levels decrease specifically at *COR* gene loci during CA. However, the two studies are difficultly comparable due to the difference in duration of their respective LT treatment (hour-long as compared to week-long time points). It is thus conceivable that H3K9ac is needed for the initial induction of *COR* gene expression and is no longer required as higher levels of transcripts are consistently produced after one and two weeks of CA treatment. Similarly, we would have expected that the levels of H3K27me3, a mark generally associated with gene silencing, to decrease during CA in order to favour *COR* gene expression. While this seems to be the case for three of the varieties tested, we observed that Yvonne shows an opposite trend with increased levels of H3K27me3 at *COR* gene loci during CA. It is possible that H3K27me3 is deposited for a similar reason that MeC is also deposited: to repress *TIL* and COR413pm that are transcribed in ample amounts at CA14. However, for MeC, the levels go much higher at DA whereas the levels of H3K27me3 return to NA levels. It appears that H3K27me3 is not required for the downregulation of *COR* genes during DA. This process may revolve around a locus-specific increase of MeC to reduce the expression of genes involved in CA. More precisely, the MeC profiles varied greatly among the varieties, but Finola and Yvonne showed a greater deposition of this mark at DA. This observation, along with the distinctive global DNA methylation profiles displayed by these two varieties, supports a more extensive use of DNA methylation in cold acclimation. These results are further substantiated by the marked increased expression of DNA methylation regulators. Using *de novo* DNA methylation, Yvonne may actively remodel its DNA methylation patterns to better respond to

its environment.

Altogether, our results shed light on the molecular mechanisms underlying cold acclimation in *Cannabis sativa* L. and reveal higher levels of complexity regarding how genetic, epigenetic, and environmental factors intertwine. By comparing several varieties that display varying cold acclimation capacities, this integrative study provides more comprehensive information than studies that focus on a single variety to define determinants of tolerance.

3.6 Figure legends

Figure 3.1: Electrolyte leakage assay in exposure to cold. This assay measured the effect of the cold acclimation treatment on the electrolyte leakage of nine varieties of *Cannabis sativa* L. NA, non-acclimated plants grown for 16 days; CA, 14 days old plants cold-acclimated for 7, 14, 21 and 28 days. Error bars show standard error of a minimum of three biological replicates. Letters above bars represents statistical significance ($P < 0.05$); different letters indicate statistically different leakage values for each variety.

Figure 3.2: Whole plant freezing test of nine hemp varieties. This test uncovered contrasting levels of cold acclimation capacity in nine varieties of *Cannabis sativa* L. (A) Plants of nine varieties were grown for 16 days at 20°C (NA) or grown for 14 days at 20°C and transferred at 4°C for 14 days (CA). (B) Survival curves of NA and CA plants from the nine varieties subjected to a freeze test performed at -3°C, -5°C, -7°C, -9°C. Survival count was

recorded after a recovery period of 2 weeks. Error bars show standard error of a minimum of three biological replicates. * $P < 0.05$. (C) CFX-2, CRS-1, Finola and Yvonne NA and CA plants subjected to a freeze test performed at -3°C, -5°C, -7°C, -9°C. The pictures were captured after a recovery period of 2 weeks.

Figure 3.3: SPAD assay and water-soluble sugar quantification. NA, non-acclimated plants grown 16 days; CA, 14 days old plants cold-acclimated for 7 and 14 days; DA, CA14 deacclimated for one day. Error bars show standard error of a minimum of three biological replicates. Letters above bars represents statistical significance ($P < 0.05$); different letters indicate statistically different values for each variety.

Figure 3.4: *COR* gene mRNA abundance in response to cold. This figure depicts changes in relative mRNA abundance of cold-responsive genes *COR413pm*, *COR413tm*, *TIL* and *PGIP/AFP* in response to cold acclimation in nine varieties of *Cannabis sativa* L. Cold-regulated 413 plasma and thylakoid membrane (*COR413pm* and *COR413tm*), Temperature-induced lipocalin (*TIL*), polygalacturonase-inhibitor/anti-freeze (*PGIP/AFP*). NA, non-acclimated plants grown 16 days; CA, 14 days old plants cold-acclimated for 7 and 14 days, DA, CA14 plants deacclimated for one day. Error bars show standard deviation of a minimum of three biological replicates. Letters above bars represents statistical significance ($P < 0.05$); different letters indicate statistically different expression values for each variety.

