GENOMIC AND EPIGENOMIC CHANGES IN TRANSGENERATIONAL RESPONSE TO COLD STRESS IN *ARABIDOPSIS THALIANA*

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DEDICATION

This dissertation is dedicated to the almighty Allah (subhanahu wa ta'ala). Furthermore, to all the people, friends, and family, who have helped me in my pursuits, especially—my parents, Md. Abdur Rahman and Aleya Sharmin, who taught me to be a better person, and my brother, Md. Ashikur Rahman Shamim, as a source of my inspiration, and to my grandparents. Finally, I also would like to dedicate this thesis to my supervisor, Dr. Igor Kovalchuk, for his continued care, support, guidance, and believe in me; without him, this endeavour would not have been possible.

ABSTRACT

Plants are continuously exposed to various environmental stresses. They employ numerous strategies of resistance and develop a memory of stress exposure for future generations. Abiotic stress, like cold, can prompt the changes in phenotype, genotype, and epigenotype of plants. Plants can establish these as somatic and transgenerational memories. We studied the *Arabidopsis thaliana* plants exposed to multiple generations of cold stress. We hypothesized that the progeny of plants exposed to 25 generations to cold stress would be genetically and epigenetically more diverse than the parental plants. Our study reveals that multigenerational exposure to cold stress resulted in the physiological changes, as well as changes in the genomic and epigenomic (DNA methylation) patterns across generations. The main changes in the progeny were due to the high frequency of genetic mutations rather than epigenetic changes. Our work supports the existence of transgenerational stress response in plants and demonstrates that genetic changes prevail.

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LIST OF ABBREVIATIONS

15d8 recombination reporter gene
ANOVA Analysis of variance
C control lines
Cd cold-stressed lines
CHG cytosines at cytosine- any nucleotide-guanine trinucleotide
CHH cytosines at cytosine- any nucleotide-any nucleotide trinucleotide
Col-0 *Arabidopsis thaliana* Columbia ecotype
CpG cytosine and guanine separated by a phosphate
CTAB cetyl trimethylammonium bromide
DEL deletions
DMCs differentially methylated cytosine
DMRs differentially methylated regions
DNA deoxyribonucleic acid
dpg days post germination
F25C samples of Generation 25 grown in non-stressed conditions
F25Cd samples of Generation 25 grown in cold stressed conditions
F2C samples of Generation 2 grown in non-stressed conditions
GATK Genome Analysis Toolkit
gDNA genomic DNA
GO gene ontology
INDELs insertions or deletions
INS insertions
LUC luciferase recombination reporter gene carrying a copy of a direct repeat of the luciferase recombination transgene.
miRNAs micro RNAs
mRNA messenger RNA
MS Microsoft
nt nucleotides
PCR polymerase chain reaction
RdRP RNA-dependent RNA polymerase ribosomal DNA (rDNA).
RNA ribonucleic acid
RNAi RNA Interference
SE standard error
siRNAs small interfering RNA
SnpEff program for annotated variants and coding effects
SNPs single nucleotide polymorphism
sRNAs small RNAs
TAIR10 The *Arabidopsis* Information Resource 10
TEs Transposable Elements
UVC ultraviolet-C
WGBS whole genome bisulfite sequencing
WGS whole genome sequencing

CHAPTER 1: INTRODUCTION

Environmental factors actively influence the growth as well as the reproductive and biological fate of plants. Plants must endure environmental stresses during most of the time of their life cycle because they cannot escape them. In broad, stresses that plants endure can be categorized primarily into two sections—biotic and abiotic. Biotic stresses are connected to biological entities like bacteria, viruses, fungi, abiotic stresses include temperature extremes, water scarcity and salinity, among other factors. In response to stress, plants exhibit physiological, cellular, molecular and morphological changes for the sake of their survival. More importantly, plants develop the ability to adapt or tolerate those changes due to stress (Lämke and Bäurle 2017). Since plants are frequently exposed to various environmental stresses, the molecular- and cellular-level changes in the physiology and morphology are observed in plants grown under stressed conditions, but they seem to disappear particularly when stress conditions are no longer present (Rejeb, Pastor et al. 2014). Several studies suggest that environmental stresses may lead to an increase in the genomic diversity in the plants' progeny, even in the untreated generations, and they potentially result in the adaptation to adverse conditions (Molinier, Ries et al. 2006).

Even though stresses can trigger massive changes in the development of plants, they can show certain degrees of tolerance to stress. As a result, plants develop a complex system to identify and respond to different stresses to minimize the damage. At the same time, they can maintain their cellular and biological resources for growth and reproduction (Atkinson and Urwin 2012). Several studies also suggest that abiotic stresses can lead to an increase

in homologous recombination frequency and point mutation frequency (Yao and Kovalchuk 2011). Sometimes those changes are heritable. For instance, it has been demonstrated that exposure of *Arabidopsis thaliana* plants to ultraviolet-C (UVC) increases the homologous recombination frequency in the progeny at least for two consecutive non-stressed generations (Molinier, Ries et al. 2006). Such capability of plants to pass the memory from their previous experience to their progeny is called the transgenerational response (Pecinka, Rosa et al. 2009). Abiotic stresses can trigger changes in methylation patterns, genomic stability and stress tolerance. For instance, in the progeny of salt-stressed plants, changes have been reported in the genome stability, DNA methylation, histone modifications, and gene expression (Bilichak, Ilnystkyy et al. 2012).

The phenotypic plasticity and adaptability help plants in shaping their morphology under different environmental conditions and thus maintains the relative fitness of plants. Moreover, several reports found that the previous experience in the past environmental conditions can be passed on and reflected in the progeny for several generations (Latzel, Janeček et al. 2014, Lampei, Metz et al. 2017). Transgenerational effects can refer to the passing of responses to chemicals and/or pathogens from parents to offspring (T. E. Huxman, T. N. Charlet et al. 2001). Several studies suggest that the preprogramming of phenotypes of the offspring can be inherited through epigenetic mechanisms (Thellier and Lüttge 2013, Müller-Xing, Xing et al. 2014). The environmentally induced and inherited epigenetic marks can facilitate plants' adaptation to the changing environments. It can cause short-term microevolution in clonal plants (Latzel, Janeček et al. 2014, Verhoeven and Preite 2014, Dodd and Douhovnikoff 2016).

Plants experience many environmental stresses (e.g., drought, salinity, extreme temperatures) for more than once in their lifespan. Undesirable conditions could induce morphological, physiological and molecular changes of plants and adversely affect plant growth. In response to the recurring stresses, plants often show improved stress resistance or enhanced adaptation. However, most of the environmentally induced memories are relatively short and can exist only as somatic memories. Only a few memories can be transmitted to the subsequent generations as transgenerational memories (Avramova, 2015; Kinoshita & Seki, 2014; Martinez-Medina et al., 2016; Sani, Herzyk, Perrella, Colot, & Amtmann, 2013). However, now it has been well reported that a transgenerational memory can play a role in generating epigenetic variants that can allow plants to exhibit a certain degree of tolerance to the environmental stresses and consequently lead to adaptation, microevolution and potentially speciation (Lämke & Bäurle, 2017; Rasmann et al., 2012).

Epigenetic mechanisms in plants primarily consist of DNA modifications, e.g., DNA methylation, small non-coding RNAs (regulating gene expression) and chromatin structures such as histone modification. DNA methylation is environmentally inducible and, in many cases, inheritable (Lämke and Bäurle 2017). However, due to the reprogramming of the environmentally induced epigenetic marks in meiosis, in most of the cases, epigenetic modifications are maintained within generations and infrequently passed onto the sexually derived offspring (Heard and Martienssen 2014, Tricker 2015). In terms of studies of the epigenetic inheritance, the majority of studies focus on DNA methylation. DNA methylation occurs in CG, CHG and CHH contexts in plants where H represents the

nucleotides A, T or C. CHH methylation is relatively unstable since it is asymmetrical and can only be maintained via the guidance of non-coding RNAs, such as small interfering RNAs (siRNAs) (Law & Jacobsen, 2010). In plants, epigenetic regulation such as DNA methylation is meiotically stable and can be transmitted either through maintenance methyl transferases at symmetrical cytosines (CG and CHG) or at asymmetrical CHH via small RNAs that can further guide in the re-establishment of DNA methylation patterns to their progeny acquired from the parents (Bond & Baulcombe, 2014; M. Iwasaki & J. Paszkowski, 2014b). For the successful transmission of the transgenerational information to progeny, epigenetic marks need to be transmitted by the settings of genome reprogramming during gametogenesis and zygote development (Bond and Baulcombe 2014, Iwasaki and Paszkowski 2014). In plants, epigenetic regulation such as DNA methylation is meiotically stable and can be transmitted via small RNAs that can further guide the system to the re-establishment of DNA methylation patterns to their progeny acquired from the parents (Bond and Baulcombe 2014, Iwasaki and Paszkowski 2014). Even though recent studies suggested some estimated rates of transgenerational stability in DNA methylation modifications (Becker, Hagmann et al. 2011), it is still unclear what extent of stress exposure can trigger the stable methylation patterns, how many generations of DNA methylation persist, and what subsequent level of epigenetic persistence is required for adaptive processes by epigenetic regulation (Rapp and Wendel 2005, Herman and Sultan 2011, Herman, Spencer et al. 2013).

Since abiotic stress, such as cold, could play a crucial role in the changes of the phenotypic, genetic, and epigenetic diversity of plants, it has been planned to explore this further.

Although plants exposed to cold stress exhibit changes in the transposon expression and recombination frequency (Migicovsky and Kovalchuk 2015) data gathered during experiments on the prolonged exposure to cold stress are yet to be analyzed, especially data on the investigation of multigenerational genetic variations. A widely considered model plant, *Arabidopsis thaliana*, had been selected for this study. For instance, in response to heat stress, changes in the phenotypes and epigenotypes have already been reported in *Arabidopsis thaliana* (Migicovsky, Yao et al. 2014). Therefore, there arises a great interest in examining the evidence of response to cold stress in this model plant by studying its phenotypic profiling, genetic diversity, and epigenetic traits across multiple generations. Therefore, we anticipate elucidating further the phenotypic, genomic, and epigenomic differences in response to cold stress across multiple generations in the progeny of stressed plant in comparison with the progeny of control plants.

It has been hypothesized that the progeny of plants exposed to cold stress across 25 generations would be genetically and epigenetically more diverse than the parental plants. Moreover, epigenetic diversity would be associated with the pathways and stress-specific features of epigenetic inheritance (DNA methylation) linked to cold stress.

Greater understanding is needed of whether microevolution occurs at the genomic and epigenomic level. Moreover, concerning changes in the epigenome, it has been planned to investigate whether epigenomic microevolution is more prevalent than genomic diversity. For this purpose, experiments for studying genomic and epigenomic variations in-between generation 2 and generation 25 have been developed by using computation techniques and

data obtained by whole-genome sequencing (WGS) and whole-genome bisulfite sequencing (WGBS) for the methylome. Further research is required to recognize the location and direction of mutations and the occurrence of epimutations and determine whether they are random or non-random. Differentially Methylated Regions (DMRs) and Differentially Methylated Cytosines (DMCs) have been investigated both in 100 base pairs and 1000 base pairs windows to find out whether those DRMs and DMCs are associated with cold stress-related genes. Finally, the bioinformatics-based analysis has been made to determine the biological functions associated with changes in DNA methylation.

The following questions are planned to be answered:

Are the progenies of the plants that had the previous experience of being exposed to stress genetically and epigenetically more diverse? Are these changes random or non-random in nature? If genetic changes occur in the progeny of plants in response to cold stress, are there any patterns associated with the genomic and epigenomic changes and pathways involved in the stress response? The patterns associated with the epigenetic changes and pathways are considered to be investigated.

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CHAPTER 2: REVIEW OF LITERATURE

2.1 Introduction

Faithful transmission of the phenotypic traits of an organism from one generation to another is a crucial part of life. Inheritable traits of an organism are primarily mediated by copying and transmitting the genomic DNA. In addition to the genetic information of an organism, the epigenetic information is equally essential for determining cell fate (Gurdon 2006). As for multicellular organisms, in most of the cases, many traits can be inherited during the mitotic cell division. Let us consider a classic example in mammals where it has been demonstrated that one out of the two X chromosomes is randomly chosen by cells in the early embryo for the silencing activity, and the X chromosome remains inactive in females (Lyon 1961). Cells can remember this choice in the subsequent cell divisions as somatic memory that is responsible for large fur colour patches in calico cats. In the germline, most of the cell's information is erased or reprogrammed in multicellular organisms because all the distinct cell types are re-established in each organismal generation. However, in more than a decade of study, many special cases revealed the phenomena of transgenerational epigenetic inheritance from one organismal generation to the next: by transmitting the germline epigenetic information (epigenome) from one generation to another.

2.2 The Concept of Transgenerational Memory

The term “transgenerational inheritance” denotes the transmission of information from one generation to at least next two generations of offspring, which implies multiple generations of inheritance without re-exposure to stress at every generation. On the other hand, “intergenerational inheritance” refers to the inheritance affecting only the next generation. The concept of the establishment of heritable traits induced by the environmental conditions is often referred to as soft inheritance (Holliday 2006, Dickins and Rahman 2012). Several studies tried to decipher the molecular mechanisms behind the transgenerational inheritance in plants (Boyko, Blevins et al. 2010, Bilichak, Ilnytskyy et al. 2015). Now it has been well established that if the phenotypic characteristics are transferred to the offspring without any intervention of the gene sequences, the underlying mechanism is likely epigenetic in nature. There are three types of mechanisms well considered for the epigenetic gene regulation: DNA methylation, the expression of small RNAs and histone modifications. In the dynamic response to environmental stresses, plants exhibit both immediate and delayed responses at the somatic level of an individual plant (Leyva-Pérez, Valverde-Corredor et al. 2015). To fight back properly, plants may acquire a certain memory of stress exposures for further encounters to similar and dissimilar stresses, and responses are applicable for both the abiotic and biotic stress response (Kachroo and Robin 2013, Zhang, Lv et al. 2018). The memory acquired from the stress exposure can be passed onto the progeny. The acquired memory of stress exposure and successful passing it onto the progeny is often referred to as transgenerational inheritance or transgenerational memory when conceptually, an organism can respond and remember

the environmental conditions at the molecular level and may pass the memory of alterations in the phenotypes of the subsequent progenies. (Tricker 2015).

2.3 Physiological Responses in Plants

2.3.1 Embryogenesis

Embryogenesis is a critical stage of the life cycle of a flowering plant; it begins with the formation of a single cell zygote resulting from the fusion of the male and female gamete. Flowering plants (angiosperms) exist primarily as sporophytes. In angiosperms, such as *Arabidopsis thaliana*, the female gametophyte develops in the ovule. It is originated from a single haploid spore consisting of seven cells that are generated mitotically, including the haploid egg cell and the diploid cell. In *Arabidopsis*, all embryonic developmental stages are named by the shape of the embryo at that stage. The embryonic developmental stages of *Arabidopsis* can be categorized into several stages, such as mature, bent, linear, heart and globular stages. Moreover, development of endosperms also occurs in several distinct stages (Berger 1999, Li and Berger 2012). When embryo matures, the endosperm is gradually depleted; only a single layer of endosperm cells remains because when an *Arabidopsis thaliana* seed matures, the embryo occupies nearly the whole space of it (Berger 1999).

2.3.2 Control of Multicellular Gene Expression

Most cells are genetically identical in multicellular organisms, even though there are substantial phenotypic variations that exist between and among cells which eventually formulate very different forms and functions. Therefore, the question is, how can a single

DNA sequence show such verities? It is well established that DNA sequences are organized and compacted with the protein scaffold which can be marked with different modifications that sometimes determine the function of the cell's DNA without changing the original DNA sequence. These marks can concert with the cellular pieces of machinery and proteins to effectively turn on or off gene expression. These marks are known as epigenetic marks which determine the cellular forms and functions without changing the actual DNA sequence. Another important genetic material is Transposable Elements (TEs). TEs are important pieces of moveable genetic information. They can shape the genome architecture and phenotypic differences by moving genetic information/elements (Vicient and Casacuberta 2017)**.** As such, TEs could be a source of genetic variations (McClintock 1984). The existence of TEs affects various biological processes (Chuong, Elde et al. 2017)**.** Although TEs insertions might have adaptive advantages, transpositions could be highly mutagenic. TEs could be regulated by the epigenetic mechanisms, such as epigenetic silencing. Mobilization of TEs and changes of the epigenetic landscapes altogether could allow the species in rapid phenotypic adaptations to the environmental changes (Lerat, Casacuberta et al. 2019).

2.3.3. Control of Gene Expression in Plants

Like other multicellular organisms, plants must retain the genomic stability in every stage of their development. The genomic DNA of an organism is always threatened by a constant pressure of internal and external factors. Also, cellular processes like photosynthesis and respiration consistently challenge the plant genome. Free radicals produced by internal stresses that can cause damage of the DNA can directly pose additional challenges in

maintaining the genomic stability. Since plants are sedentary organisms, they are sometimes vulnerable to challenging environmental conditions. Chemical and physical stresses from the external environment factors such as temperature fluctuations, light intensity, wind, nutrients, water availability as well as various biotic stresses produced by pathogens can have a significant impact on the genomic expression and stability of plants. To fight against stresses and for better survival rates, plants usually display responses that are controlled by genetic and epigenetic mechanisms. More importantly, plants can develop mechanisms of tolerance and resistance (Boyko and Kovalchuk 2011) to cope with stresses and develop new adaptive mechanisms. Plants can maintain genomic stability in the ever-challenging growth environment (Dassler, Roscher et al. 2008) because they contain additional copies of various DNA repair genes in their genome that can have redundant functions (Singh, Roy et al. 2010). The genome integrity is controlled by different mechanisms which include utilizing different DNA repair pathways and a proper maintenance of the nuclear and chromatin architecture. DNA damage repair mechanisms can be maintained at several levels, including scanning and identification of DNA damage, DNA damage repair by synthesizing and proofreading the newly added DNA sequences and by relaxing the chromatin structure globally or locally. Moreover, different or similar epigenetic factors like DNA methylation and histone modifications can have an impact on the DNA damage repair mechanisms. The genome stability is regulated by a different chromatin compaction when chromosomal regions can be relaxed through numerous epigenetic modifications and by choosing different DNA repair pathways (Downey and Durocher 2006).

2.4 Epigenetic Mechanisms of Gene Expression

Epigenetic mechanisms of gene expression are broadly mediated by three distinct mechanisms: DNA methylation, chromatin architecture (histone modifications), and utilizing small non-coding RNAs.

2.4.1 DNA Methylation

Methylation of cytosine at position 5 is central to many epigenetic mechanisms and inheritance. The copying mechanisms in cytosine methylation patterns are conceptually simple for the epigenetic inheritance. Heritable cytosine methylation mostly occurs in the framework of symmetric CpG dinucleotide, where replication results in two daughter genomes each carrying hemi methylated CpG which eventually gives a substrate for the proper maintenance of methylation by methyltransferase, e.g., MET1 in *Arabidopsis*. It is also common for plants to methylate cytosines in the sequence context of CHG and CHH, but mechanisms that are generally needed for the ongoing reestablishment of epigenetic modifications are mostly guided by small RNAs or heterochromatin-directed methylation pathways (Feng, Jacobsen et al. 2010, Stroud, Greenberg et al. 2013). Inherited CpG methylation patterns can be stable in dividing mammalian cells, but they are largely erased from one generation to the next organismal generation. However, in plants, DNA methylation epialleles can be transmitted over hundreds of generations (Paszkowski and Grossniklaus 2011, Quadrana and Colot 2016). Additionally, recent studies reported that modifications in the adenine 6-methylation could be a potential carrier of the epigenetic memory (Luo, Blanco et al. 2015).

2.4.2 Histone Modifications

In eukaryotic genomes, chromatin architecture is primarily composed of nucleosomes consisting of DNA wrapped around a histone octamer. The mechanisms of replication of chromatin states are vaguely understood and remain subject to ongoing research (Kaufman and Rando 2010, Alabert and Groth 2012). Parental histones are disbursed to both daughter chromosomes in replication. They are retained within 400 base pairs downstream or upstream of the locus from which they were evicted. At a small number of genomic loci, the newly synthesized histones fill in the gaps between parental nucleosomes. These histones eventually establish the covalent modification state of the previous old nucleosomes. The central concept behind this mechanism is that many modifying enzymes bind to the very modifications which they catalyse (Campos, Stafford et al. 2014). Most modifications that occur in response to an environmental stimulus are rapidly erased or diluted when the inciting stimulus is removed. (Coleman and Struhl 2017, Wang and Moazed 2017). But several studies show that chromatin states often need the production of local RNAs that might either recruit or activate chromatin regulators (Huang, Fejes Toth et al. 2017).