Figure 3.5: DNA methylation in response to cold: global profiles and regulators. This figure shows global DNA methylation profiles and relative mRNA abundance of genes involved

in RdDM during cold acclimation in four varieties of *Cannabis sativa* L. with contrasting cold acclimation abilities. (A) Relative global DNA methylation. (B) Relative mRNA abundance of *Methyltransferase* 1 (*MET1*), *Chromomethylase* 3 (*CMT3*) and *Domains-rearranged methyltransferase 2* (*DRM2*) in response to cold acclimation. (C) Relative mRNA abundance of genes involved in de novo DNA methylation *RNA-dependant RNA polymerase 2* (*RDR2*), *Dicer-like 3* (*DCL3*), *HUA enhancer 1* (*HEN1*) and *Argonaute 4* (*AGO4*) in response to cold acclimation. NA, non-acclimated plants grown 16 days; CA, 14 days old plants cold-acclimated for 7 and 14 days; DA, CA14 deacclimated for one day. Error bars show standard error (A) and standard deviation (B, C) of a minimum of three biological replicates. Letters above bars represents statistical significance ($P < 0.05$); different letters indicate statistically different values for each variety.

Figure 3.6: Locus-specific histone modifications and DNA methylation in response to cold. This figure outlines modulations in H3K9ac and H3K27me3 and methylcytosine levels at *TIL and COR413pm* loci in four varieties of *Cannabis sativa* L with contrasting cold acclimation capacities. (A) Target regions of *TIL* and *COR413pm* loci used for ChIP-qPCR and MeDIP-qPCR analyses. Respective levels of (B) Histone 3 lysine 9 acetylation (H3K9ac), (C) Histone 3 lysine 27 tri-methylation (H3K27me3), and (D) Cytosine methylation (meC) at *TIL* and *COR413pm* loci at NA, CA7, CA14 and DA. NA, non-acclimated plants grown 16 days; CA, 14 days old plants cold-acclimated for 7 and 14 days; DA, CA14 deacclimated for one day. The CT values were normalised to total histone 3 levels (B, C) or to input DNA (D). Error bars show standard deviation of a minimum of three biological replicates. Letters above bars represents statistical significance ($P < 0.05$); different letters indicate statistically different values for each variety.

3.7 Table and Figures

Table 3.1: *COR* genes and primer sequences

Target mRNA		Primer sequence	Amplicon size	Query accession	Identity (%)
CsCOR413pm	F	AACAGCCGATGATGAGGCAT	116	NP_001189533.1	50
	R	TTGGTTGGAGTTGCCAGGTT			
CsCOR413tm	F	AGCATTCCAGCATTTTACACGA	71	NP_973936.1	75
	R	TGGTGGCAACAATTGAACCT			
CsTIL	F	GATGCTGCAACAAACAGGCT	114	AED96994.1	76
	R	ACCAAGGGCATTTGGTGGAT			
CsPGIP/AFP	F	CTCCACACAAGCACCGGTTA	77	AFW20019.1	65
	R	TGGGAAGATTCCTATGGGTGG			
CsRDR2	F	CCTGCTTGCAGCTCTTCTCT	105	NP_192851.1	51
	R	CCCAAAGGGTCGTGTGCTTA			
CsDCL3	F	ACAACTTTCGGGTCTTCGGT	108	NP_001154663.2	48
	R	GGTCGCTCCAGGACAACATT			
CsHEN1	F	TGGCTCTGGAAGTTTGTTGGA	99	NP_001190782.1	40
	R	GCTGCACGTATAAGACTTTTGT			
CsAGO4	F	GGAGTTGAGGAACAGGTGGA	102	NP_565633.1	79
	R	CTGCACAGGTTGGTCAGTTT			
CsCMT3	F	CGTGTCAAGGAGTAAGCGGT	112	NP_177135.1	31
	R	GGCTTCAAATACTCAATAATGCTCA			
CsMET1	F	CCAGAATGGCCAGAACCGAT	93	NP_199727.1	74
	R	AGTGCTCTTAGCAGCAGCAT			
CsDRM2	F	ACGTGCAACAACTGAACAGC	91	NP_196966.2	66
	R	TTATTGCATGGGCTCCCACC			
Cs18S JF317360.1	F	TATTCTGTTGGCCTTCGGGATC	128	N/A	N/A
	R	TCCTTGGCAAATGCTTTCGC			

Target gene			
CsCOR413pm R1	F	AACAGCCGATGATGAGGCAT	140
	R	TTGGTTGGAGTTGCCAGGTT	
CsCOR413pm R2	F	AGCATTCCAGCATTTTACACGA	116
	R	TGGTGGCAACAATTGAACCT	
CsTIL	F	GATGCTGCAACAAACAGGCT	114
	R	ACCAAGGGCATTTGGTGGAT	

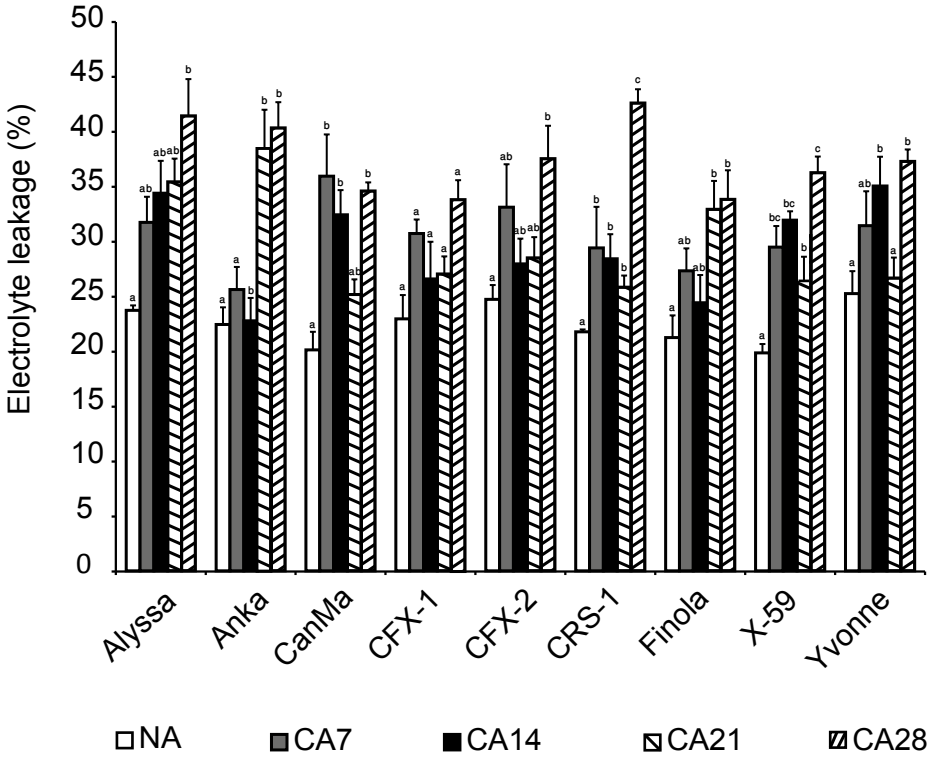
Electrolyte leakage (%)
50
45
40
35
30
25
20
15
10
5
0
Alyssa
Anka
CanMa
CFX-1
CFX-2
CRS-1
Finola
X-59
Yvonne
NA
CA7
CA14
CA21
CA28

Figure 3.1

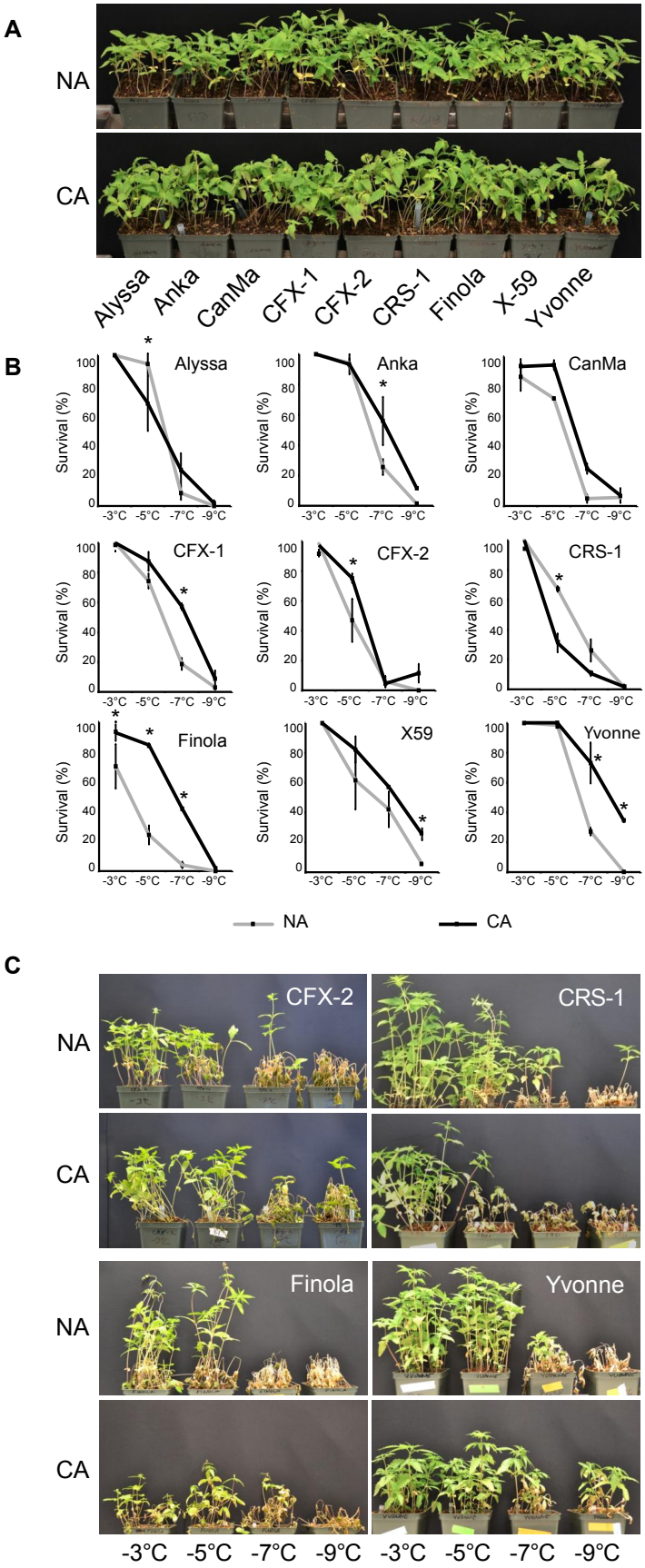
A
NA
CA
Alyssa
Anka
CanMa
CFX-1
CFX-2
CRS-1
Finola
X-59
Yvonne
B
Survival (%)
-3°C -5°C -7°C -9°C
X59
NA
CA
C
-3°C -5°C -7°C -9°C -3°C -5°C -7°C -9°C