2.4.3 Small RNAs

There are different kinds of small non-coding RNAs including microRNAs, tRNAs, rRNAs, snoRNAs and other that differ in both biogenesis and the mechanism of action (Ghildiyal and Zamore 2009, Heard and Martienssen 2014, Holoch and Moazed 2015). Small RNAs, such as small interfering RNAs (siRNAs), are fundamental for the mechanisms of establishing the transgenerational epigenetic memory. In the mediated

mechanisms, RNA levels are maintained by the involvement of an RNA-dependent RNA polymerase (RdRP), where small RNAs are a key to copying the host transcripts from which, consequently, various secondary RNA species are produced (Guérin, Palladino et al. 2014). Overall, an epigenetic inheritance paradigm usually depends on the interplay between two or more various mechanisms in the epigenetic pathways. Small RNAs, for instance, can direct the de novo cytosine methylation at the homologous genomic loci in both plants and mammals (Zilberman, Cao et al. 2003, Aravin, Sachidanandam et al. 2008). Likewise, small RNAs also can direct the formation of H3K9-based heterochromatin (Volpe, Kidner et al. 2002). In this case, long non-coding RNAs facilitate a complex mechanism of the recruitment and modulation of H3K4/Trithorax and H3K27/Polycomb chromatin pathways (Rinn and Chang 2012). As a result, DNA modifications and heterochromatin jointly can affect the expression of small RNA-generating loci.

2.5 Stress and the Physiological Response to Stress

During their dynamic life cycle, plants face different environmental factors that are broadly grouped into biotic and abiotic stresses. Different types of stresses can have a powerful impact on the growth, reproduction, and yield potentials of plants. Abiotic stresses relate to the non-living stresses and include light intensity, temperature fluctuations, availability of water, heat, and salinity. In contrast, biotic stresses refer to biological entities like bacteria, viruses, or fungi. For successful reproduction, plants respond to stresses by switching from the normal developmental program to the stress-response program. Response to stresses could be tissue and organ-specific and can be contingent on the developmental stage of plants (Gray and Brady 2016). Even though normal development

of plants can be affected by stresses that can radically disturb their healthy development, plants can also exhibit certain degrees of tolerance to stresses. By a very long evolutionary history, plants developed a complex network in their genome to identify stresses and respond to them by minimizing damages while preserving resources for their growth and reproduction (Atkinson and Urwin 2012). Thousands of years of evolution enabled plants to shape their genome to make a robust defence system against biotic stresses and to develop mechanisms of tolerance against abiotic stresses (Alvarez, Nota et al. 2010). In response to stresses, in most of the cases, plants either change their gene expression so that they can produce proteins that can support them to cope with stresses or they change the molecular reconfiguration of the genome (also known as genome reprogramming) which directs the expression pattern. Genome reprogramming takes place at the transcriptional and post-transcriptional levels during the natural development or under certain degrees of stress (Arnholdt-Schmitt 2004).

2.5.1 Plant Response to Cold Stress

In nature, environmental stresses are commonly observed as combined. Periodically, cold stress can be a significant factor in the determination of crop production as one of the responsible agents that causes crop yield losses. Plant exposure to low temperatures triggers the phenotypic responses like poor germination rates, wilting, the reduction in organ expansion, and impaired reproductive development (Hussain, Hussain et al. 2018). Cold stress in *Arabidopsis* can be persistent in low temperatures or low temperature oscillation, both having an immense impact on the regulation of the plant's healthy development and fitness. Similarly, in rice (*Oryza sativa*), cold-priming can prevent the

cold-induced impaired water uptake in roots, colour bleaching, and leaf wilting (Ahamed, Murai-Hatano et al. 2012). At the same time, cold-priming can lead to a certain degree of tolerance to subsequent exposure to a similar stress or other stresses (Vyse, Pagter et al. 2019).

2.5.2 Epigenetic Modifications Induced by Stress

Understanding how epigenetic factors can influence the cell identity and gene expression during gametogenesis and cell development, and how it is transmitted and affect the offspring is very important for answering a wide range of biological questions. In recent years, several stress-induced epigenetic changes have been demonstrated. Over the past decades, considerable achievements have been observed in the development of cutting-edge technologies for studying the differentially expressed genes at the transcription level (e.g., high-throughput RNA and sRNA sequencing). These technologies are also necessary for studying the complexity of the regulation of histone modifications and whole-genome cytosine methylation profiles (Castellano, Martinez et al. 2016, Wibowo, Becker et al. 2016). Several biotic stresses like bacteria (Li, Mukherjee et al. 2015) and viroid infections (Martinez, Castellano et al. 2014), and abiotic stresses like dehydration (van Dijk, Ding et al. 2010) and salinity (Chen, Luo et al. 2010) can induce and cause the relaxation of the epigenetic control of transposable elements (TEs) or ribosomal DNA (rDNA). Since the epigenetic control over repetitive regions like TEs in the genome has been reported as an extra layer of gene expression control (Lisch 2013), TEs near the regulatory parts of the gene can influence the epigenetic regulation at the transcriptional level (Wang, Weigel et al. 2013).

Likewise**,** abiotic stresses can lead to changes in the epigenetic regulation; for example, drought can induce changes in the genome at the methylation levels which are strongly correlated with responses of the transcriptome. Another well-studied abiotic stress is heat which also induces changes in the methylome of the genome. Heat stress also causes the reactivation of ONSEN retrotransposons in *Arabidopsis thaliana* (Ito, Gaubert et al. 2011). Recently, a study of 1001 *Arabidopsis thaliana* epigenomes has shown changes at the methylation level that are robustly correlated with the environmental response and expression of defence genes (Kawakatsu, Huang et al. 2016).

2.6 Epigenetic Mechanisms of Transgenerational Memory

It has been identified that epialleles in plants could be stable for hundreds of years. The peloria mutant of *Linaria vulgaris* identified by Linneus may be an excellent example of this. Due to changes in the promoter of the flower morphogenesis gene, flowers of this mutant are symmetrical compared to wild-type flowers that are dorsoventrally asymmetrical (Cubas, Vincent et al. 1999). Transgenerational epigenetic inheritance and stress memory are meiotically stable and can extend their activity over at least a single stress-free generation (Luna, Bruce et al. 2012).

2.6.1 Mechanisms of Stress Memory Inheritance

At low temperatures, mutants lacking demethylase activity usually pass the vernalized state onto their progeny. It has been suggested that a histone-based epigenetic state could be transmitted by gametogenesis and meiosis (Crevillen, Yang et al. 2014). For example, in yeast, in the absence of EPE1 demethylase, H3K9me is stable over many mitotic

generations at the transgene locus (Audergon, Catania et al. 2015, Ragunathan, Jih et al. 2015). Even though genetic analysis reveals the involvement of DNA methylation and siRNA pathways in several cases of transgenerational stress memory, our understanding of a mechanistic basis for transgenerational or even intergenerational memory remains unknown.

2.6 Conclusion

In the 19th century, Lamarck first coined a hypothesis that traits acquired in one generation can be transmitted to the following generations. The proposed idea of the inheritance of acquired traits was viewed with considerable scepticism until this century when rapid progress in genomics and epigenomics studies came into light. Since the life cycle of *Arabidopsis thaliana* is short, it is a valuable model for studying adaptation and transgenerational effects because several generations could be obtained in just one year. In this study, the transgenerational inheritance of *Arabidopsis* had been studied to decipher complex mechanisms in stress response. However, in most of the cases, the stress memory is reset in plants after a single stress-free generation (Wibowo, Becker et al. 2016). Since transgenerational memory is likely inherited through epigenetic mechanisms, including changes in DNA methylation, histone modifications and chromatin states, and it is likely in part triggered by non-coding RNAs, in *Arabidopsis*, it would be exciting to investigate if stress responses trigger the stress memory and inheritance. If we find some meaningful insights, it might be helpful to the breeders for a universal practice for growing economically important crops.

CHAPTER 2 - BIBLIOGRAPHY

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CHAPTER 3: MATERIALS AND METHODS

3.1 Plant Material:

Arabidopsis thaliana plants from different generations were used to examine the transgenerational stress response to cold. Experimental plant seeds were obtained from a single inbred Columbia plant (Columbia ecotype, Col-0). The F1 progeny of parental seeds from the 15D8 line was obtained from our laboratory (Plant Biotechnology Laboratory, University of Lethbridge). The 15D8 line is a special transgenic plant for the luciferase (LUC) recombination reporter gene carrying a copy of a direct repeat of the luciferase recombination transgene. Dr. Kovalchuk's laboratory has already propagated 25 generations of plants exposed to cold stress (12 hours at +4°C for seven days) and control plants grown under normal conditions. Seeds from the parental and progeny sampling generations were kept at 4°C for the stratification and then planted in all-purpose potting soil prepared with water containing a generic fertilizer (Miracle-Gro, Scotts Canada) made to field capacity in 4 x 4 pots. Plants were grown in a growth chamber (BioChambers) at 22°C under extended day conditions of 16 hours light and 8 hours dark (18°C). The pots containing plants were placed in trays measuring ~ 25 cm x 50 cm and watered from the tray. A total of eight (8) plants per pot and two pots per sample group were designed for the phenotypic profiling, thus resulting in approximately sixteen (16) plants per treatment group. Three independent stress groups and two independent control groups were generated and propagated for 25 generations by replanting seeds harvested at each generation.

3.1.1 Key Concepts Relevant to The Study

i. Two set of seeds were collected: 25 (F1 to F25) generations from plants propagated under cold stress conditions (S) and 25 generations under non-stress conditions (NS). Control non-stressed lines were named F1C to F25C and cold-stressed (Cd) lines - F1Cd to F25Cd.

ii. Two sets of independently propagated (for 25 generations) lines were used:

   a. Progeny of cold stressed plants: two different populations, Cd-4 and Cd-5.

   b. Progeny of control plants: two different populations, C-12 and C-13.

iii. Two sets of conditions:

   The propagation of all plants under two different conditions: Cold Stress Condition (S) and Non-stress Condition (NS).

iv. The experimental plan: Study the phenotypic, genomic and epigenomic profiles of plants to find differences in their progenies.

v. For the phenotypic analysis: Generations were used as follows F1, F10, F15, F20, and F25.

vi. For the genomic and epigenomic analysis: All plants were propagated in non-stress condition. Generations used for the sequencing were: F2C (Control), F25C (Control) and F25Cd (Cold).

vii. The method used for the Genomic Analysis: Whole Genome Sequencing (WGS).

viii. The method used for the Epigenomic Analysis: Whole Genome Bisulfite Sequencing (WGBS).

3.2 Growth Conditions and the Phenotypic Profiling Setup:

For this study, there were two sets of samples (i.e., stressed and control plants) and two types of conditions (i.e., normal and cold) to perform the phenotypic profiling. Seeds from the generations F1, F10, F15, F20, and F25 were collected from our lab for the phenotypic profiling. Seeds were planted in all-purpose potting soil with a ratio of 9:1 to vermiculite and kept at 4°C for 72 hours for the stratification. Approximately after three days, the plants were transferred into the growth chamber, and after five days post germination (dpg), the plants were transplanted into individual pots. At 5 dpg, only the plastic lids covering the plant pots were kept and let them be opened for 20 minutes for allowing the natural airflow. At 10 dpg, cold stress was applied to one treatment group, and it was counted as day-1 for the cold stress. Cold stress was applied for seven consecutive days by placing plants at 4°C overnight, from 8 pm to 8 am. At 21 dpg, the number of true leaves was counted. Next, bolting and flowering time observations were made by observing the plants daily. Usually, in between 22 to 40 dpg, data collection from flowering and bolting observations was done. At 43 dpg, to measure the fresh weight of plants, individual plants were uprooted and weighed on a five-digit balance (Mettler Teledo), and then we continued to dry the plants by putting them in the incubator at 55°C. Then a few days later, when the plants dried completely, the dry weight of individual plants was taken. Finally, seeds were harvested, and seed size measurements were done by using a microscope (Hund WETZLAR). The height and width of an individual seed were measured in micrometer scales using a photograph from the microscope; approximately 25 seeds were measured for each generation. Overall, the phenotypic profiling includes data from true leaf counts, bolting time, flowering time and measurements of seed length and width.

3.2.1 Statistical Analysis of Phenotypic Data

Phenotypic data were obtained, recorded, and graphically represented by using Microsoft (MS) Excel® 2016. Statistical analyses were performed by using an R Studio and MS program. Standard errors or standard deviations were calculated, and significant differences between the means were compared using a two-way ANOVA and Tukey-Kramer HSD test. Significant differences between experimental groups were analyzed using p-values. All statistical tests were conducted at a 95% level of confidence.

3.3 Genomic and Epigenomic Profiling:

3.3.1 Molecular Techniques Used

The parental F2C plants and the progeny of F25 plants (both from the progeny of cold stressed plants, F25Cd and parallel control plants, F25C) were grown to about 21 dpg, and then the leaf tissue was harvested for molecular analysis. Methylation profiles of the generation F2C allowed us to consider it as parental control progeny, F25C as parallel control progeny and F25Cd as the progeny of cold-stressed plants to find the methylation profile differences among progeny generations. Methylation profiles of sample groups used were F25Cd versus F2C, F25Cd versus F25C, and F25C versus F2C.

3.3.2 DNA Isolation

The whole rosette leaves of F2 and F25 plants were collected, frozen in liquid nitrogen, and stored at -80°C for DNA extraction. Total genomic DNA was extracted from approximately 100 mg of leaf tissue homogenized in liquid nitrogen using a CTAB protocol with some modifications. A DNA extraction buffer consisted of 31.8 g Sorbitol,

6 g Trizma base (Tris), 0.84 g EDTA in 500 ml of DDW, pH adjusted to 7.5 with HCl. A nucleic lysis buffer was prepared using 30.29 g Tris, 23.27 g EDTA, 73.05 g NaCl, 5 g CTAB dissolved in ~ 250 ml DDW, pH adjusted to 7.5. The total extraction buffer used was prepared with Na-bisulfite (38 mg/10 ml) added before use, 10 ml of nucleic lysis buffer, and 4 ml of 5% Sarkosyl. 700 µl of the total extraction buffer was used per sample. Samples were incubated at 65°C for 1 hour and inverted periodically. 700 µl of chloroform was added to the samples and shaken by hand for 5 minutes. The samples were centrifuged at 16,000 g for 10 minutes at 4°C with a supernatant phase transferred to a new tube; this chloroform step was repeated. Two-third volume of isopropanol was added and incubated at room temperature for about 24 hours to precipitate DNA. All samples were then centrifuged at 12,000 g for 15 minutes at 4°C, the pellet of precipitated gDNA was rinsed twice with 70% ethanol and once in 100% ethanol, then air-dried at room temperature for about 10 minutes. 100 µl of P1 buffer (Qiagen Kit) mixed with RNAase was added, and the samples were incubated for 10 minutes at 37°C.

An additional extraction was performed at this stage by adding 100 µl of Phenol: Chloroform mixture, centrifugation at 16,000 g for 10 minutes at 4°C and transferring 90 µl of the supernatant phase to a new tube. 9 µl of Sodium acetate and 250 µl of 100% ethanol were added and incubated at room temperature to precipitate DNA. The samples were centrifuged at 12,000 g for 10 minutes at 4°C to obtain pellets of precipitated DNA and washed twice with 1ml of 70% ethanol and once in 1ml of 100% ethanol, then they were air-dried at room temperature. The DNA pellets were dissolved in distilled water and

quantified using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc.). Also, agarose gel electrophoresis was performed to verify the integrity of DNA samples.

3.3.3 Whole Genome Sequencing (WGS) and Whole Genomic Bisulfite Sequencing (WGBS)

The isolated genomic DNA was used for whole-genome sequencing (WGS) and whole-genome bisulfite sequencing (WGBS) (Illumina) to assist in identifying the genomic and epigenomic (associated with changes in DNA methylation) profiles and variations. Data obtained were analyzed using several toolkits found in the methylKit package. WGBS allows for the investigation of genome-wide patterns of DNA methylation at a single base resolution. It involves the sodium bisulfite conversion of unmethylated cytosine to uracil. The resulting cytosine residues in the sequence represent the methylated cytosine in the genome, which is then mapped to a reference genome (Clark, Harrison et al. 1994). Binomial tests were applied and used to determine the observed methylation frequency against a bisulfite conversion reaction, and the percentage methylation (%methylation) levels were calculated at each base (Schultz, Schmitz et al. 2012)

3.3.4 Bioinformatics Based Analysis of WGS Data

Adapter trimming was done by using Trim Galore software with the "-q 30" option. Then reads were mapped to the Tair10 genome using the bwa-mem of BWA software, and duplicates were marked by using the Picard software. Local realignments around SNPs and INDELs were performed using GATK (a Genome Analysis Toolkit) which accounts for genome aligners, mapping errors and gives the consistent regions that contain SNPs and

INDELs. The resulting reads were quality controlled by Haplotype scores, and sample variant sites were called individually and jointly by using Haplotypercaller with GATK. The sites marked as a low-quality score by GATK were filtered out and used. The effects of variants in the genome sequences were classified using the SnpEff program (Cingolani, Platts et al. 2012). Toolkits used included genomation to obtain a biological understanding of genomic intervals and the Functional Classification SuperViewer to create gene expression profiles and show the difference between samples. The genes nearest to the non-overlapping SNPs and INDELs sites were annotated.

3.3.5 Bioinformatics Based Analysis of WGBS Data

Raw sequencing reads were quality controlled and trimmed using Trim Galore software similar to the WGB analysis initiation. The trimmed reads were subsequently aligned to the TAIR10 reference genome using the bisulfite mapping tool Bismark (Krueger and Andrews 2011). The methylated cytosines (Cs) were extracted from the aligned reads with the Bismark methylation extractor using default parameters followed by the computation of methylation frequency using the R package software, methylKit. The %methylation was calculated by counting the ratio of the frequency of Cs divided by reads with C or T at each base and computed at bases with coverage ≥ 10 (Akalin, Kormaksson et al. 2012).

$$\%\text{Methylation} = \{\text{Frequency of C} \div \text{read coverage}\} \times 100$$

Common bases covered across all samples were extracted and compared, and the differential hyper- and hypomethylated bases in each chromosome were extracted. The

differentially methylated cytosines (DMCs) overlapping with genomic regions were assessed (in the preference for a promoter > exon > intron), and the average percentage methylation of DMCs around genes with distances of DMCs to the nearest transcription start sites (TSSs) were also calculated. Annotation analysis was performed with the genomation package within a methylKit to obtain a biological understanding of genomic intervals over the pre-defined functional regions like promoters, exons, and introns (Akalin, Franke et al. 2015). Functional commentary of the generated gene expression profiles was performed using the SuperViewer tool with Bootstrap to show the difference between samples (Provart and Zhu 2003). Hierarchical clustering of samples was used to analyze for similarities and detect sample outliners based on the percentage methylation scores and a possible molecular signature. Also, Principal Component Analysis (PCA) was utilized for variations and any biological relevant clustering of samples. Scatterplots and bar plots showing the percentage of hyper-/hypo- methylated bases, overall chromosomes and heatmaps were used to visualize similarities and dissimilarities between DNA methylation profiles.

3.3.6 Analysis of Differentially Methylated Regions (DMRs)

DMRs information was investigated over the predefined regions for all contexts; CG, CHG, and CHH on 100 bp and 1000 bp tiles across the genome to identify stochastic and treatment associated DMRs (Akalin, Kormaksson et al. 2012). The differential hyper-/hypo- methylated regions were also extracted and compared across samples. By default, DMRs were extracted with q-values < 0.01 and percent methylation difference > 25% to find out biologically relevant results; it was taken as arbitrary because > 50% would result

near to nothing significant results. The differential methylation patterns between sample groups and methylation events of these differences per chromosome were extracted too. In summary, sliding windows of 100 bp and 1000 bp were considered for both DMRs and DMCs, and values were extracted based on at least 25% and 50% differences (q-values > 0.01) to assess significant differences among samples.

3.3.7 Quality Control and Statistical Analysis of Sequencing data

Mapped reads were obtained with a quality score of <30, differential hyper- and hypomethylated bases were extracted with q-values < 0.01 and percent methylation difference larger than 25% in methylKit. Heatmaps of differentially methylated bases were quantified at q-values < 0.01 and the percent methylation difference was more significant than 50%. The distances of DMCs to the nearest TSSs obtained from genomation were run at >25% and >50%. The TSSs distance to DMCs was extracted within +/- 1000bp and annotated at DMCs >50%. DNA methylation profiles obtained from melthylKit used the pairwise correlation coefficients of the percent methylation level and the 1-Pearson's correlation distance for the hierarchical clustering of samples. Logistic regression and Fisher's exact test were used to determine differential methylation with calculations of q-values and Benjamini-Hochberg for p-values corrections. The T-test for the mean difference between groups was calculated and extracted with p-values at least < 0.05. Global genome methylation results were graphed using Microsoft Excel® (MS) and output graphs from each corresponding program used.

CHAPTER 3 - BIBLIOGRAPHY

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CHAPTER 4: RESULTS

4.1.1 The Origin of the Study

A single *Arabidopsis thaliana* plant (Col-0: 15d8) was used as a seed origin. Plants were propagated for twenty-five generations; they were either exposed to cold stress or to a control non- stress environment. Seeds of the first and second generations, F1 and F2, were used as the experimental parental progeny generations for the comparison with other advanced progeny generations where F1C was used for phenotyping and F2C for the genomic and epigenomic analysis. Progeny generations that were tested for the effects of transgenerational inheritance in phenotypes were F10, F15, F20, and F25. Whole-genome bisulfite sequencing and whole-genome sequencing were also performed from the plants propagated independently from F2C (a parental control sample), F25C (a parallel control progeny sample), and F25Cd (an advanced cold stressed progeny sample) seeds. For the genomic and epigenomic data analysis, the results of bioinformatics analysis are also represented in the results section to decipher the transgenerational inheritance mechanism.

There were two distinct lineages of plants relevant to this study:

1. Plants are propagated from F1 to F25 generations under control conditions. They are called “the control progeny (F1C to F25C)” as mentioned before in the methods section.
2. Plants are propagated from F1 to F25 generations under cold stress conditions. They are called “the cold stressed progeny (F1Cd to F25Cd)” as mentioned before in the methods section.

Both control and cold stressed progenies were used for the phenotypic analysis by exposing them to stress or by propagating them under normal conditions (figures 1 and 2).

4.1.2 Schematic Diagram of the Phenotypic Profiling

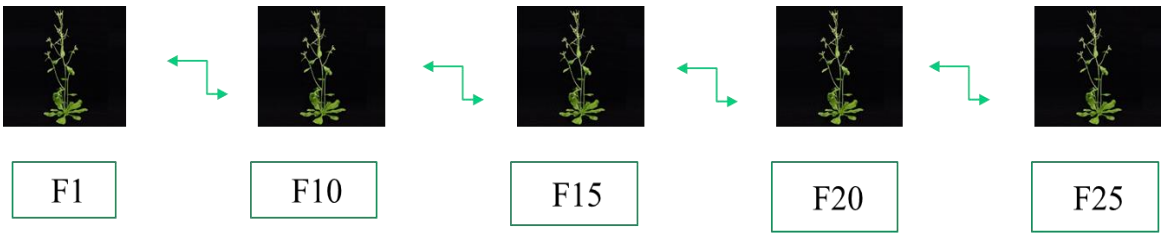


Figure 1. A schematic diagram of the phenotypic profiling experiment for plants propagated under non-stress conditions (both the control and cold stressed progenies).

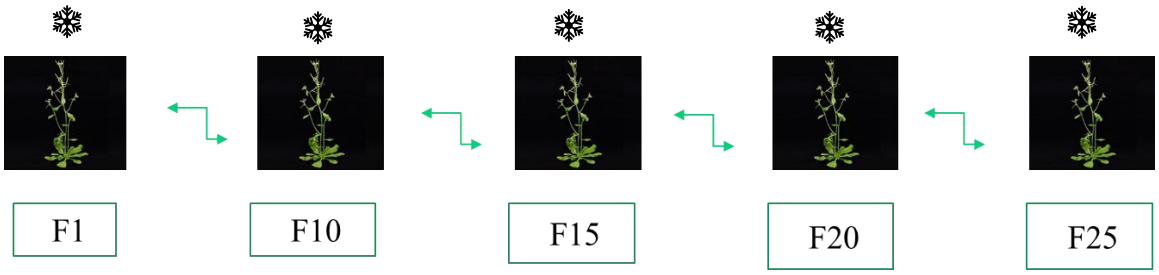


Figure 2. A schematic diagram of the phenotypic profiling experiment for plants propagated under cold-stress conditions (both the control and cold stressed progenies).

The schematic diagrams show plants of different generations propagated under control conditions (figure 1) and cold stress conditions (figure 2) applicable both for control (F1C, F10C, F15C, F20C, F25C) and cold-stressed progenies (F1Cd, F10Cd, F15Cd, F20Cd, F25Cd); the phenotypic analysis quantified the number of true leaves, bolting and flowering time and seed size measurements in height and width.

4.1.3 Schematic Diagram of the Genomic and Epigenomic Profiling

The parental generation F2C and progeny generations F25C and F25Cd were propagated under control conditions and were used for WGS and WGBS to find differences among progenies of cold (F25Cd as the advanced cold stressed progeny) and control lines (F25C as the parallel advanced control progeny) in comparison with the parental control progeny, F2C.

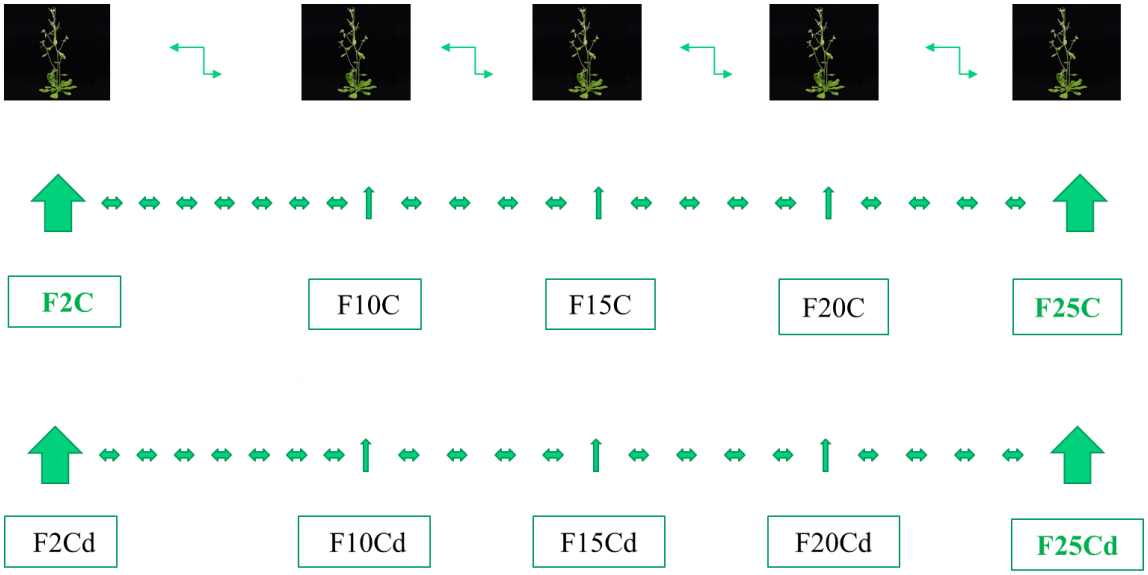


Figure 3. The design of the study for the genomic and epigenomic profiling (F2C, F25C, and F25Cd were used for the sequencing, labeled with green colored font).

4.1.4 Results of Phenotypic Profiling

4.1.4.1 Changes in the leaf numbers

Changes in the number of leaves were counted in both parental and advanced progeny generations. True leaf numbers varied from progeny to progeny. There was an overall significant difference ($p < 0.05$) in the number of leaves across all the generations when the progeny plants (F10, F15, F20, F25) are compared to parental plants (F1). Regardless of the propagation conditions, the progeny of parallel control plants (F25C) and the progeny of the cold stressed plants (F25Cd) showed a significant difference ($p < 0.05$) in the number of leaves. It is strongly supported by the data that the number of true leaves was significantly higher in the advanced progenies in comparison with the leaf number of the parental progeny, regardless of propagation conditions (figure 4).

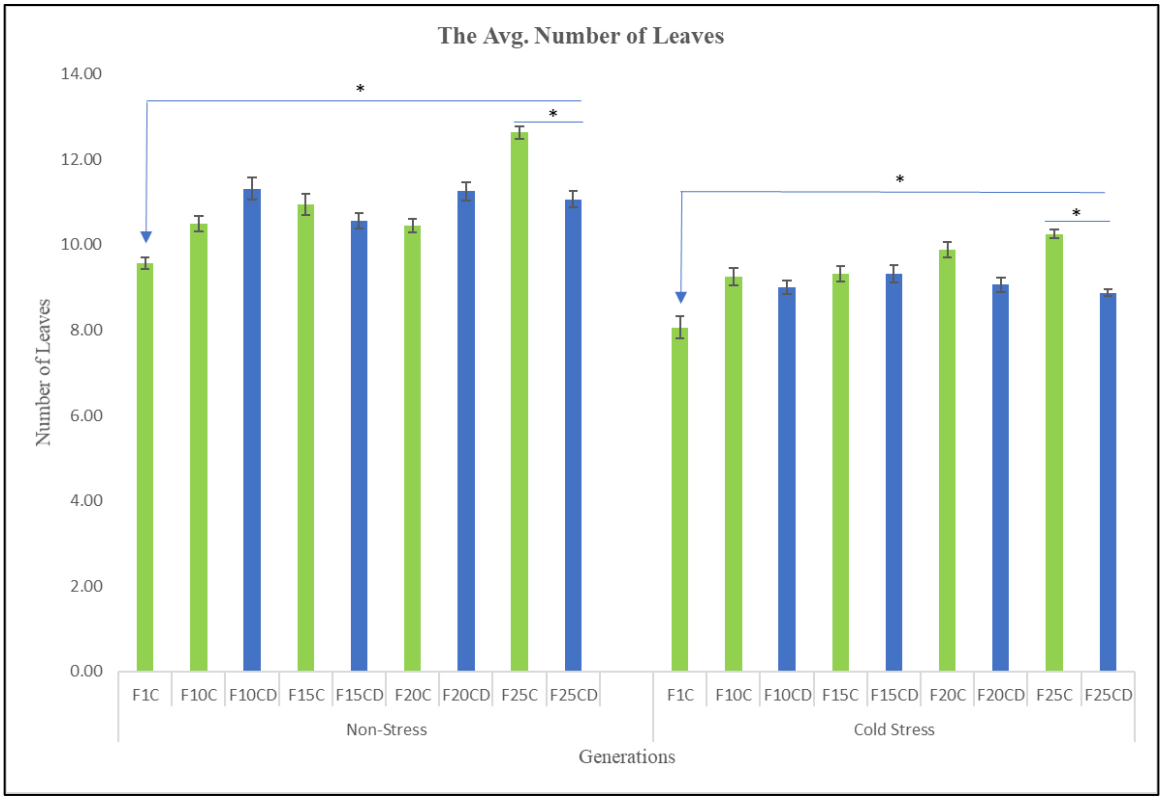


Figure 4. Data on true leaves from plants propagated under cold stress and non-stress conditions (applicable both to the control and cold-stressed progeny). Error bars represent Standard Error (SE). Leaves were counted at 21 dpg (n=16, SE). The asterisk (*) above shows a significant difference compared with the parental generation, where * indicates $p < 0.05$.

In summary, the number of leaves is significantly greater in the advanced progeny plants, regardless of the propagation conditions. The most advanced progeny of cold stressed plants (F25Cd) showed a significantly lower number of leaves compared with the progeny of parallel control plants (F25C).

4.1.4.2 The Bolting Phenotype

The percentage of bolted plants was calculated at 31 days post-germination (dpg). The bolting data were collected from both the control and cold stressed progenies plants propagated under cold stress and non-stress conditions. Surprisingly, plants propagated under cold stress conditions (both the control and cold stressed progenies) did not bolt until 35 dpg. In summary, all plants propagated under non-stress conditions showed early bolting compared with plants grown under cold stress conditions. When plants were propagated under cold-stress conditions, bolting time varies from generation to generation in both the control and cold stress progenies. However, when plants were propagated under non-stress conditions, all the progenies (both cold stressed and control) showed early bolting compared with the parental control plants (figure 5). In particular, both of the advanced progeny generations (F25C and F25Cd) showed a statistically significant

difference in the percentage of bolting compared with the parental progeny generation (F1C) when propagated under non-stress conditions. In the case of cold stress conditions, the parental progeny (F1C) did also show a statistically significant difference in the bolting percentage, in comparison with the advanced progeny of the cold stressed plants (F25Cd), but not with the advanced parallel control progeny (F25C) (figure 5).

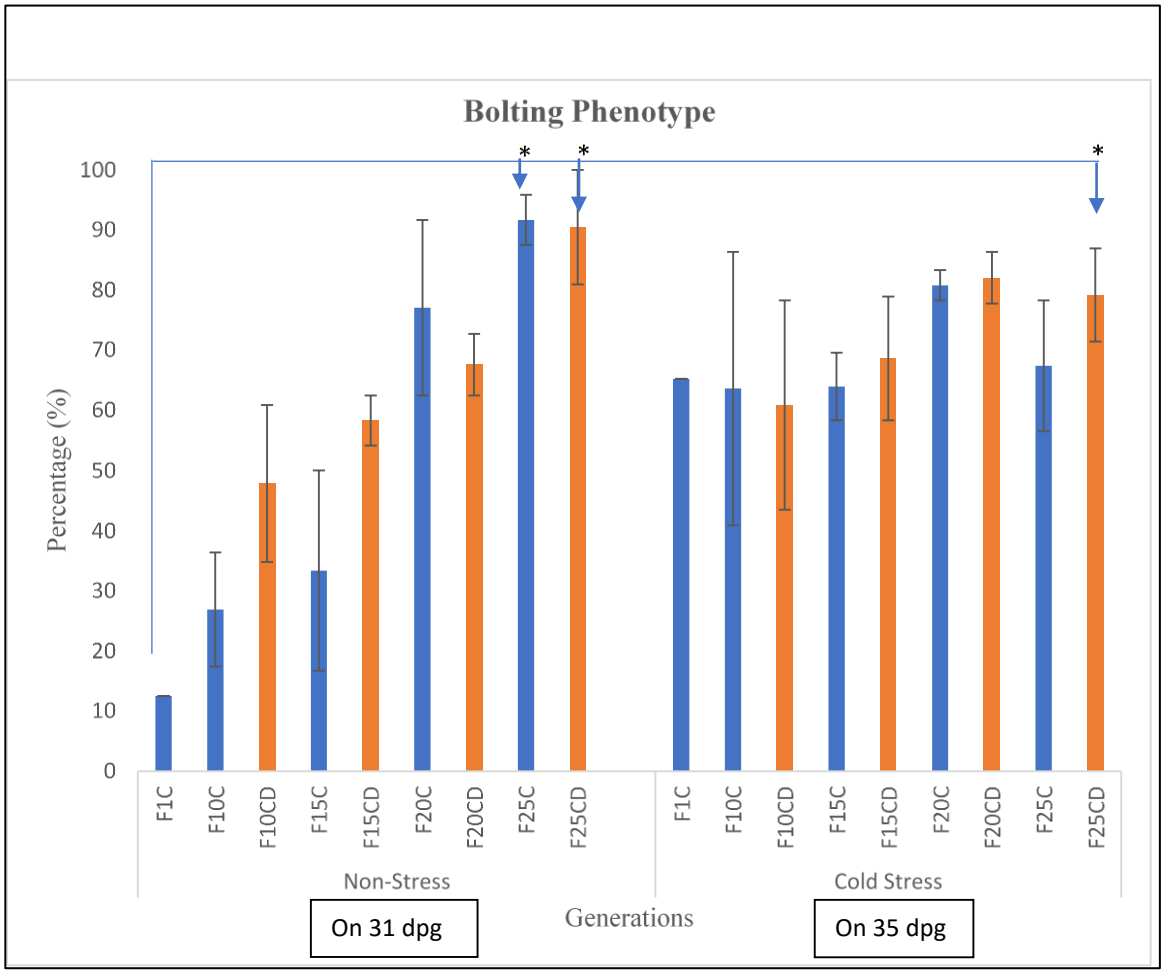


Figure 5. The percentage of plants showing bolting under non-stress and cold stress conditions (applicable for both the control and cold progenies). The percentage averages were calculated from approximately 24 plants for the control and 24 plants for cold stressed

groups. The error bars represent Standard Error (SE). The asterisk (*) above shows a significant difference compared with the parental generation, where * indicates $p < 0.05$.

In summary, non-stress conditions plants (both the control and cold stressed progenies plants) showed the earlier flowering than cold stressed plants. But the response of bolting was not consistent across generations within the same growth conditions.

4.1.4.3 The Flowering Phenotype

The percentage of flowering plants was calculated on the 35 dpg. The flowering data were collected from both the control and cold stressed progenies of plants propagated under cold stress and non-stress conditions. Surprisingly, plants propagated under cold stress conditions (both control and cold progenies) did not show any flowering until 40 dpg. In summary, all the plants propagated under non-stress conditions showed the earlier flowering compared with plants grown under cold stress conditions (figures 7, A-E). Like in the case of bolting time, in plants propagated under cold stress conditions, flowering time varied from generation to generation in both the control and cold stress progenies. However, a similar process was observed in plants propagated under non-stressed conditions where all the advanced progenies (both cold and control) showed the earlier flowering compared with the parental plants. F25C and F25Cd propagated under non-stress conditions showed a statistically significant difference (greater flowering) in the percentage of flowering compared with the parental generation (F1C). In the case of cold stress conditions, the parental progeny (F1C) showed a statistically significant difference in the flowering percentage in comparison with the advanced progeny of the cold stressed plants (F25Cd), but not with the advanced parallel control progeny (F25C) (figure 6)

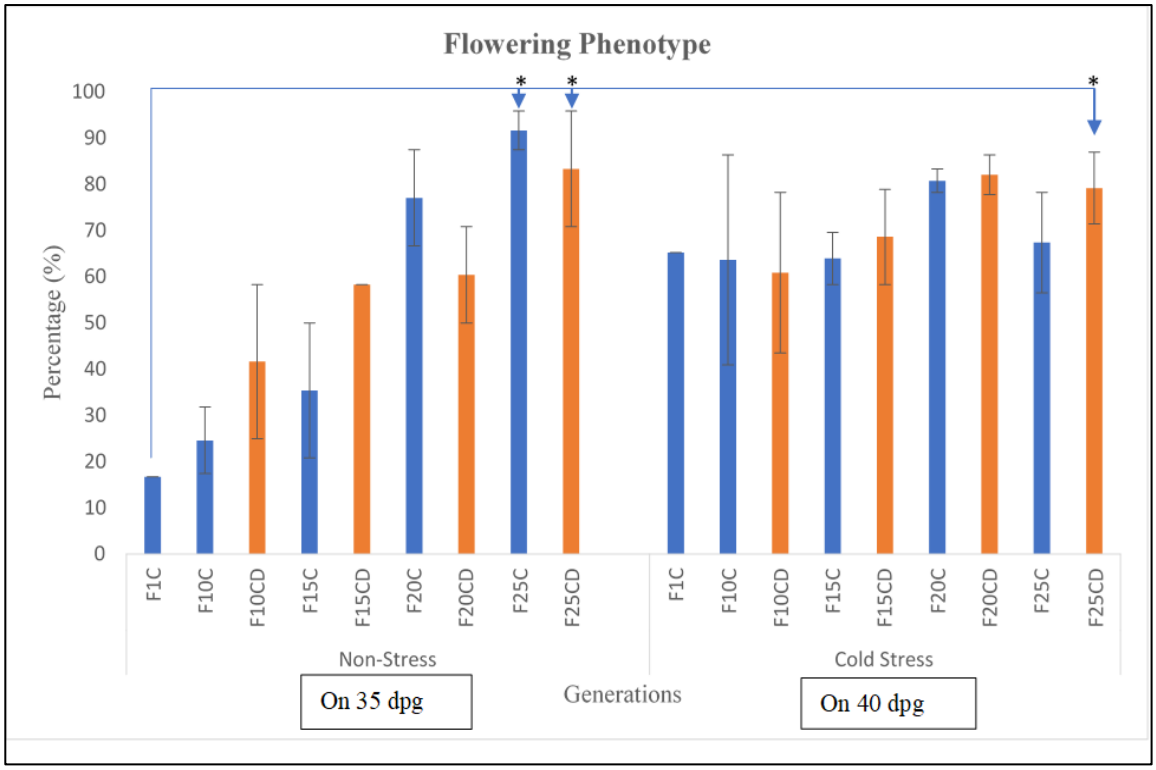
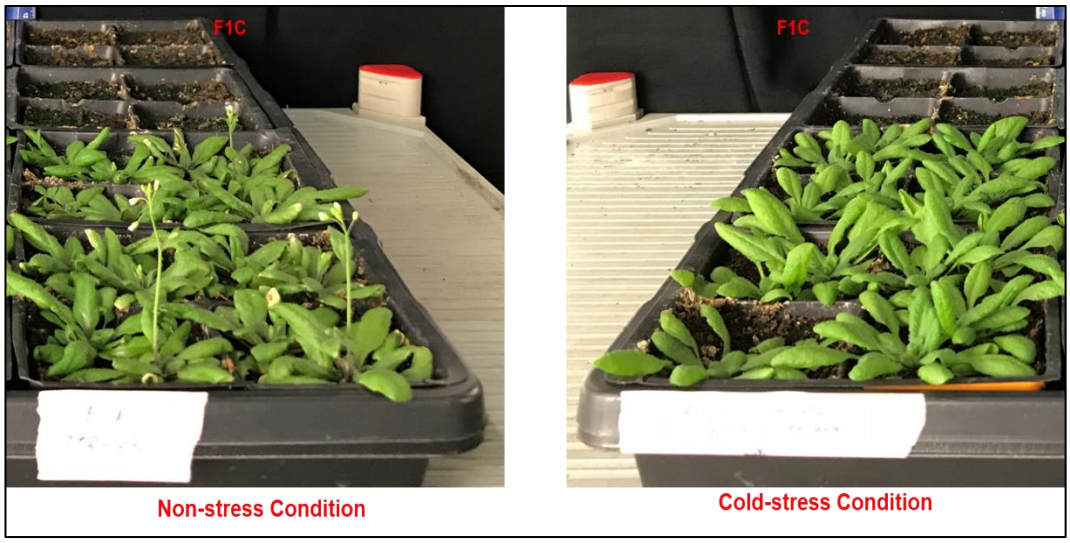


Figure 6. The percentage of plants showing flowering under non-stress and cold stress conditions (applicable for both the control and cold progenies). The percentage averages were calculated from approximately 24 plants for the control and 24 plants for the cold stressed groups. The error bars represent Standard Error (SE). The asterisk (*) above shows a significant difference compared with the parental generation, where * indicates $p < 0.05$.