Figure 3.2

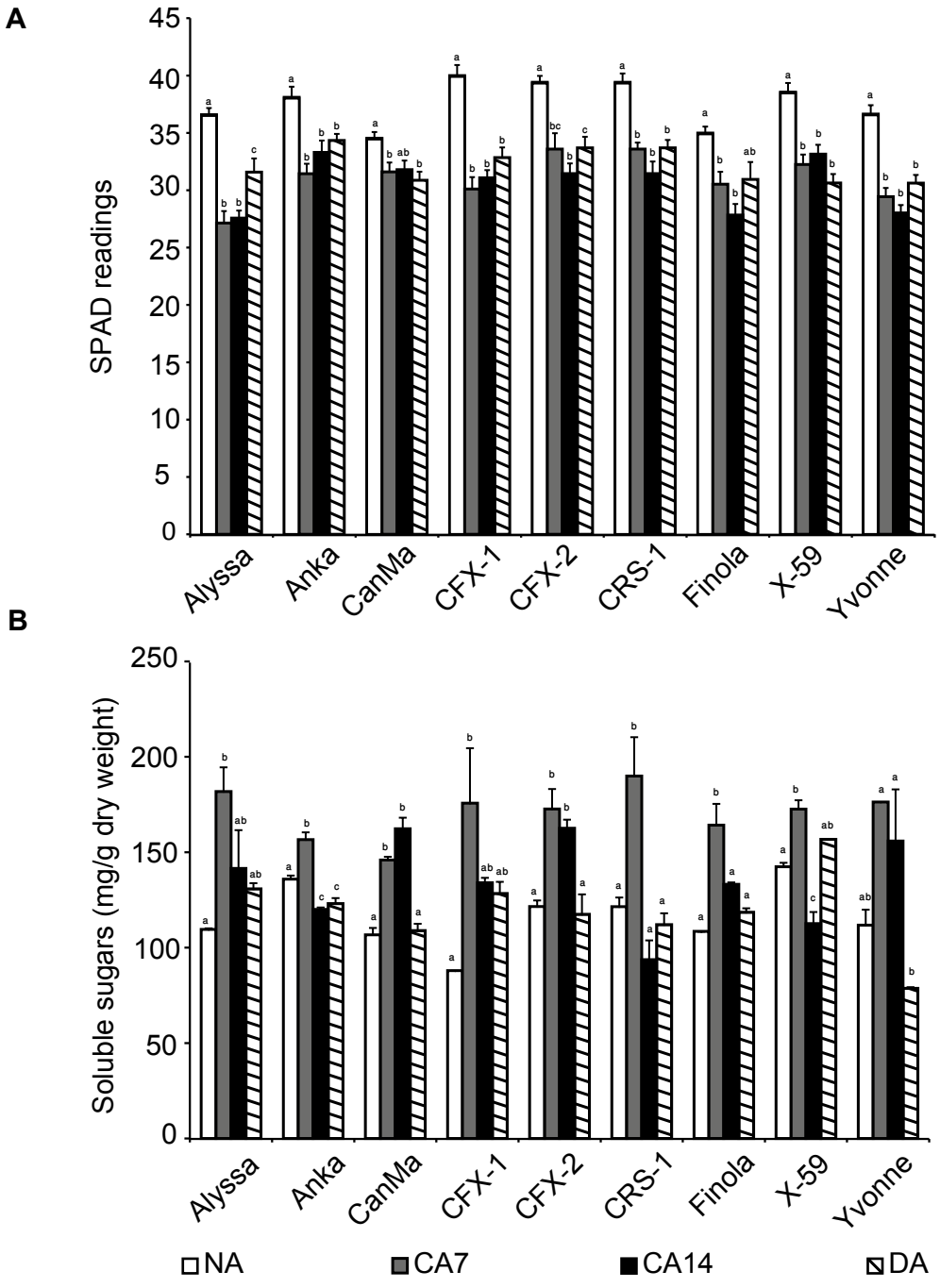
A
SPAD readings
45
40
35
30
25
20
15
10
5
0
Alyssa
Anka
CanMa
CFX-1
CFX-2
CRS-1
Finola
X-59
Yvonne
B
Soluble sugars (mg/g dry weight)
250
200
150
100
50
0
NA
CA7
CA14
DA