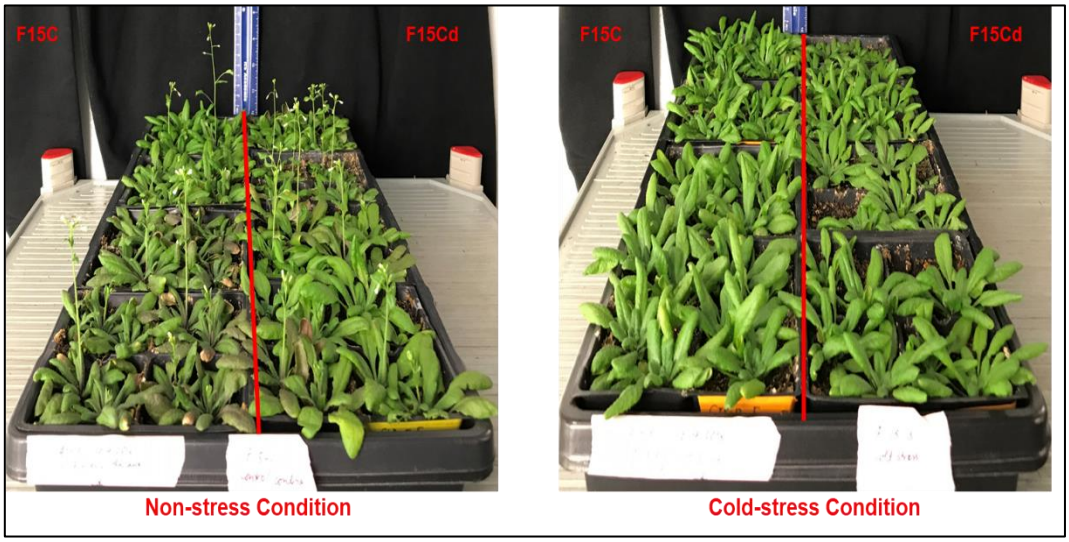
(A) F1


F1C
F1C
Non-stress Condition
Cold-stress Condition

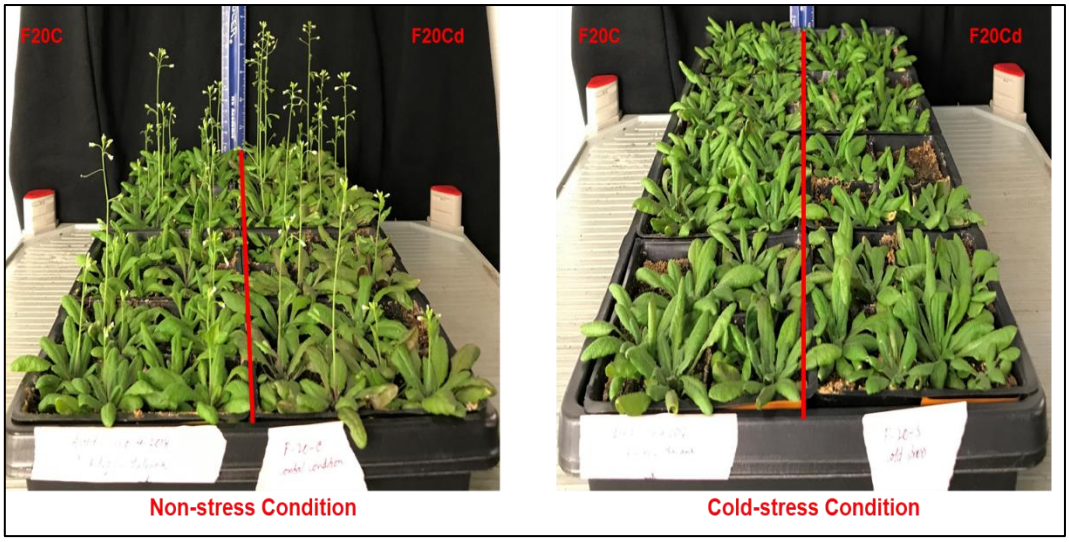
(B) F10


F10C
F10Cd
F10C
F10Cd
Non-stress Condition
Cold-stress Condition

(C) F15


F15C
F15Cd
F15C
F15Cd
Non-stress Condition
Cold-stress Condition

(D) F20


F20C
F20Cd
F20C
F20Cd
Non-stress Condition
Cold-stress Condition

(E) F25

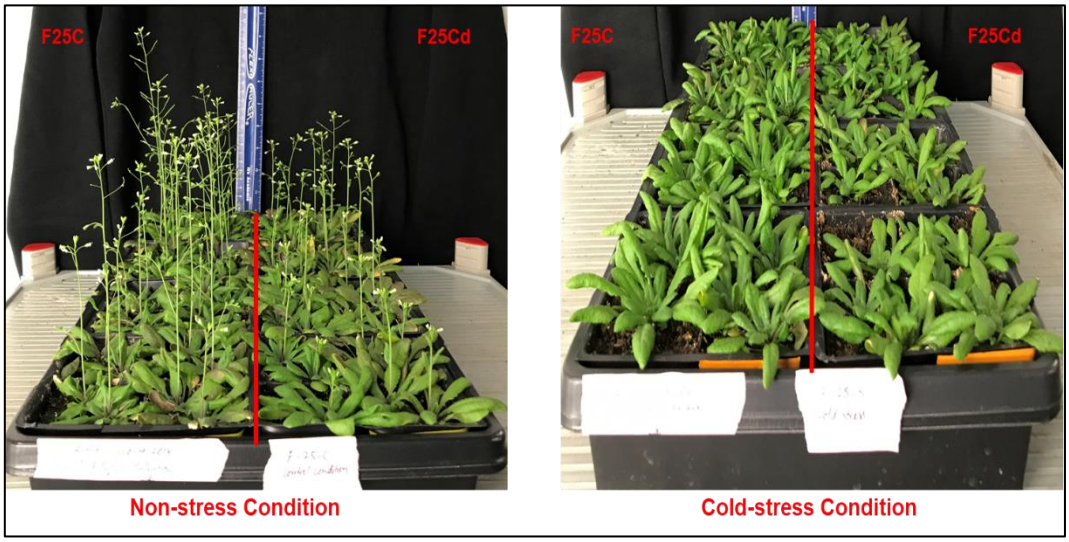


Figure 7 (A-E). Plants showing the flowering phenotype under non-stress and cold-stress conditions on the 35th day, "C" as the control and "Cd" as cold stressed progeny, where **(A)** shows F1, plants only from the control progeny **(B)** F10, plants from both the control and cold stressed progenies **(C)** F15, plants from both the control and cold stressed progenies **(D)** F20, plants from both the control and cold stressed progenies **(E)** F25, plants from both the control and cold stressed progenies.

In summary, like in the case of bolting time, plants propagated under non-stress conditions (both the control and cold stressed progenies) showed earlier flowering than cold stressed plants. But the response of flowering was not consistent across generations within the same growth condition.

4.1.4.4 Changes in the average height of seeds

The height of seeds was measured in the micrometer scale (μm) from twenty-five randomly selected seeds. When the progeny of cold-stressed plants were propagated under non-stress conditions and the seeds were collected from those plants, the average height of seeds increased in general compared to the parallel control progenies. However, when plants were propagated under cold stress conditions, the average height of seeds decreased in the cold stressed progenies compared to the parallel control progenies. No statistically significant differences were found when the height of seeds from the parental generations (F1C) was compared with F25Cd propagated under the non-stressed conditions. Statistically, significant differences ($p<0.05$) were observed when the height of seeds from the parental generation (F1C) propagated under either the control conditions or cold stressed conditions was compared with the height of F25Cd seeds propagated under cold stress conditions; but no significant difference was found in F25C propagated under cold stress conditions (figure 8).

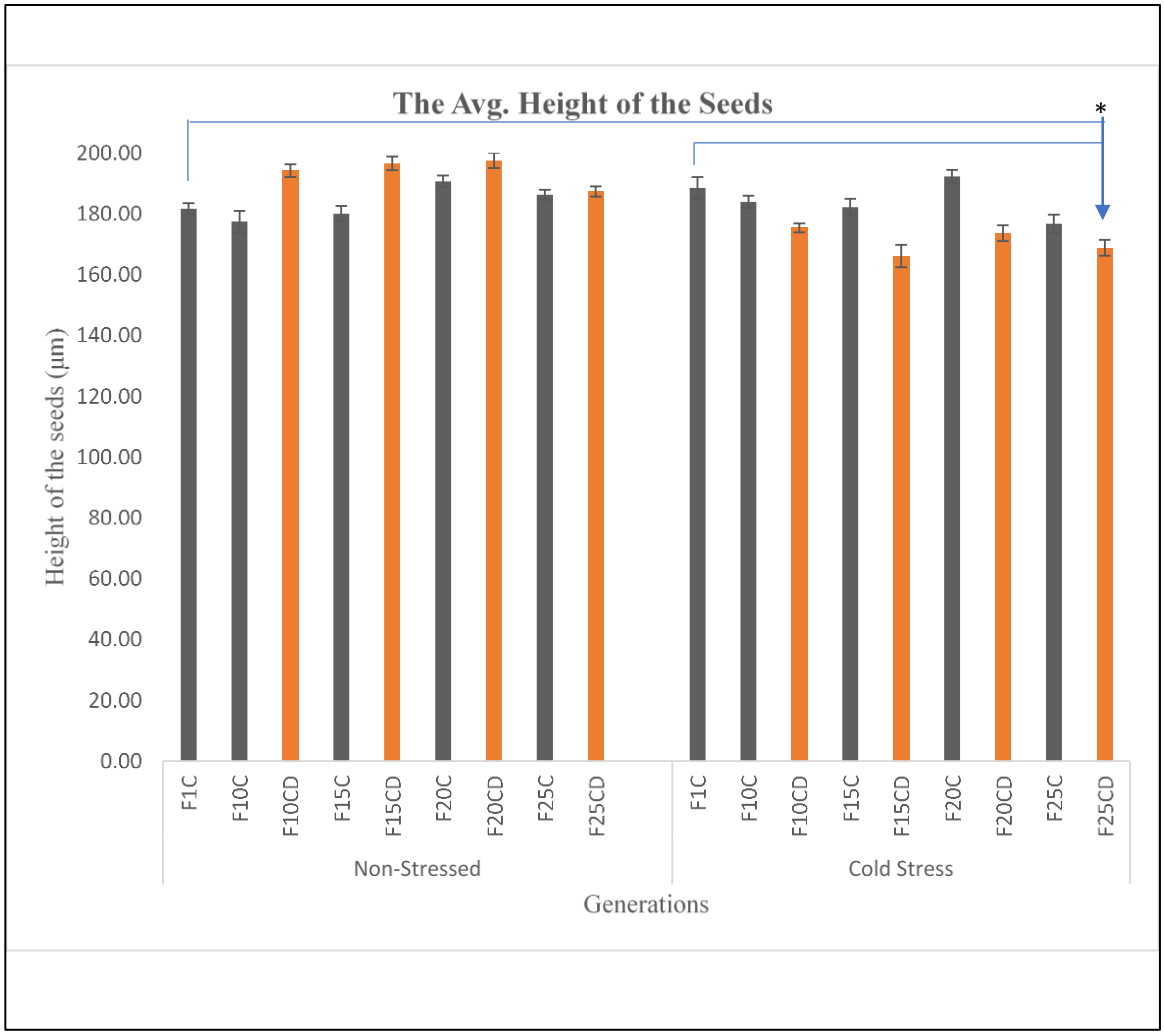


Figure 8. The height measurement of seeds, where 25 seeds were measured for each group. Error bars represent Standard Error (SE). The asterisk (*) indicates a statistically significant difference, where $p<0.05$ (using two-way ANOVA and Tukey-Kramer Test).

4.1.4.5 Changes in the average width of the seeds

The average width of seeds was measured from the same seeds which were used for the height measurement. The measurement was done in the micrometer scale (μm) from the twenty-five randomly selected seeds to represent a population of a sample generation. When plants were propagated under cold stress conditions, the average width of seeds decreased in the cold stressed progenies compared to the parallel control progenies. No statistically significant difference was found when the width of the seeds from the parental generation (F1C) was compared with the seeds from plants F25Cd propagated under non-stress conditions. However, when plants were propagated under cold stress conditions, the average width of seeds decreased in all the control and cold stressed groups. Statistically, significant differences ($p<0.05$) were observed when the width of seeds from the parental generation (F1C) was compared with the width of seeds in the F25Cd and F25C groups propagated under cold stress conditions (figure 9).

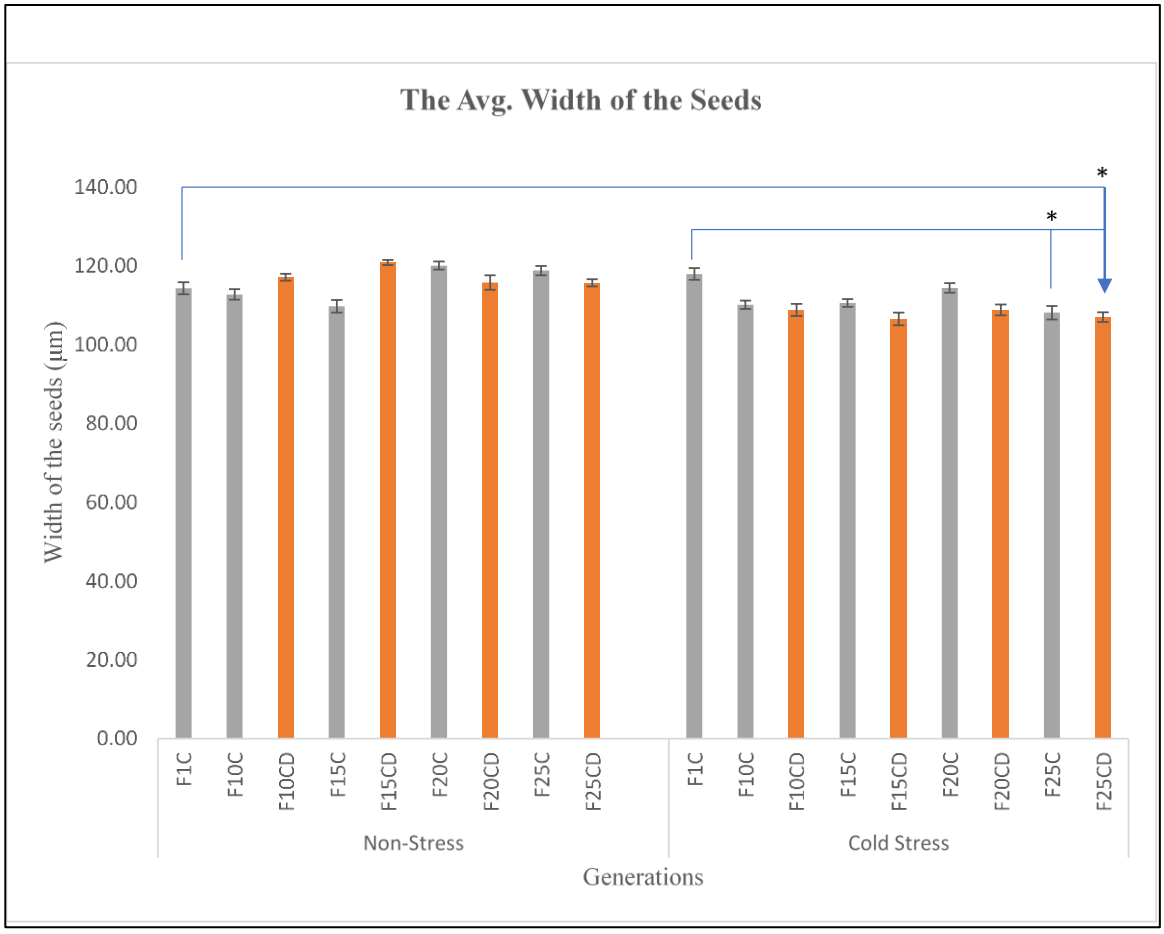


Figure 9. The measurement of seed widths, where 25 seeds were measured for each group. Error bars represent Standard Error (SE). The asterisk (*) indicates a statistically significant difference, where $p < 0.05$ (using two-way ANOVA and Tukey-Kramer Test).

4.2 Genomic and Epigenomic Changes in F2 and F25

Results from the Whole Genome Sequencing (WGS) data have been analyzed to reveal the genetic changes over multiple generations of control and cold stress groups. Similarly, Whole Genome Bisulfite Sequencing (WGBS) data reveals the epigenomic changes from the analysis of the DNA methylation profiles. Five genomes or methylomes (biological replicates) from each of the generations (F2C, F25C, and F25Cd) were considered for the study.

4.2.1 Genomic Variants between F2 and F25

4.2.1.1 Single Nucleotide Polymorphisms (SNPs)

The average number of SNPs calculated from the five biological replicates is higher in the cold stressed progeny (F25Cd) than in the parallel (F25C) and parental (F2C) control progeny. In comparison among generations, F25Cd showed a significant difference in the numbers of SNPs when F25Cd (9751) was compared with either F2C (9308) or F25C (9359), where $p < 0.05$ (figure 10).

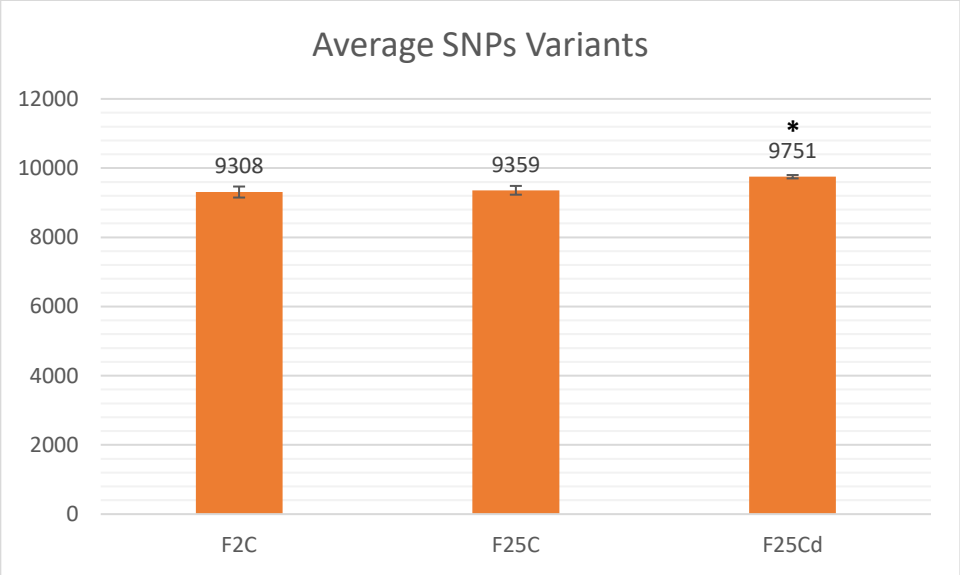


Figure 10. A comparison of average SNPs variants in the genome calculated from five independent biological repeats where F25Cd showed a significantly different number of SNPs. The asterisk above (*) shows a significant difference between the cold stressed progeny and parental or parallel control generations ($p < 0.05$).

4.2.1.2 The Number of Insertions and Deletions (INDELs)

The average number of Insertions and Deletions (INDELs) calculated from five biological replicates is higher in the cold stressed progeny (F25Cd) compared with the parallel and parental control progeny. Data comparison among generations showed a statistically significant difference in the number of INDELs in the cold stressed progeny such as F25Cd (4589) and F2C (4350), F25C (4380) where $p < 0.05$ (figure 11). On the contrary, the number of insertion events was slightly but not significantly lower in the cold stressed progeny compared to the parallel and parental control progeny (figure 12). on the other hand, deletion events in the cold stressed progeny is significantly higher compared with the parallel or parental control plants where $p < 0.05$ (figure 13).

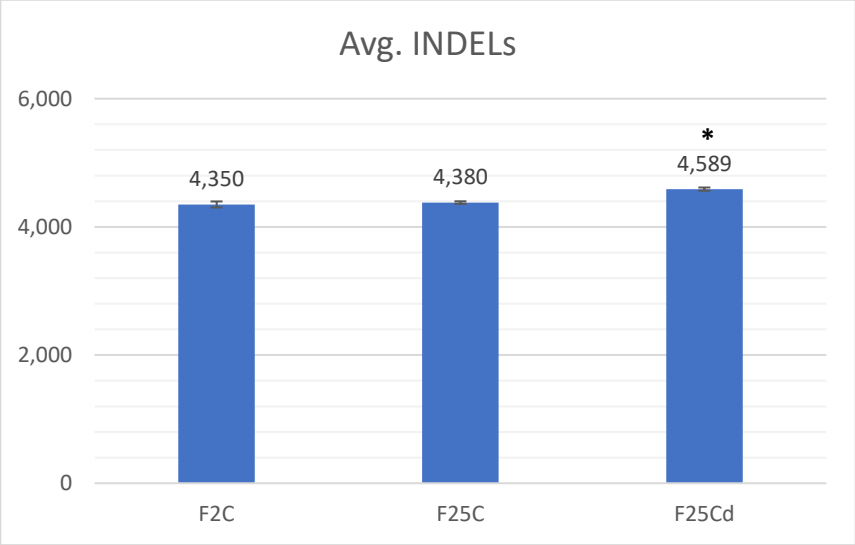


Figure 11. A comparison of the average number of INDELs (insertion and deletion events) variants in the whole genome of F2C, F25C, and F25Cd where F25Cd shows a significantly different number of INDELs. The asterisk above (*) shows a significant difference between the cold stressed progeny and the parental or parallel control generations ($p < 0.05$).

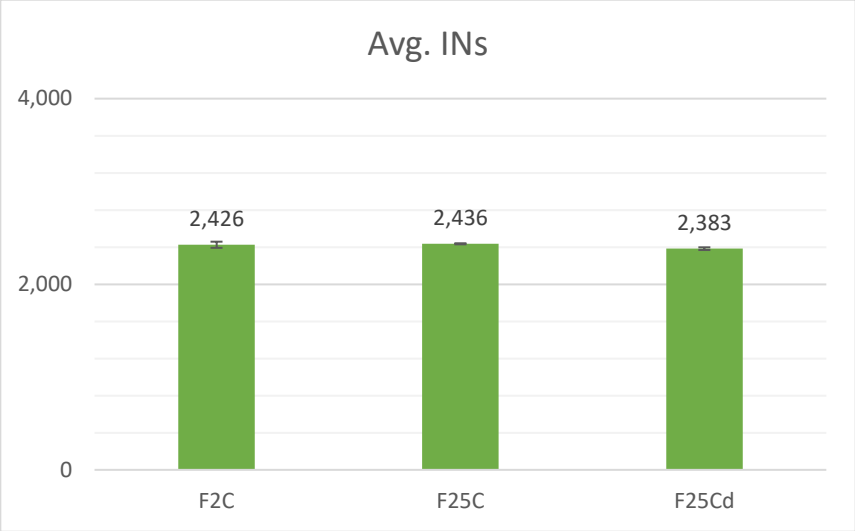


Figure 12. A comparison of the average number of insertions variants in the whole genome of F2C, F25C, and F25Cd. No group is statistically significantly different from another where $p > 0.05$.

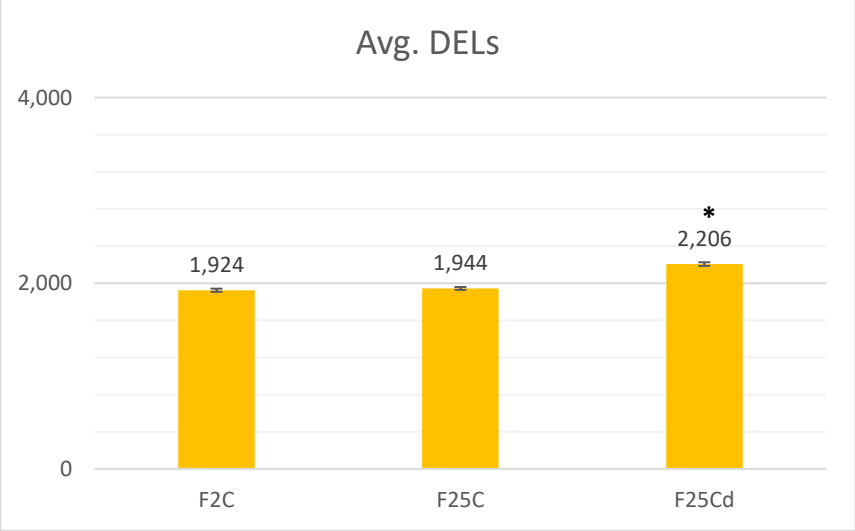


Figure 13. A comparison of the average number of deletions variants in the whole genome of F2C, F25C, and F25Cd. The numbers of deletion events in F25Cd was statistically significant in comparison with both the parental (F2C) and parallel (F25C) control progeny. The asterisk above (*) shows a significant difference between the cold stressed progeny and the parental or parallel control generations ($p < 0.05$).

4.2.2 Venn Diagram of SNPs and INDELs

SNPs and INDELs variants that are unique to each sample of the genome were extracted by common and non-overlapping sites specific to F2C, F25C, and F25Cd. The analysis of the unique number of SNPs reveals that the number of SNPs in the cold stressed progeny F25Cd is higher (705) than the number of SNPs in F2C (375) and the number of SNPs in F25C (367) (Figure 14). Similarly, the analysis of the unique number of INDELs reveals that the number of INDELs in the cold stressed progeny F25Cd is higher (158) than the number of INDELs in the parental control progeny F2C (87) and the number of INDELs in the parallel control progeny F25C (70) (Figure 15).

SNPs:

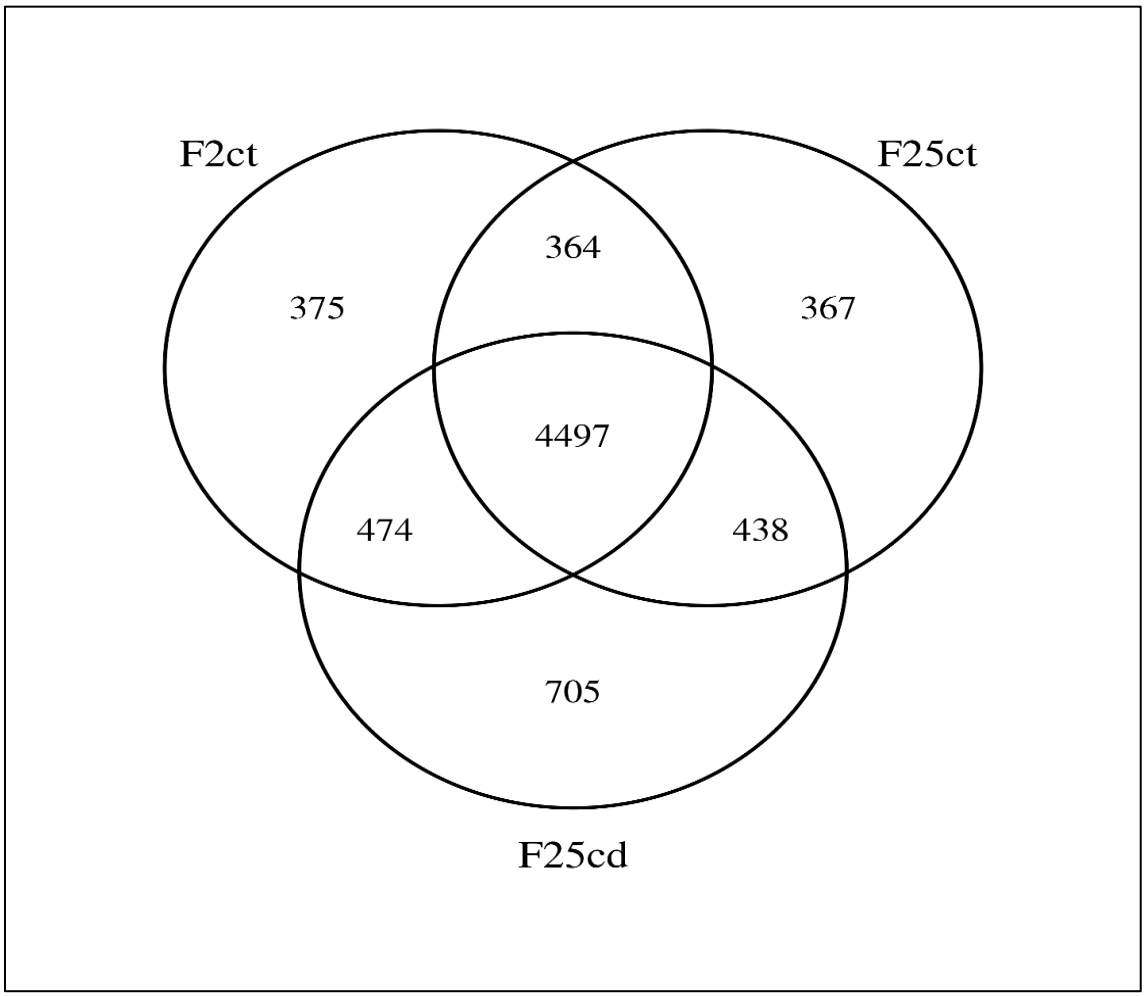


Figure 14. Venn diagram of SNPs for F2C, F25C and F25Cd progenies.

INDELs:

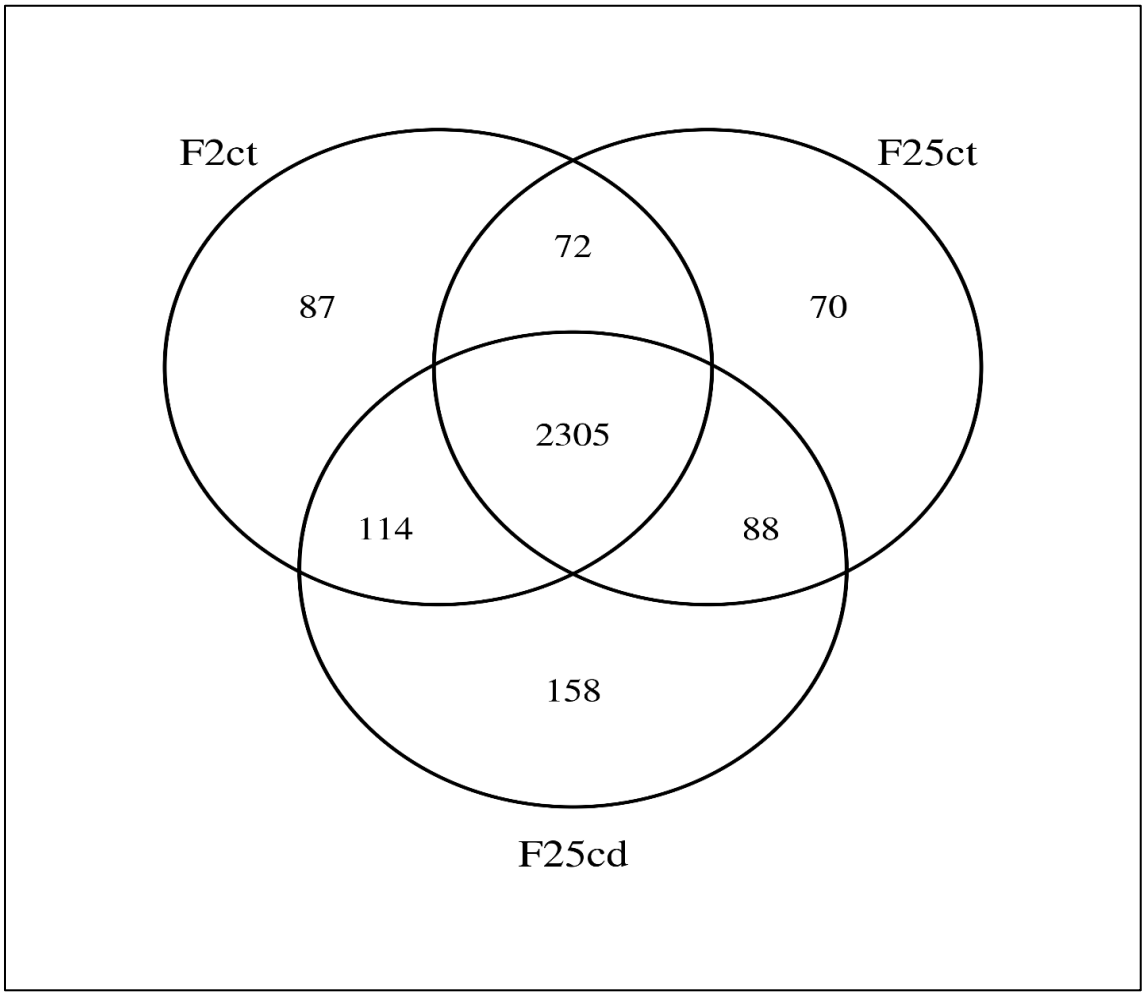


Figure 15. Venn diagram of INDELs for F2C, F25C and F25Cd progenies.

4.2.3 The Variants' Rate of SNPs and INDELs

The variants' rate of SNPs and INDELs can be calculated from the effective variants in a range of a certain genome length. The number of SNPs and INDELs variants and the rate of SNPs and INDELs variants were significantly higher in the cold stressed progeny (tables 1-2). Although the rates of variants were comparable between F2C and F25C (tables 1-2), there was a statistically significant difference between the cold stressed and control progenies ($p < 0.05$). Specifically, F25Cd had an average rate of one variant per every of ~ 12256 bases, F25C had one variant per every of ~ 12771 bases, and F2C had one variant per every of ~ 12841 bases. Therefore, it indicated that SNPs variants were occurring more frequently in F25Cd, meaning that the variants' rate is higher in the cold stressed progeny compared with the other two control groups (table 1).

Similar to SNPs, the rate of INDEL variants also showed that the INDEL occurrence rates were more frequent in F25Cd in comparison with F25C and F2C (table 2), wherein in F25Cd, there was one variant per every of ~ 26078 bases, F25C had one variant per every of ~ 27325 bases, and F2C had one variant per every of ~ 27523 bases (table 2).

Table 1. The average number of SNPs variants from five biological replicates.

Progeny	Avg. SNPs variants	Avg. fragment length for each of SNPs variants
Control Progeny (F2C)	9308	12841.8
SD	159	220.0
Control Progeny (F25C)	9359	12771.8
SD	127	172.8
Cold Progeny (F25Cd)	9751	12256.4
SD	49	61.2

Table 2. The average number of INDELs variants from five biological replicates.

Progeny	Avg. INDELs variants	Avg. fragment length for each of INDELs variants	INS	DEL
Control Progeny (F2C)	4349.8	27523.4	2425.6	1924.2
SD	104.9	665.1	74.0	38.3
Control Progeny (F25C)	4379.6	27325.8	2435.8	1943.8
SD	44.2	276.0	12.9	34.1
Cold Progeny (F25Cd)	4589.2	26078.4	2383.2	2206.0
SD	56.3	321.7	32.5	43.7

4.2.4.1 The functional classification of SNPs

To analyze the potential meaning of differences in SNPs, the functional classification of genes associated with these SNPs was performed. Enrichment in three categories was analyzed: biological process, molecular function, and cellular component. Enrichment would represent a statistically significantly higher or lower number of SNPs in the specific category as compared to a random occurrence in the *Arabidopsis* genome.

Analyzing functional enrichment of SNPs in the cellular components, the enrichment analysis revealed that the cold stressed progeny (F25Cd) uniquely showed the statistically significant over-representations in mitochondria, extracellular and other cellular components. However, only the parallel control progeny (F25C) showed the over-representation in the nucleus and plasma membrane, whereas the parental control progeny showed the over-representation in the cytosol. All three-progenies showed the over-representation in other intracellular and cytoplasmic components and in other membranes; and the difference between the progenies was not significant (figure 16).

In the case of molecular functions analysis, only the parallel control progeny (F25C) showed the over-representation in other types of enzymatic and hydrolase activities. The transcription factor activity is over-represented only in the parental control progeny (F2C). The cold stressed progeny (F25Cd) showed a significant over-representation in other binding and transferase activities (figure 16).

From the biological process analysis, protein metabolism was found statistically over-represented only in the cold stressed progeny (F25Cd). However, other biological processes showed over-representation only in parallel control (F25C); and interestingly,

the parental control progeny (F2C) showed over-representation in response to stress. Also, F2C showed the over-representation in transcription, DNA dependent process. All threes progenies showed the over-representation in other cellular and metabolic processes; and there was no difference among progenies (figure 16).

Table 3. Functional Annotations of SNPs.

Enrichment		**F2C SNPs**	**F25C SNPs**	**F25Cd SNPs**
Biological process				
transcription, DNA-dependent	frequency	1.3	-	-
	SD	0.261		
	p-value	0.045		
other metabolic processes	frequency	0.87	0.82	0.88
	SD	0.08	0.134	0.077
	p-value	0.024	0.042	0.026
other cellular processes	frequency	0.82	0.79	0.81
	SD	0.076	0.13	0.077
	p-value	9.44E-03	0.029	5.69E-03
cell organization and biogenesis	frequency	0.47	-	0.51
	SD	0.164		0.163
	p-value	9.26E-03		0.012
response to stress	frequency	0.69	-	-
	SD	0.162		
	p-value	0.03		
other biological processes	frequency	-	0.33	-
	SD		0.193	
	p-value		0.012	
protein metabolism	frequency	-	-	0.76
	SD			0.165
	p-value			0.037
Molecular function				
transcription factor activity	frequency	1.53	-	-
	SD	0.334		
	p-value	0.026		

protein binding	frequency	0.74	-	0.74
	SD	0.163		0.168
	p-value	0.036		0.034
nucleotide binding	frequency	0.7	0.13	0.61
	SD	0.185	0.11	0.187
	p-value	0.047	3.27E-03	0.022
other binding	frequency	-	0.73	1.1
	SD		0.189	0.125
	p-value		0.043	0.048
transferase activity	frequency	-	0.32	0.72
	SD		0.169	0.185
	p-value		0.011	0.037
hydrolase activity	frequency	-	0.23	-
	SD		0.156	
	p-value		5.26E-03	
other enzyme activity	frequency	-	0.21	-
	SD		0.138	
	p-value		2.02E-04	
Cellular component				
other membranes	frequency	0.8	0.55	0.85
	SD	0.132	0.165	0.12
	p-value	0.025	9.75E-03	0.039
other cytoplasmic components	frequency	0.77	0.53	0.79
	SD	0.108	0.162	0.122
	p-value	0.013	3.60E-03	0.016
chloroplast	frequency	0.72	-	0.8
	SD	0.16		0.151
	p-value	0.029		0.048
other intracellular components	frequency	0.71	0.64	0.72
	SD	0.124	0.189	0.133
	p-value	8.18E-03	0.027	8.68E-03
cytosol	frequency	0.49	-	-
	SD	0.204		
	p-value	0.02		
plasma membrane	frequency	-	0.5	-
	SD		0.227	
	p-value		0.036	
nucleus	frequency	-	1.26	-
	SD		0.169	
	p-value		0.023	

other cellular components	frequency	-	-	0.46
	SD			0.233
	p-value			0.041
extracellular	frequency	-	-	1.38
	SD			0.244
	p-value			0.021
mitochondria	frequency	-	-	0.72
	SD			0.184
	p-value			0.036

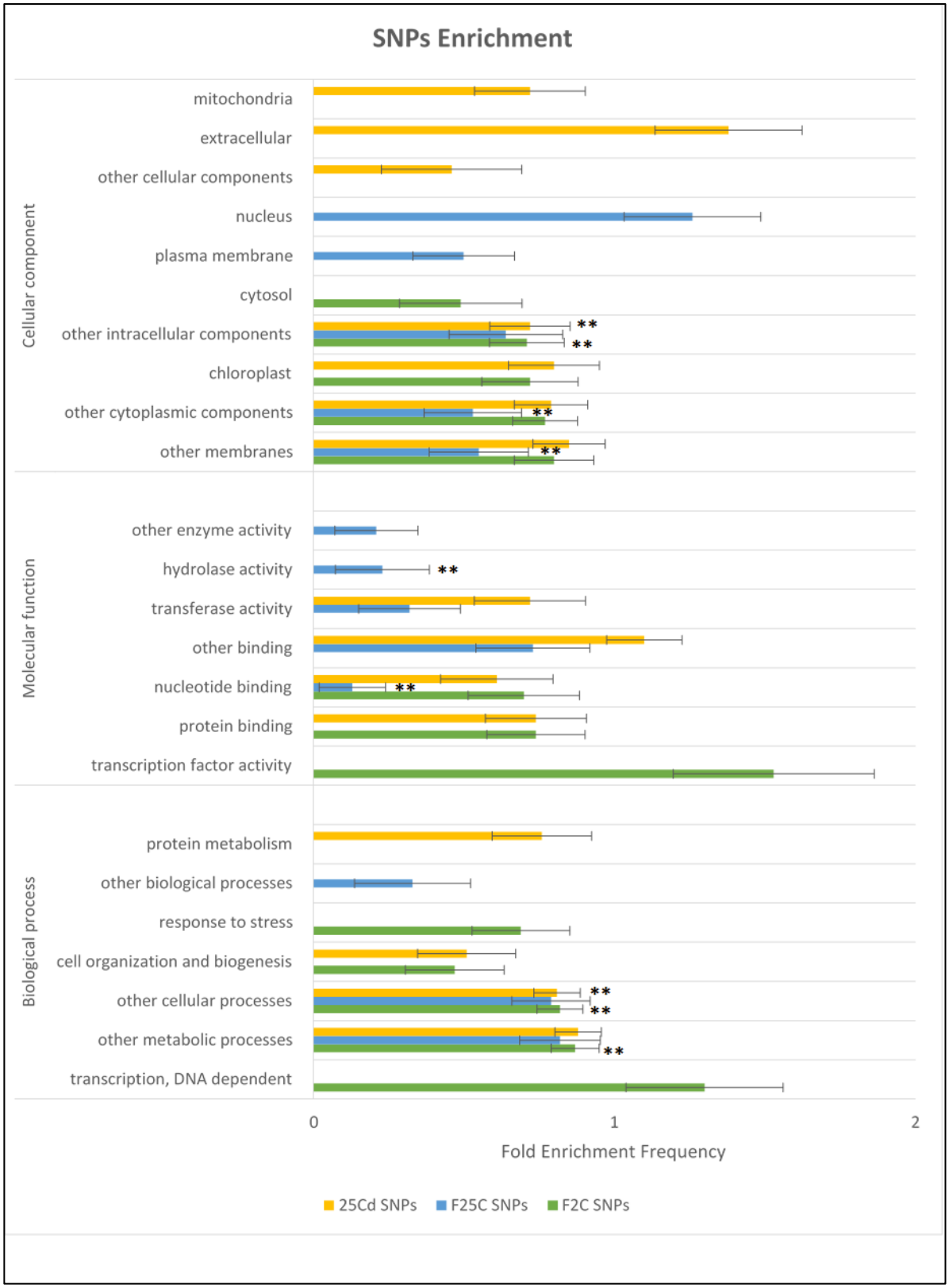

SNPs Enrichment
Cellular component
mitochondria
extracellular
other cellular components
nucleus
plasma membrane
cytosol
other intracellular components
chloroplast
other cytoplasmic components
other membranes
Molecular function
other enzyme activity
hydrolase activity
transferase activity
other binding
nucleotide binding
protein binding
transcription factor activity
Biological process
protein metabolism
other biological processes
response to stress
cell organization and biogenesis
other cellular processes
other metabolic processes
transcription, DNA dependent
**
0
1
2
Fold Enrichment Frequency
25Cd SNPs
F25C SNPs
F2C SNPs

Figure 16. An enrichment analysis of SNPs and associated genes and their classification based on biological processes. The X-axis represents the normalized frequency with binomial coefficients as calculated by SuperViewer. Genes are classed with p-values < 0.05, and two asterisks (**) is with $p < 0.01$, ±bootstrap StdDev.