Figure 3.3

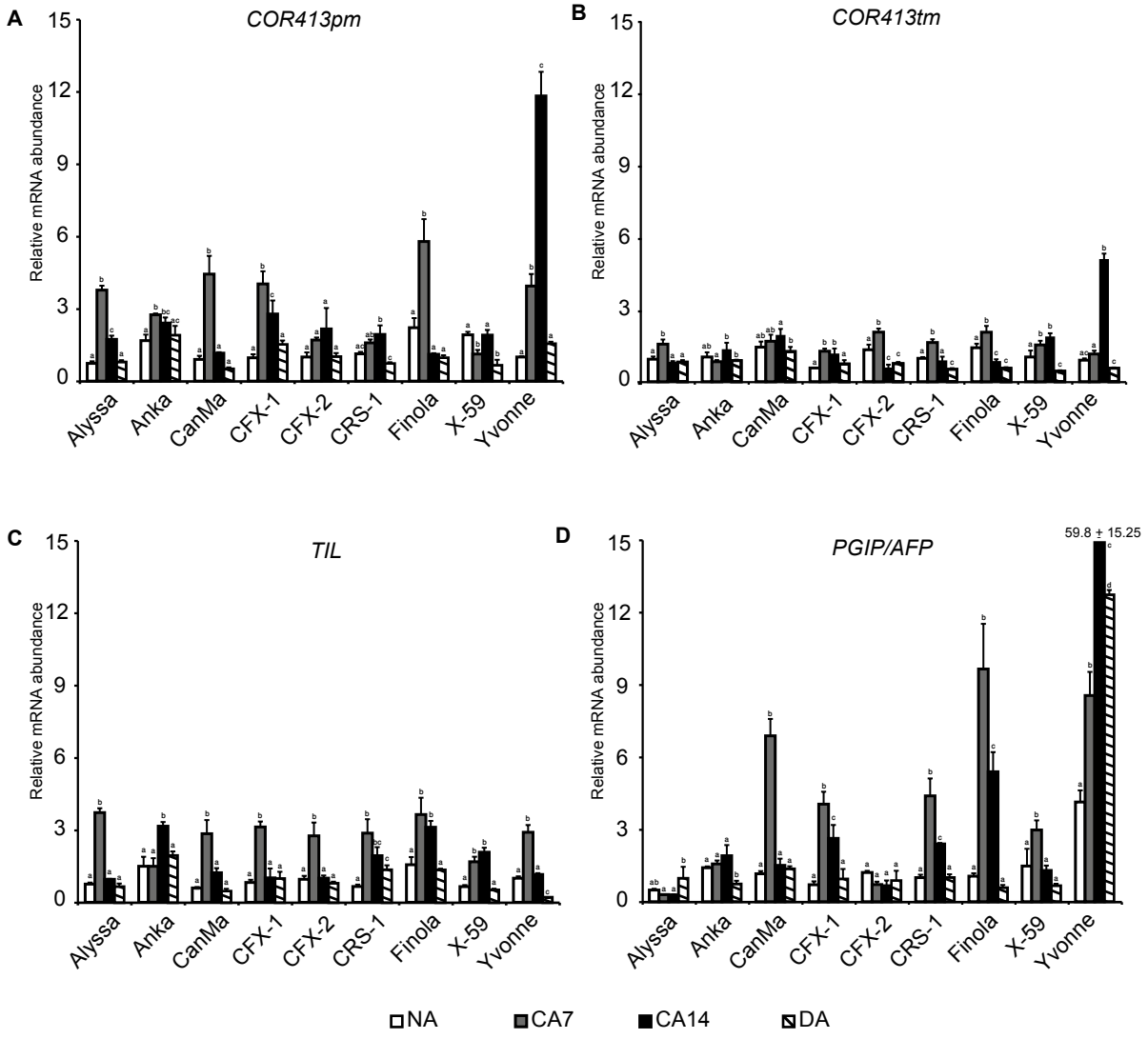
A
COR413pm
B
COR413tm
C
TIL
D
PGIP/AFP
59.8 ± 15.25
Relative mRNA abundance
0
3
6
9
12
15
Alyssa
Anka
CanMa
CFX-1
CFX-2
CRS-1
Finola
X-59
Yvonne
NA
CA7
CA14
DA

Figure 3.4

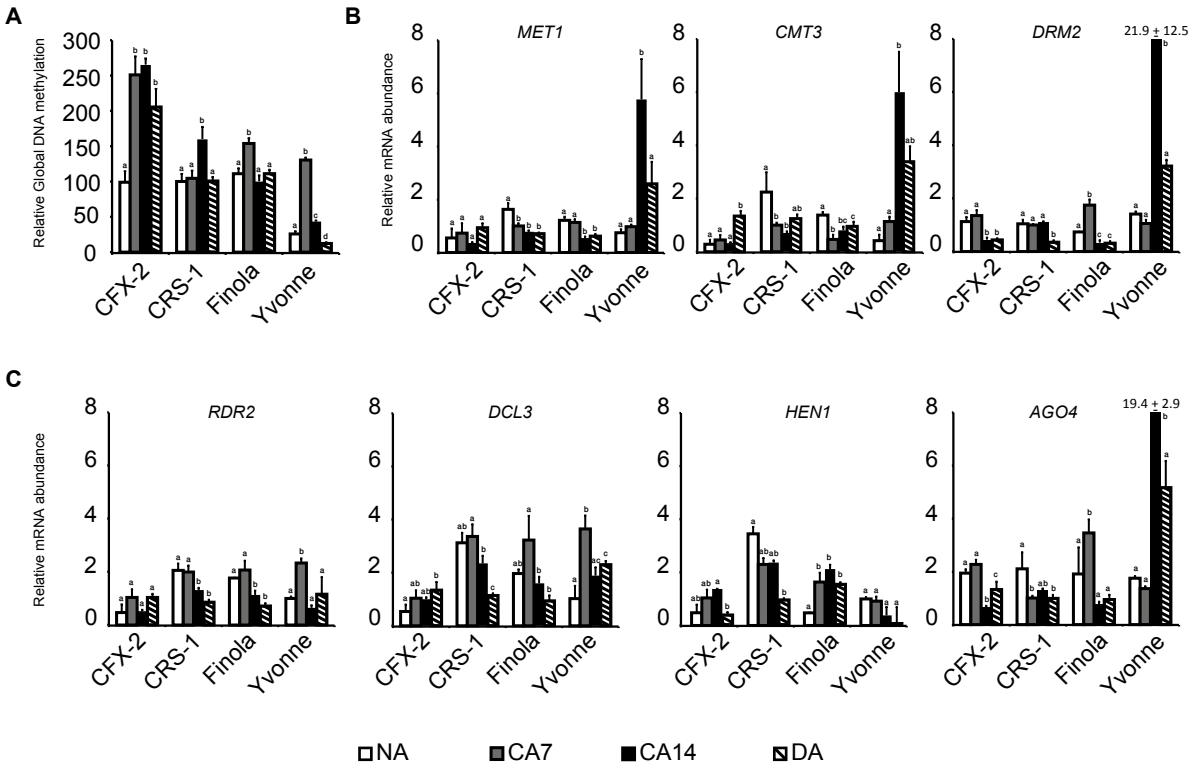
A
Relative Global DNA methylation
300
250
200
150
100
50
0
CFX-2
CRS-1
Finola
Yvonne
B
Relative mRNA abundance
MET1
CMT3
DRM2
21.9 + 12.5
8
6
4
2
0
C
RDR2
DCL3
HEN1
AGO4
19.4 + 2.9
NA
CA7
CA14
DA