When we further analyzed the SNPs to reveal the functions of the cold stressed progeny (F25Cd) specifically, we found that in the case of cellular components, other intracellular components showed the over-representation ($p<0.01$ scale) in the cold stressed progeny F25Cd compared with the parallel control progeny F25C. Similarly, with an interest in the biological process, F25Cd showed the over-representation ($p<0.01$) in other cellular processes, compared with F25C. However, other metabolic processes showed the over-representation ($p<0.01$) in the parental control progeny, F2C (figure 16).

4.2.4.2 INDELs Functional Enrichment Analysis in F2C, F25C, and F25Cd progenies

To analyze the potential meaning of differences in INDELs, the functional classification of genes associated with these INDELs was performed. Enrichment in three categories was analyzed: biological process, molecular function, and cellular component. Enrichment would represent a statistically significantly higher or lower number of INDELs in the specific category compared with a random occurrence in the *Arabidopsis* genome.

From the analysis of functional annotation of INDELs, in the case of cellular components analysis, only the cold stressed progeny (F25Cd) showed a statistically significant over-representation in cell walls and plastids. However, all three progenies showed over-representation in the chloroplast, other membranes, extracellular, other cytoplasmic components, other intracellular components, the nucleus, and other cellular components (figure 17).

In the case of molecular function, the cold stressed progeny (F25Cd) uniquely showed over-representation in kinase activity, whereas the parental control progeny (F2C) showed over-representation in transporter activity. All three progenies showed over-representation in other enzyme activity, transferase activity, other bindings, DNA or RNA bindings, and transcription factor activity (figure 17).

In the case of the biological process analysis, only the cold stressed progeny (F25Cd) showed over-representation in other biological processes, cell organization and biogenesis, and response to stress. All three progenies showed over-representation in other cellular processes, other metabolic processes, protein metabolism, transcription, DNA dependent, and signal transduction (figure 21).

Table 4. Functional Annotations of INDELs.

Enrichment		F2C INDELs	F25C INDELs	F25Cd INDELs
Biological process				
signal transduction	frequency	1.23	0.62	1.20
	SD	0.22	0.18	0.15
	p-value	0.034	0.018	0.021
transport	frequency	1.20	-	1.08
	SD	0.20		0.14
	p-value	0.031		0.042
transcription, DNA-dependent	frequency	1.12	1.38	1.38
	SD	0.15	0.20	0.13
	p-value	0.047	7.74E-03	3.22E-04
protein metabolism	frequency	1.10	0.89	0.91
	SD	0.14	0.13	0.08
	p-value	0.034	0.048	0.026
other metabolic processes	frequency	0.99	1.00	1.01
	SD	0.06	0.07	0.04
	p-value	0.039	0.045	0.027
other cellular processes	frequency	0.99	0.93	1.00
	SD	0.05	0.07	0.04
	p-value	0.039	0.028	0.03
developmental processes	frequency	0.87	-	1.19
	SD	0.14		0.12
	p-value	0.046		9.82E-03
response to abiotic or biotic stimulus	frequency	-	0.72	1.09
	SD		0.13	0.11
	p-value		0.016	0.031
response to stress	frequency	-	-	1.25
	SD			0.11
	p-value			1.30E-03
cell organization and biogenesis	frequency	-	-	0.85
	SD			0.12
	p-value			0.027
other biological processes	frequency	-	-	1.15
	SD			0.13
	p-value			0.013
Molecular function				
other molecular functions	frequency	1.54	-	1.28
	SD	0.33		0.23
	p-value	0.014		0.03
nucleic acid binding	frequency	1.39	1.36	-
	SD	0.24	0.26	
	p-value	0.011	0.025	
transporter activity	frequency	1.32	-	-
	SD	0.28		
	p-value	0.027		

transcription factor activity	frequency	1.22	1.62	1.53
	SD	0.21	0.26	0.17
	p-value	0.038	2.18E-03	1.08E-04
DNA or RNA binding	frequency	1.18	1.19	1.17
	SD	0.13	0.15	0.09
	p-value	0.015	0.021	5.66E-03
protein binding	frequency	1.17	-	1.11
	SD	0.12		0.09
	p-value	0.019		0.019
other binding	frequency	0.99	0.87	1.00
	SD	0.07	0.09	0.07
	p-value	0.044	0.023	0.034
transferase activity	frequency	0.89	0.70	0.90
	SD	0.12	0.13	0.08
	p-value	0.045	9.63E-03	0.028
other enzyme activity	frequency	0.86	0.89	0.87
	SD	0.08	0.11	0.07
	p-value	0.021	0.039	0.011
hydrolase activity	frequency	-	0.81	0.87
	SD		0.15	0.09
	p-value		0.034	0.023
nucleotide binding	frequency	-	0.58	0.91
	SD		0.14	0.10
	p-value		2.81E-03	0.037
kinase activity	frequency	-	-	1.15
	SD			0.17
	p-value			0.04
Cellular component				
other cellular components	frequency	1.22	1.63	1.34
	SD	0.26	0.31	0.20
	p-value	0.049	5.35E-03	8.00E-03
mitochondria	frequency	1.13	-	1.08
	SD	0.13		0.10
	p-value	0.032		0.031
nucleus	frequency	1.07	1.03	1.03
	SD	0.06	0.07	0.05
	p-value	0.019	0.042	0.024
other intracellular components	frequency	1.01	0.87	0.90
	SD	0.10	0.10	0.07
	p-value	0.045	0.025	0.014
other cytoplasmic components	frequency	0.94	0.96	0.97
	SD	0.07	0.08	0.06
	p-value	0.033	0.047	0.031
extracellular	frequency	0.84	0.83	1.05
	SD	0.14	0.14	0.11
	p-value	0.037	0.045	0.042
other membranes	frequency	0.83	0.91	0.89
	SD	0.09	0.10	0.06
	p-value	6.65E-03	0.036	9.86E-03
chloroplast	frequency	0.78	0.76	0.83

	SD	0.11	0.12	0.09
	p-value	9.92E-03	0.014	6.54E-03
Golgi apparatus	frequency	0.66	-	0.67
	SD	0.19		0.15
	p-value	0.035		0.013
plasma membrane	frequency	-	0.77	0.87
	SD		0.13	0.08
	p-value		0.019	0.018
cytosol	frequency	-	0.56	0.85
	SD		0.17	0.11
	p-value		6.65E-03	0.032
plastid	frequency	-	-	0.79
	SD			0.14
	p-value			0.027
cell wall	frequency	-	-	1.27
	SD			0.23
	p-value			0.042

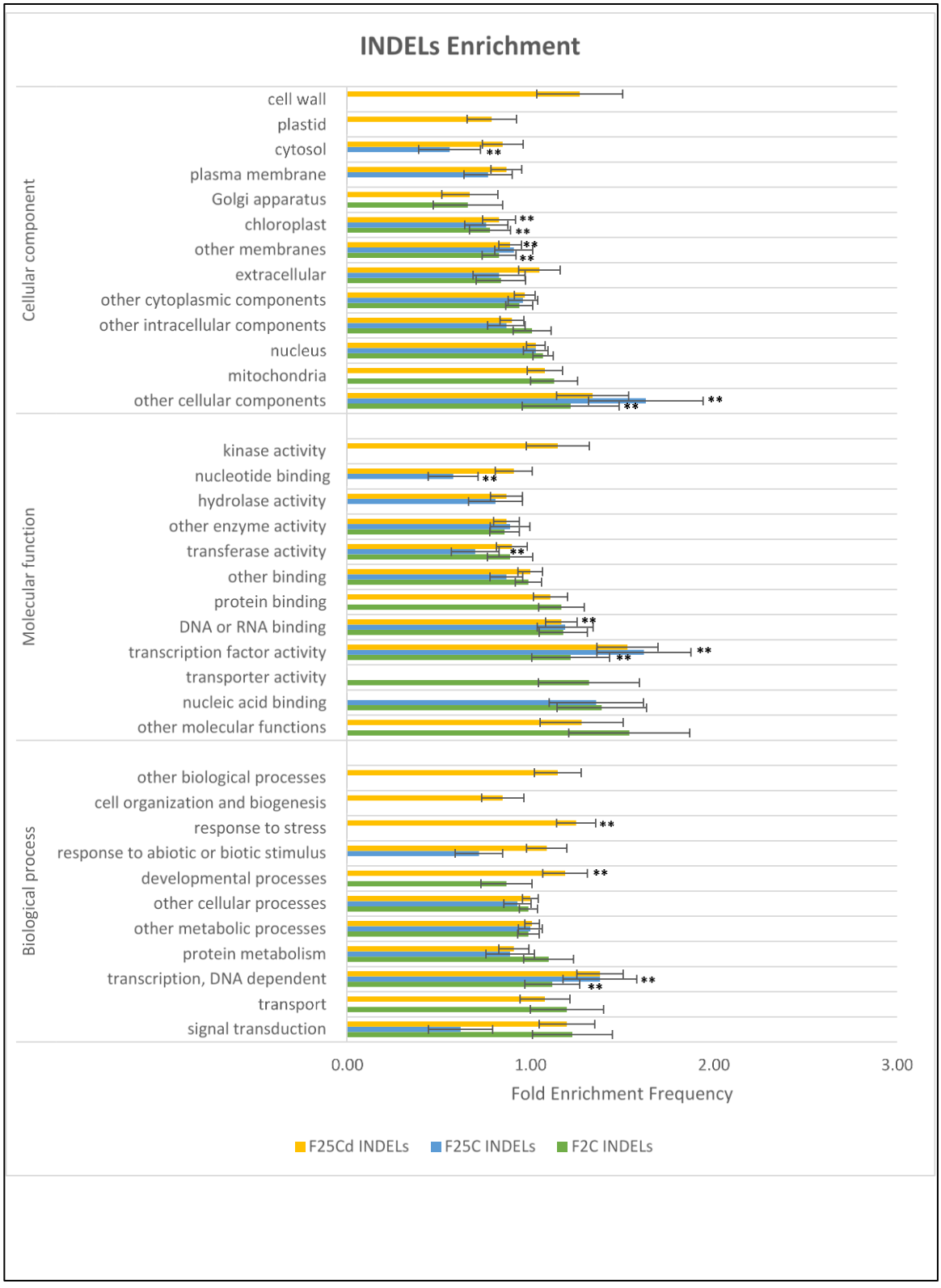

INDELs Enrichment
Cellular component
cell wall
plastid
cytosol
plasma membrane
Golgi apparatus
chloroplast
other membranes
extracellular
other cytoplasmic components
other intracellular components
nucleus
mitochondria
other cellular components
Molecular function
kinase activity
nucleotide binding
hydrolase activity
other enzyme activity
transferase activity
other binding
protein binding
DNA or RNA binding
transcription factor activity
transporter activity
nucleic acid binding
other molecular functions
Biological process
other biological processes
cell organization and biogenesis
response to stress
response to abiotic or biotic stimulus
developmental processes
other cellular processes
other metabolic processes
protein metabolism
transcription, DNA dependent
transport
signal transduction
0.00
1.00
2.00
3.00
Fold Enrichment Frequency
F25Cd INDELs
F25C INDELs
F2C INDELs

Figure 17. The enrichment analysis of INDELs and associated genes and their classification based on biological processes. The X-axis represents the normalized frequency with binomial coefficients as calculated by SuperViewer. Genes are classed with p-values < 0.05, and two asterisks (**) is with p < 0.01, ±bootstrap StdDev.

By further analyzing the functional annotations of INDELs with a particular interest in the cold stressed progeny (F25Cd), we found that in the case of biological process, a response to stress and developmental processes showed the over-representation ($p<0.01$) only in F25Cd compared with both F25C and F2C. Transcription, DNA-dependent function also showed the over-representation ($p<0.01$) in both F25Cd and F25C compared with the parental progeny control line, F2C. Moreover, the analysis done in the context of molecular functions associated with F25Cd, showed another notable over-representation ($p<0.01$) in F25Cd was DNA/RNA binding (figure 17).

4.2.6 SNPs and INDELs associated with transposons

Transposable elements are frequently activated in response to stress. Previous reports suggest evidence of changes in the regulation and activity of transposons in the progeny of plants exposed to stress. The average number of transposons associated with SNPs and INDELs revealed that the cold-stressed progeny (F25Cd) showed significantly higher numbers in comparison with the control progenies (F2C and F25C). But, when comparing controls (F2C and F25C), no statistically significant differences were found in SNPs and INDELs (tables 5-6). The data in Tables 5 and 6 show the summary of the average number of variant positions in all regions, the promoter and gene regions of transposable element genes, the enhancer regions, and the remaining other regions (tables 5-6).

The results revealed a higher number of SNPs associated with gene body compared with the promoter and enhancer, wherein in almost all cases, the cold stressed progeny showed a higher number of SNPs, except in the enhancer region (table 5). However, INDELs primarily occurred in the promoter region compared with the gene body and enhancer, wherein in all cases, the cold stressed progeny showed a higher INDELs number (table 6).

Table 5. The average number of transposons associated with SNPs.

Sample Type	Sample Id	All	Transposons				Others	Transposons% in SNPs
			Promoter	Gene	Enhancer	Total		
Control Progeny (F2C)	F2_ct1	9416	2437	2979	47	5463	3953	58.02
	F2_ct2	9151	2315	2782	39	5136	4015	56.13
	F2_ct3	9505	2356	2941	45	5342	4163	56.20
	F2_ct4	9324	2359	2971	29	5359	3965	57.48
	F2_ct5	9146	2279	2935	31	5245	3901	57.35
Average		9308	2349	2922	38	5309	3999	57.03
SD		159	59	80	8	124	100	0.83
Control Progeny (F25C)	F25_ct6	9532	2415	2976	48	5439	4093	57.06
	F25_ct7	9343	2334	2918	35	5287	4056	56.59
	F25_ct8	9348	2376	2937	29	5342	4006	57.15
	F25_ct9	9178	2358	2912	33	5303	3875	57.78
	F25_ct10	9392	2357	2986	42	5385	4007	57.34
Average		9359	2368	2946	37	5351	4007	57.18
SD		127	30	34	8	62	82	0.43
Cold stressed Progeny (F25Cd)	F25_cd1	9771	2458	3015	35	5508	4263	56.37
	F25_cd2	9735	2535	3098	35	5668	4067	58.22
	F25_cd4	9805	2407	3181	35	5623	4182	57.35
	F25_cd5	9676	2422	3110	36	5568	4108	57.54
	F25_cd6	9768	2491	3115	35	5641	4127	57.75
Average		9751	2463	3104	35	5602	4149	57.45
SD		49	52	59	0	64	76	0.68

Table 6. The average number of transposons associated with INDELs.

Sample Type	Sample Id	All	Transposons				Others	Transposons% in INDELs
			Promoter	Gene	Enhancer	Total		
Control Progeny (F2C)	F2_ct1	4295	385	347	124	856	3439	19.93
	F2_ct2	4213	355	320	125	800	3413	18.99
	F2_ct3	4424	405	343	127	875	3549	19.78
	F2_ct4	4479	396	328	133	857	3622	19.13
	F2_ct5	4338	397	335	115	847	3491	19.53
Average		4350	388	335	125	847	3503	19.47
SD		105	20	11	6	28	85	0.40
Control Progeny (F25C)	F25_ct6	4438	390	365	124	879	3559	19.81
	F25_ct7	4316	368	323	128	819	3497	18.98
	F25_ct8	4395	382	327	136	845	3550	19.23
	F25_ct9	4380	396	315	122	833	3547	19.02
	F25_ct10	4369	396	305	124	825	3544	18.88
Average		4380	386	327	127	840	3539	19.18
SD		44	12	23	6	24	24	0.37
Cold Progeny (F25Cd)	F25_cd1	4609	397	329	142	868	3741	18.83
	F25_cd2	4555	411	343	143	897	3658	19.69
	F25_cd4	4647	421	364	137	922	3725	19.84
	F25_cd5	4509	406	357	122	885	3624	19.63
	F25_cd6	4626	410	353	130	893	3733	19.30
Average		4589	409	349	135	893	3696	19.46
SD		56	9	14	9	20	52	0.40

4.3.1 Epigenomic Profiling

Whole Genome Bisulfite Sequencing (WGBS) was used to profile the epigenomic changes in the form of changes in DNA methylation patterns. For epigenomics, the distribution of Differentially Methylated Cytosines (DMCs) and Differentially Methylated Regions (DMRs) was analyzed to study responses to cold stress over multiple generations. Plants propagated independently from F2C (the parental control progeny), F25C (the parallel control progeny), and F25Cd (the cold-stressed progeny) have been studied. Five methylomes (biological replicates) stemming from individual plants in each of the generations (F2C, F25C, and F25Cd) were analyzed. In total, fifteen methylomes were analyzed in this study.

4.3.2 The Percentage of Global DNA Methylation

Global DNA Methylation data were obtained to explore the difference in total methylation in CpG, CHG, and CHH contexts in the tested generations. Bisulfite sequencing data revealed that the average percentage of global genome methylation was higher in F25Cd in the case of the CpG, and CHG contexts, but in the CHH context, F25Cd showed lower global methylation. The average percentage of global genome methylation in the CpG context was 23.46%, 23.64%, and 26.14%, respectively, in F2C, F25C, and F25Cd (figure 18). In the CHG context, the average percentage of global genome methylation was 6.92%, 6.77%, and 7.17% in F2C, F25C, and F25Cd, respectively (figure 18). Finally, the CHH context showed the average percentage of global genome methylation as 2.24%, 2.11%, and 2.01% in F2C, F25C, and F25Cd, respectively (figure 18). Furthermore, statistical tests revealed that only F25Cd showed a significant difference ($p<0.05$) compared with F2C in

the percentage of CpG global methylation (figure 18). Among all other comparisons in the CpG, CHG, and CHH contexts, global methylation showed no significant difference ($p > 0.05$).