Figure 3.5

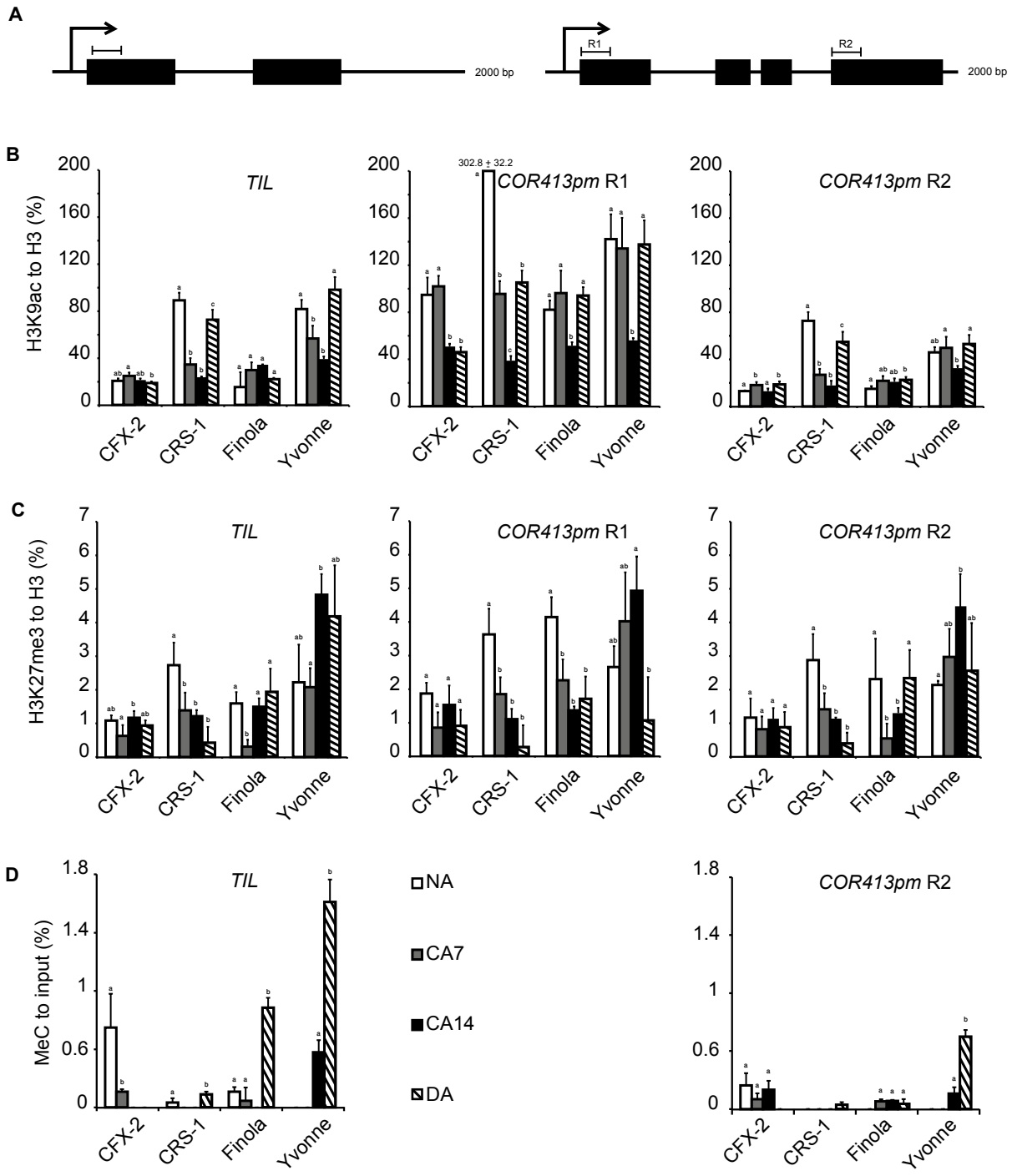
A
2000 bp
R1
R2
2000 bp
B
H3K9ac to H3 (%)
TIL
COR413pm R1
302.8 ± 32.2
COR413pm R2
CFX-2
CRS-1
Finola
Yvonne
C
H3K27me3 to H3 (%)
TIL
COR413pm R1
COR413pm R2
D
MeC to input (%)
TIL
COR413pm R2
NA
CA7
CA14
DA

Figure 3.6

CHAPTER IV: GENERAL CONCLUSION

Cannabis stands as an exceptionally resourceful crop and renewable resource. The rediscovery of *Cannabis* in the 1990's gradually placed this historically important plant in the forefront of agronomical research. Probably the first medicinal plant whose genome got published, it is now possible to answer a growing need to understand the biology of *Cannabis*. Over thirty countries now grow industrial hemp and following the 1998 licensing system, the cultivated surface of industrial hemp increased by 10 fold between 2003 and 2013 in Canada (over 26000 hectares in 2013) (CHTA, 2014). This study provided insight into the mechanisms of cold-response in hemp and is a stepping-stone for the sustainable development of *Cannabis* culture in Canada and other northern countries.

Cannabis sativa L. cold acclimates by heavily relying on the expression of cold-responsive genes. Answering the first objective, we classified nine hemp varieties according to their cold acclimation capacity and freezing tolerance into three categories: sensitive, moderately tolerant and tolerant. Indeed, the whole plant freezing test uncovered differences in cold acclimation capacities with varieties that were able to greatly increase their survival to freezing while others were damaged by an exposure to low temperature. The varieties responded to cold with very similar photosynthesis rate profiles and soluble sugars accumulation, although the accumulation of soluble sugar seems to negatively correlate with sensitivity. Thus, the difference of cold acclimation capacity we observed appeared partly relies on photosynthesis and the accumulation of soluble sugars. For the second objective, we successfully identified cold-responsive (*COR)* genes in hemp. Interestingly, the transcript abundance of *COR* genes accounted for the differences in cold acclimation capacity. The varieties that acclimated the most

efficiently accumulated *COR* gene transcripts to higher levels compared to varieties with less efficient mechanisms. Therefore, superior cold acclimation in hemp translates into a higher expression of *COR* genes.

Chromatin dynamics are involved in cold acclimation mechanisms of *Cannabis sativa* L.. In a third objective, we observed that low temperature induced epigenetic responses in all varieties regardless of their cold acclimation capacity. These changes were observed globally at the genomic DNA methylation levels and specifically on *COR* genes with two histone modifications and DNA methylation. Nevertheless, the varieties that could cold acclimate showed a greater range of epigenetic changes, with higher fold-differences between the highest and lowest levels. All varieties hypermethylated their genome in response to low temperature but only the varieties that could cold acclimate responded with a subsequent hypomethylation during the low temperature treatment. This decrease in DNA methylation levels measured during the stress may suggestively reflect an adaptation to the conditions – in this case an epigenetic reorganisation that leads to cold acclimation – especially because this change was absent in the cold-sensitive varieties. Moreover, in the most tolerant variety, we observed an upregulation of methyltransferases and genes involved in the RNA-directed DNA methylation pathway during the low temperature treatment, which coincided with a genomic hypomethylation. These two observations support the presence of an epigenetic component in cold acclimation in hemp. Overall, the varieties that were able to cold acclimate arguably use epigenetics to a greater extent than the other varieties, suggesting a more refined or regulated response network.

In brief, we evidenced different cold acclimation capacities in nine varieties of hemp that involve the expression of *COR* genes and implicate epigenetic modifications.

Altogether, this study is the first to report cold acclimation capacity and freezing tolerance differences in *Cannabis sativa* L. at the physiological, metabolic and molecular levels. Our results shed light on the molecular mechanisms underlying cold acclimation in *Cannabis sativa* L. and reveal higher levels of complexity regarding how genetic, epigenetic, and environmental factors intertwine. By comparing several varieties that display varying capacities of cold acclimation, this integrative study provided more comprehensive information than studies focusing on a single variety to define determinants of tolerance.

Future directions on this work should investigate the genetic, epigenetic and mechanistic differences we observed in these varieties of hemp. One could characterise of the genetic differences between the varieties that contrast in their cold acclimation capacity, which would hopefully highlight epigenetic actors/chromatin modifiers that are at the base of the stronger epigenetic response we observed in the successful cold acclimating varieties. Moreover, it is almost certain that other histone modifications are involved in the regulation of *COR* genes in hemp, and their identification may reveal a specialised set involved in cold acclimation. Also, the *COR* gene cascade can be further investigated in *Cannabis sativa* L., starting with the identification of the *CBF/DREB* transcription factors. Such work may involve sequencing techniques like RNA sequencing and ChIP sequencing.

Suggestively, future work should investigate further mechanisms of stress response in hemp, as this crop can grow in a wide range of climates. Studies of this type are useful because they help determine the most suitable varieties for specific sets of conditions, which provides direct valuable information for growers. Moreover, the hardy nature of the plant suggests that *Cannabis* possesses numerous adaptation abilities and stress-responsive mechanisms which can serve as a suitable organism in fundamental research, especially by contributing to the

current understanding of stress-response mechanisms in plant. *Cannabis* is very useful as a crop, but could also prove itself as a useful model organism and contribute to the development of solutions to the challenges the agricultural industry is currently facing.

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