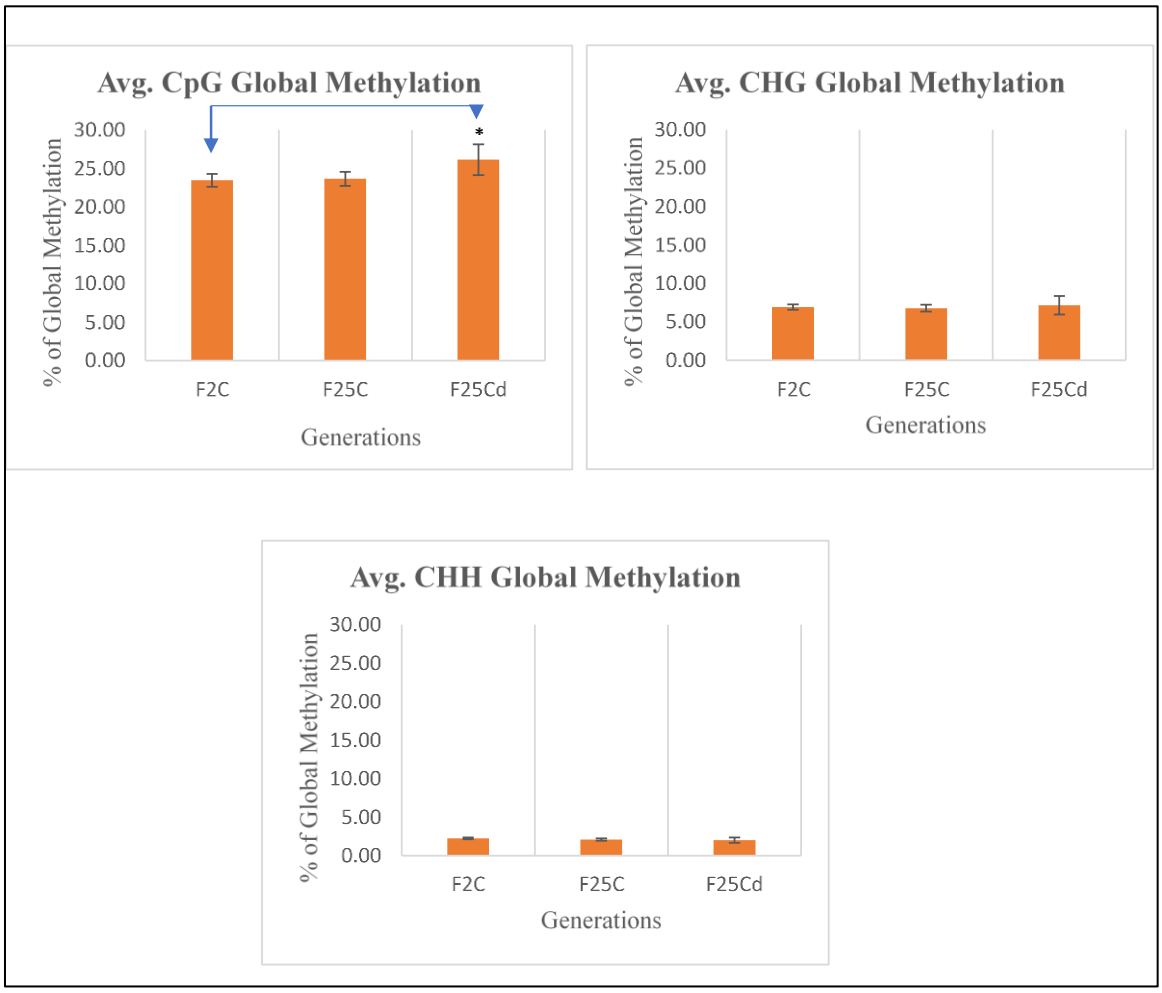


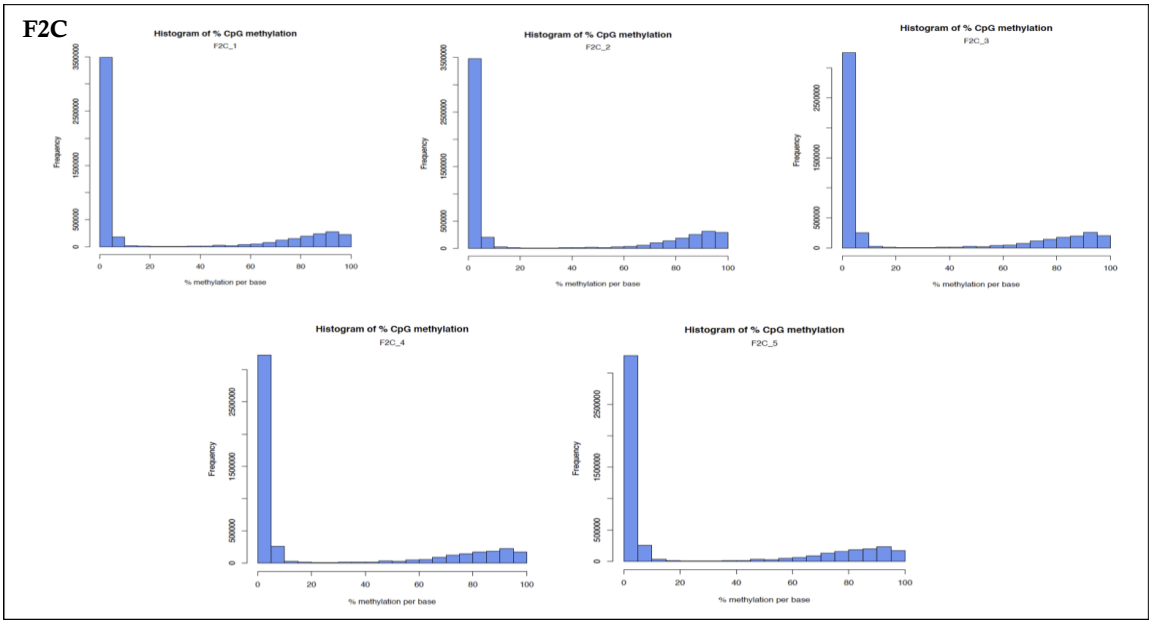
Figure 18. The average number of methylated cytosine (bases) identified with the corresponding percentages in F2C, F25C, and F25Cd in the CpG, CHG, and CHH sequence contexts (H=A, T, C and mean, n=5). F25Cd in the CpG region showed a statistically significant difference compared with the F2C, p-value < 0.05. Methylation levels were determined from reads with minimum coverage ≥ 10 mapped to TAIR 10 reference; data were analyzed by using Bismark.

Overall, the percentage of global methylation was higher in F25Cd in the CpG and CHG contexts. But only the percentage of global CpG methylation in F25Cd showed a statistical significance compared with F2C.

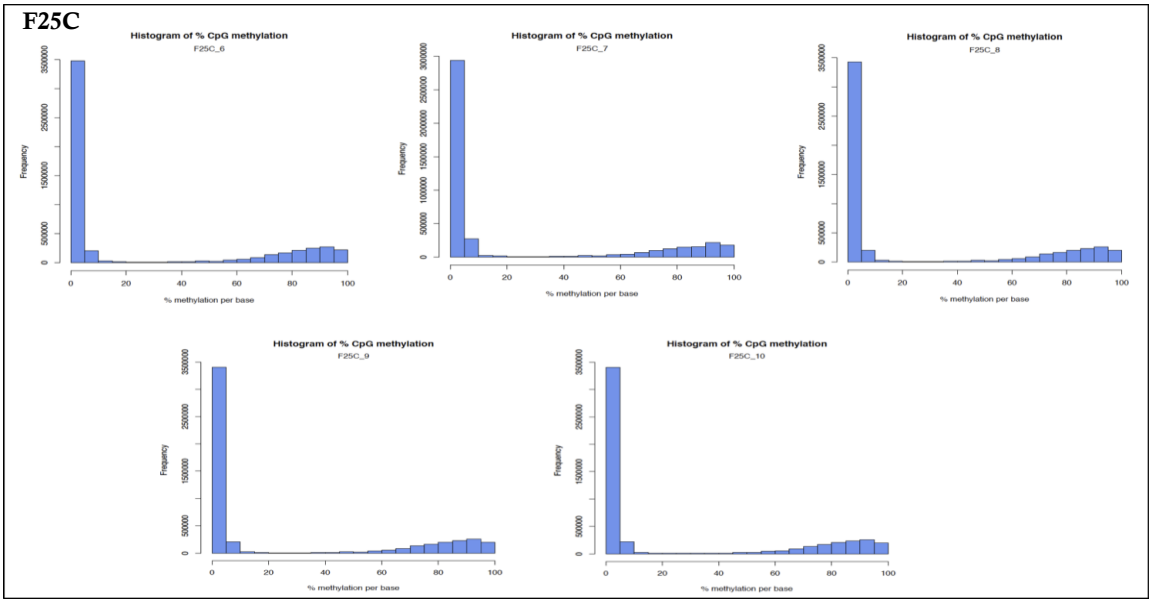
4.3.3 The Frequency Distribution of Methylation Percentages

The frequency distribution of methylation percentages in F2C, F25C, and F25Cd showed that most of the bases had either a high (70-100%) or a low (0-10%) level of methylation in the methylated CpG context (figure 19). Cytosine methylation in the CHG context was much lower in frequency compared with the CpG context; most sites had 0-20% of methylation level, and sites with high levels of methylation (70-100%) were not observed (figure 20). Similarly, methylation in the CHH context was much lower than in CpG or CHG; there was an even lower frequency of occurrence of sites with 10-20% methylation level (figure 21). In general, F2C, F25C, and F25Cd showed a similar distribution pattern of methylation percentages in the CpG, CHG, and CHH contexts (figures 19-21).

A.


F2C
Histogram of % CpG methylation
F2C_1
Histogram of % CpG methylation
F2C_2
Histogram of % CpG methylation
F2C_3
Histogram of % CpG methylation
F2C_4
Histogram of % CpG methylation
F2C_5
Frequency
% methylation per base

B.


F25C
Histogram of % CpG methylation
F25C_6
Histogram of % CpG methylation
F25C_7
Histogram of % CpG methylation
F25C_8
Histogram of % CpG methylation
F25C_9
Histogram of % CpG methylation
F25C_10
Frequency
% methylation per base

C.

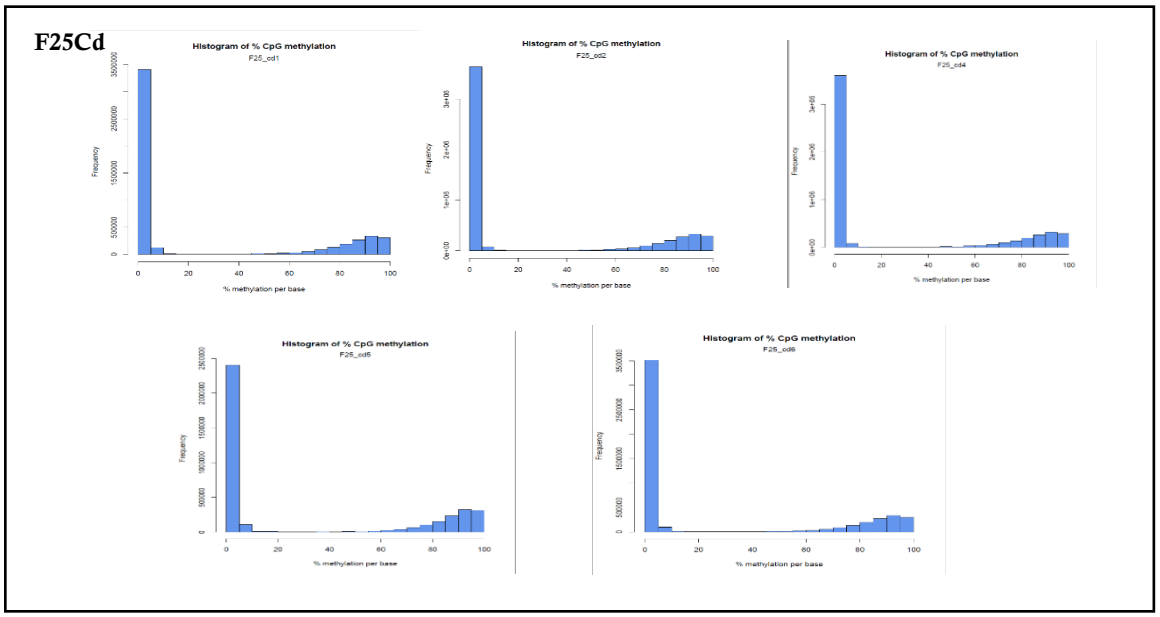
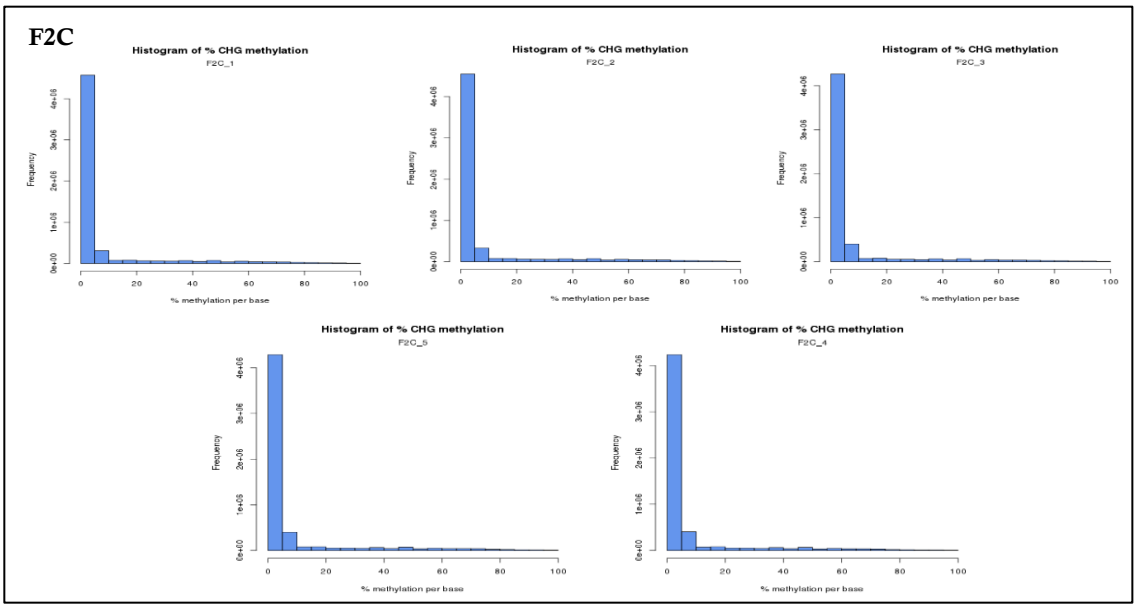
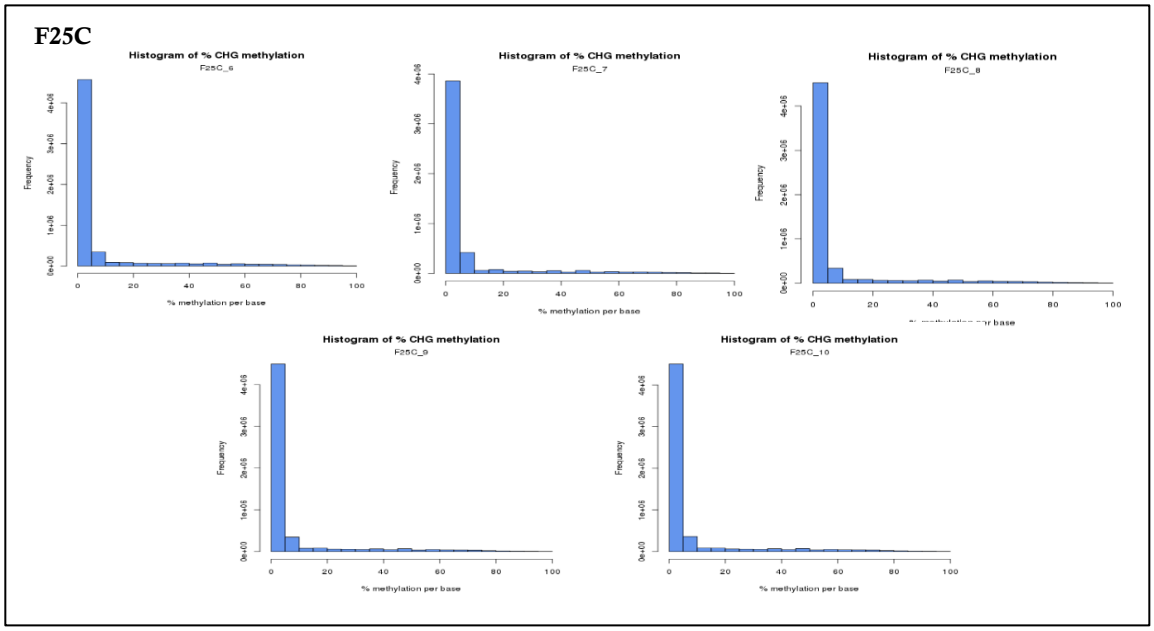


Figure 19. The frequency distribution of methylation percentage in the CpG sequence context of (A) F2C, (B) F25C, and (C) F25Cd; most of the bases have either a high or a low methylation based on bimodal distribution. The data have been normalized in Methylkit to account for clonal reads (PCR duplication bias), and each histogram represents a biological replicate where n=5 for F2C, F25C, and F25Cd.

A.


F2C
Histogram of % CHG methylation
F2C_1
Histogram of % CHG methylation
F2C_2
Histogram of % CHG methylation
F2C_3
Histogram of % CHG methylation
F2C_5
Histogram of % CHG methylation
F2C_4
Frequency
% methylation per base

B.


F25C
Histogram of % CHG methylation
F25C_6
Histogram of % CHG methylation
F25C_7
Histogram of % CHG methylation
F25C_8
Histogram of % CHG methylation
F25C_9
Histogram of % CHG methylation
F25C_10
Frequency
% methylation per base

C.

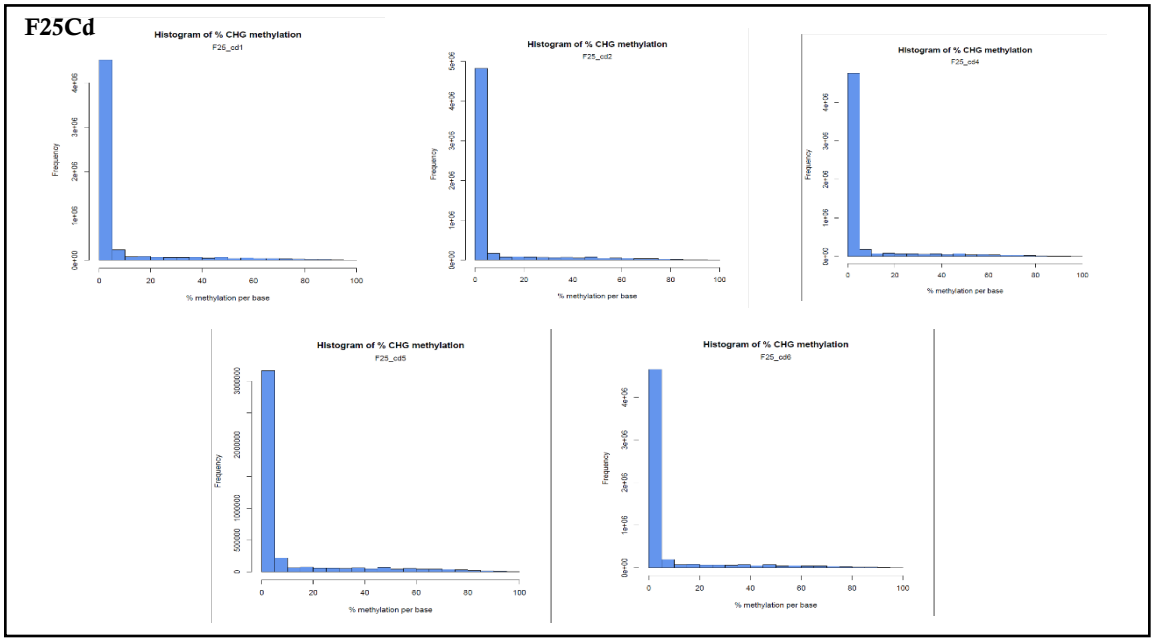
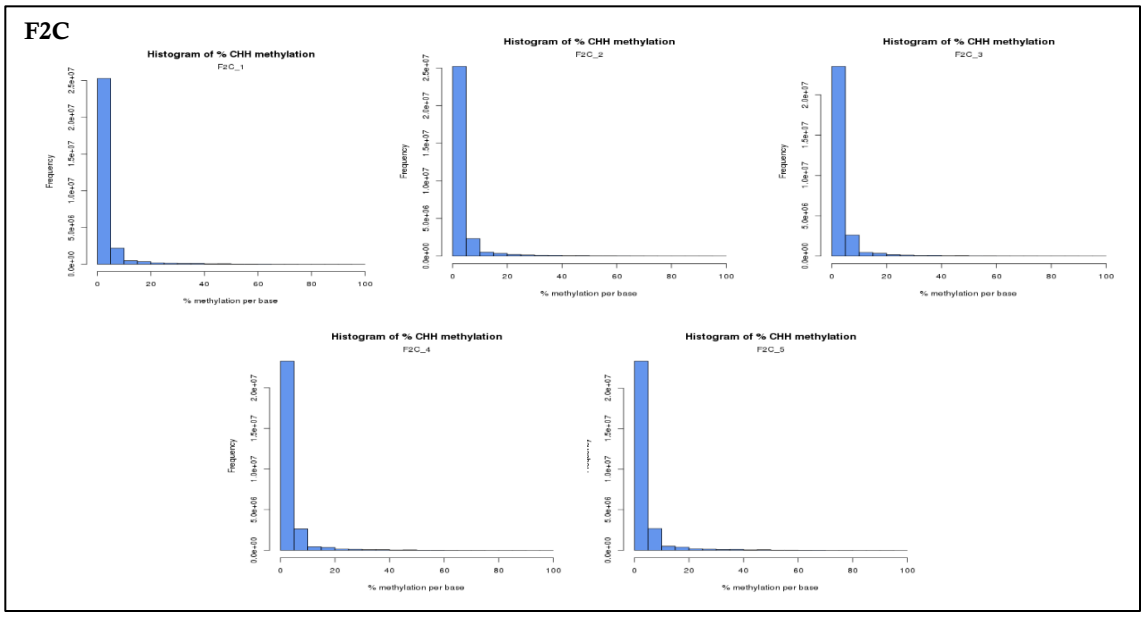
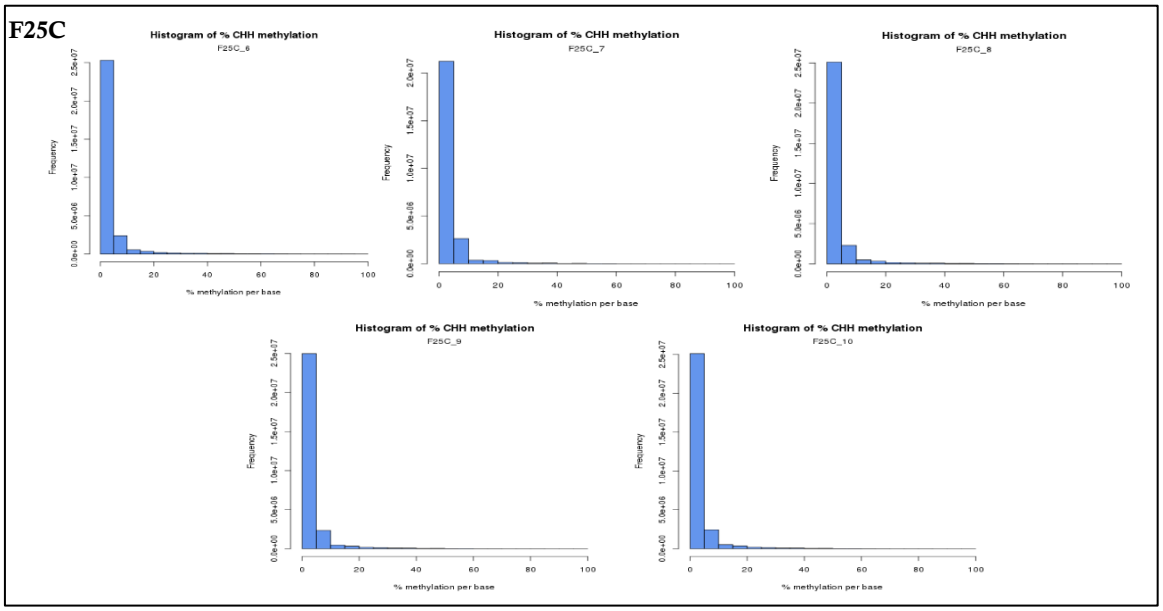


Figure 20. The frequency distribution of methylation percentage in the CHG sequence context (A) F2C, (B) F25C, and (C) F25Cd samples; most of the bases have low methylation levels based on bimodal distribution. The data have been normalized in Methylkit to account for clonal reads (PCR duplication bias), and each histogram represents a biological replicate mean n=5 for F2C F25C and F25Cd.

A.


F2C
Histogram of % CHH methylation
F2C_1
F2C_2
F2C_3
F2C_4
F2C_5
Frequency
% methylation per base

B.


F25C
Histogram of % CHH methylation
F25C_6
F25C_7
F25C_8
F25C_9
F25C_10
Frequency
% methylation per base

C.

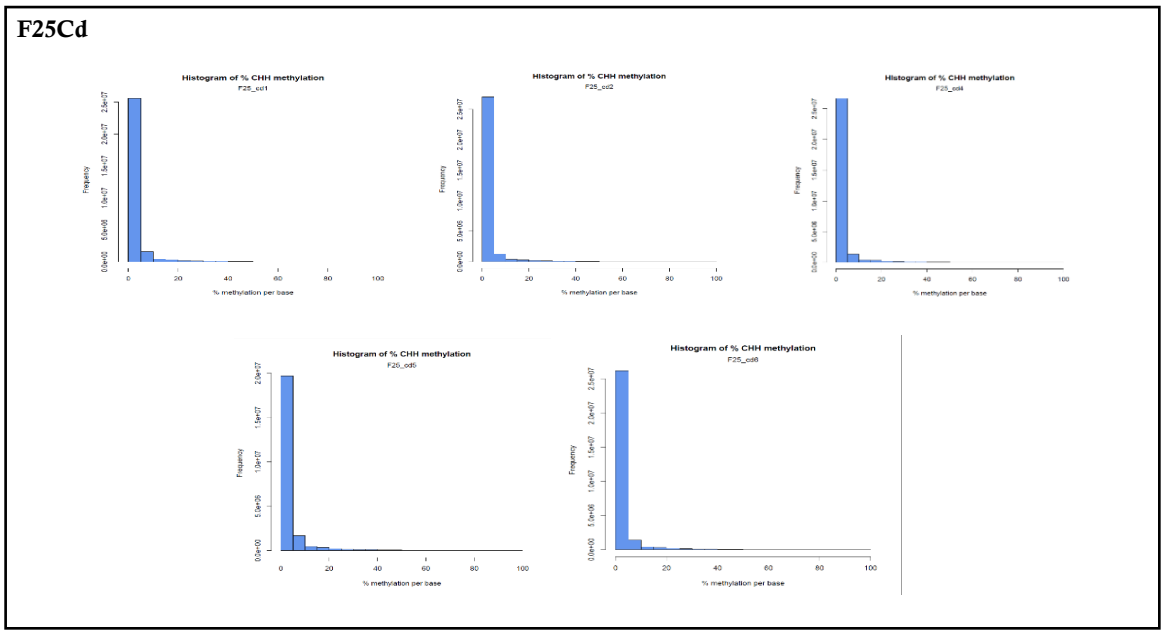


Figure 21. The frequency distribution of methylation percentage in the CHH sequence context for (A) F2C, (B) F25C, and (C) F25Cd samples; most of the bases have low methylation levels based on bimodal distribution. The data have been normalized in Methylkit to account for clonal reads (PCR duplication bias), and each histogram represents a biological replicate mean n=5 for F2C F25C and F25Cd.

4.3.4 The Total Number of Differentially Methylated Cytosines (DMCs) and Differentially Methylated Regions (DMRs)

The analysis of the total number of DMCs showed that the largest number of DMCs was observed in F25C vs. F2C comparison group – 80,464. Other comparisons showed 63,524 in F25Cd vs. F2C group and 77,648 in F25Cd vs. F25C group (table 7). This was somewhat surprising as we hypothesized that there would be more changes in methylation levels observed in the progeny of stressed plants (F25Cd) as compared with the controls.

The analysis of DMCs in a specific sequence context revealed a different pattern. While DMCs in the CpG context were more numerous in F25C vs. F2C comparison groups, in the CHG and CHH context, they were more numerous in comparison groups involving F25Cd (table 7). In fact, over a 10-fold difference was observed in the hypomethylated DMCs in the CHH context in F25Cd vs. F2C as compared with F25C vs. F2C – 1,894 vs. 176 DMCs, respectively (table 7). A similar picture was observed in the CHG context where over a 5-fold difference was observed – 1,189 vs. 230 DMCs.

These data suggest that the cold stress-induced epigenetic variations are primarily associated with changes in the CHG and CHH contexts, and that hypomethylation is a prevalent mechanism.

The analysis of the total number of DMRs in a 100-bp window showed 10,722 DMRs in F25Cd vs. F2C group and 14,524 DMRs in F25Cd vs. F25C group. Whereas comparison among the control groups F25C vs. F2C showed 12,619 DMRs. The analysis of the total number of DMRs in a 1,000-bp window showed 86 DMRs in F25Cd vs. F2C group, 67 DMRs in F25Cd vs. F25C group and 32 DMRs in F25C vs. F2C group (tables 8). In the

case of a 1,000-bp window, around a 3-fold difference was observed in DMRs in F25Cd vs. F2C group compared with F25C vs. F2C group. Similarly, a 2-fold difference was observed in DMRs in F25Cd vs. F25C group compared with F25C vs. F2C group (table 9).

Table 7. A comparison table of the total number of DMCs.

Samples Group	**Methylation Type**	**F25Cd vs. F2C**	**F25Cd vs. F25C**	**F25C vs. F2C**
CpG	Hypermethylation	37036	42058	44620
	Hypomethylation	21715	32535	34856
CHG	Hypermethylation	1006	893	495
	Hypomethylation	1189	768	230
CHH	Hypermethylation	684	1175	87
	Hypomethylation	1894	219	176
Total		63524	77648	80464

Table 8. A comparison table of the total number of DMRs (in 100-bp windows).

DMRs (100bp)	F25Cd vs. F2C	F25Cd vs. F25C	F25C vs. F2C
Hypermethylation	6859	8131	6991
Hypomethylation	3863	6393	5628
Total	10722	14524	12619

Table 9. A comparison table of the total number of DMRs (for 1000 bp windows).

DMRs (1000bp)	F25Cd vs. F2C	F25Cd vs. F25C	F25C vs. F2C
Hypermethylation	76	44	25
Hypomethylation	10	23	7
Total	86	67	32

4.3.5 Hierarchical Clustering in DMCs

Clustering of DMCs in F25Cd vs. F2C comparison group in the CpG and CHG contexts showed a clear separation of F25Cd samples and F2C samples (figure 22 and 24). In contrast, F25Cd vs. F25C group did not show such a clear separation (figure 23 and 25).

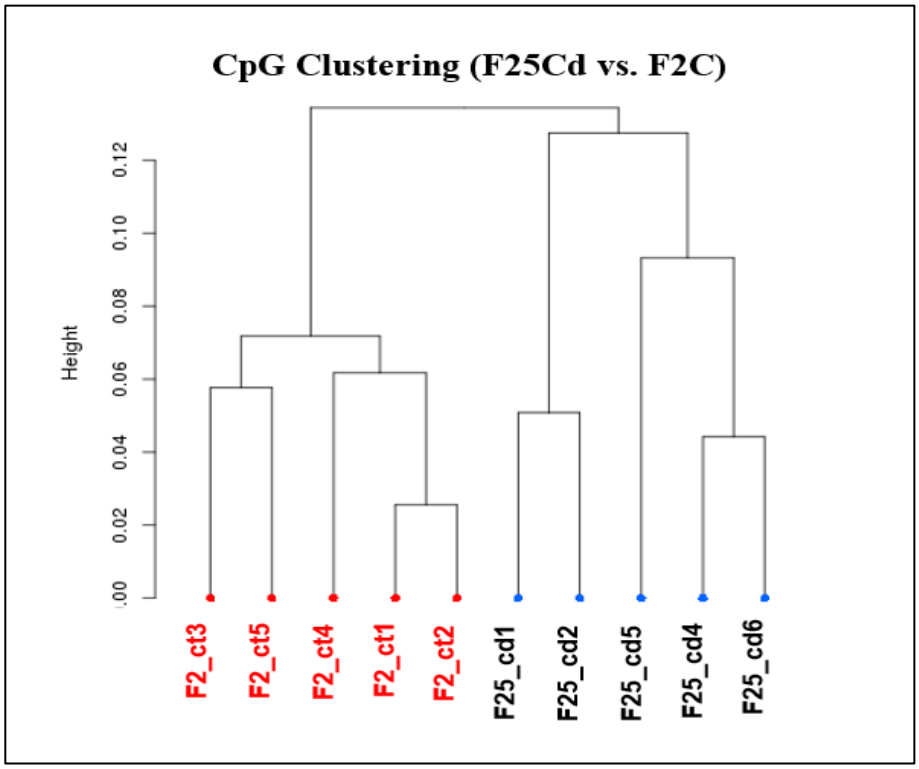


Figure 22. Global methylation clustering of DMCs in the CpG context (F25Cd vs. F2C).

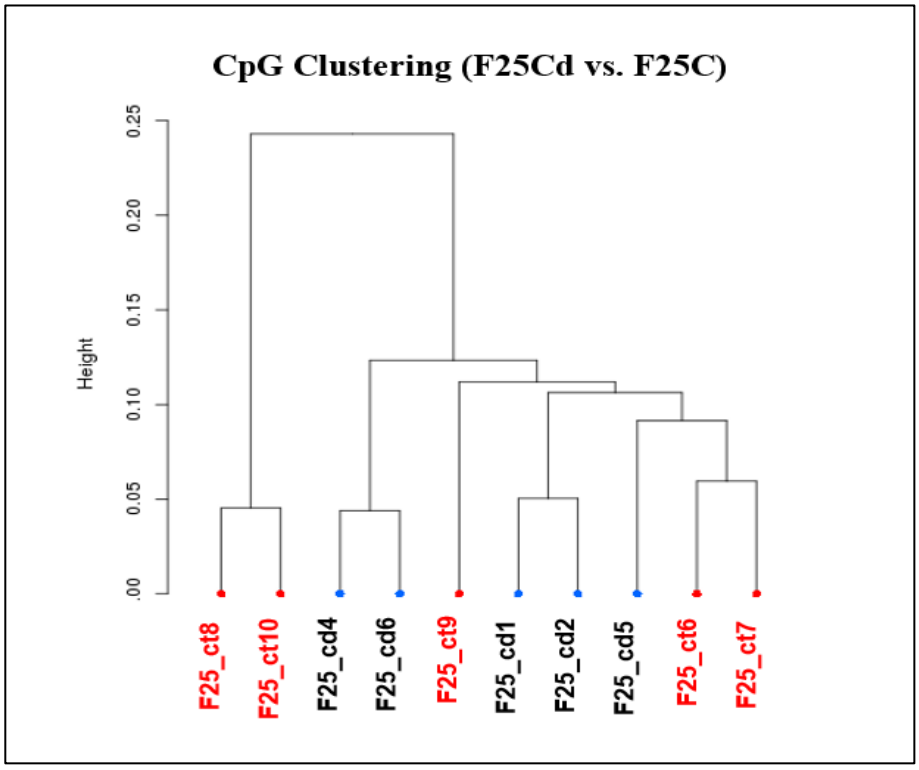


Figure 23. Global methylation clustering of DMCs in the CpG context (F25Cd vs. F25C).

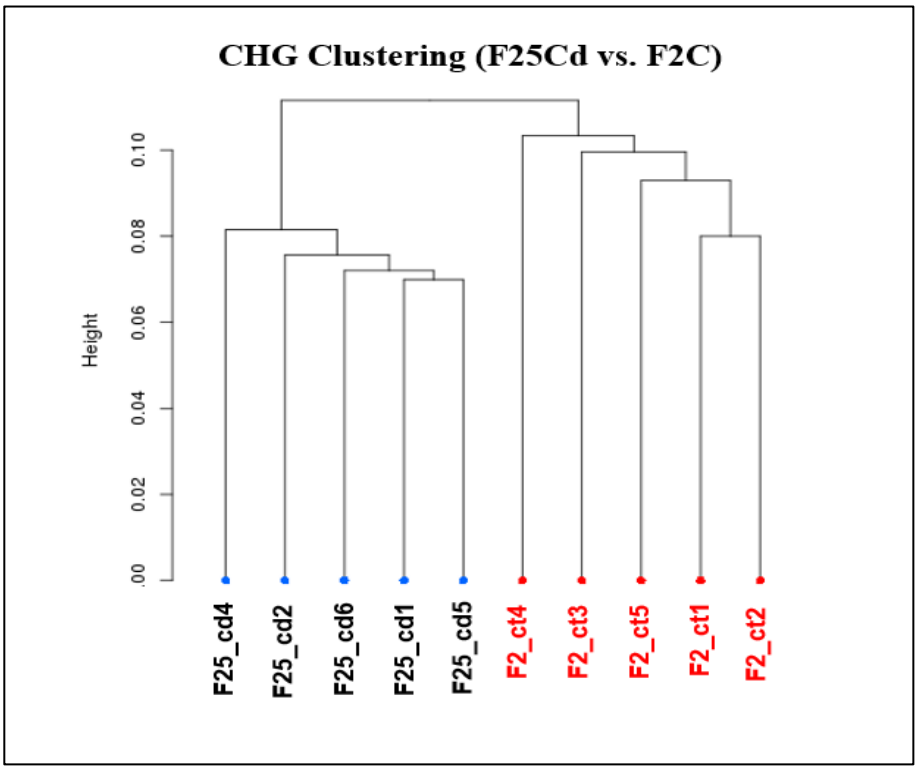


Figure 24. Global methylation clustering of DMCs in the CHG context (F25Cd vs. F2C).

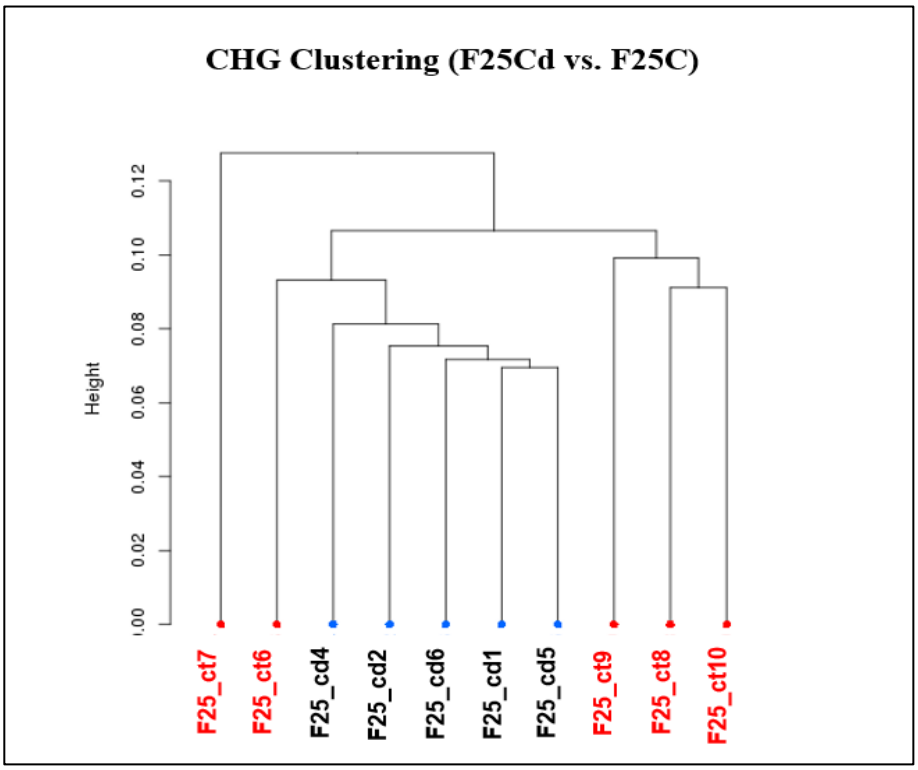


Figure 25. Global methylation clustering of DMCs in the CHG context (F25Cd vs. F25C).

4.3.6 Hierarchical Clustering (DMRs in 100-bp windows)

Clustering of DMRs in 100-bp windows in F25Cd vs. F2C comparison group in the CpG and CHG contexts showed a clear separation of F25Cd samples and F2C samples (figures 26 and 28). In contrast, the separation was not that clear in F25Cd vs. F25C group (figures 27 and 29).

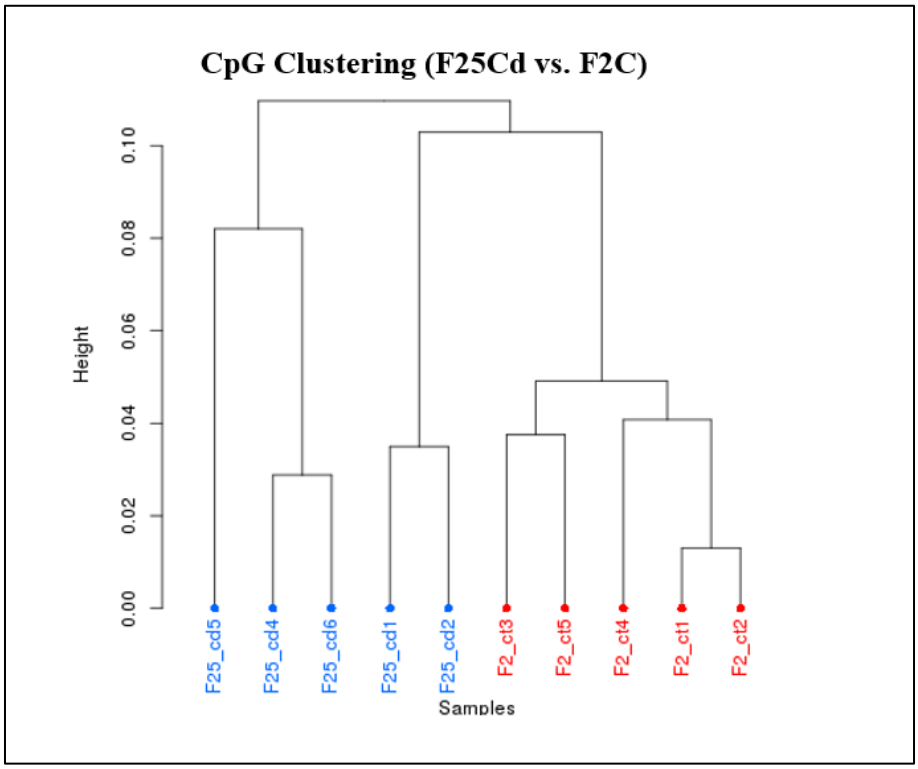


Figure 26. Global methylation clustering of DMRs in 100-bp windows in the CpG context (F25Cd vs. F2C).

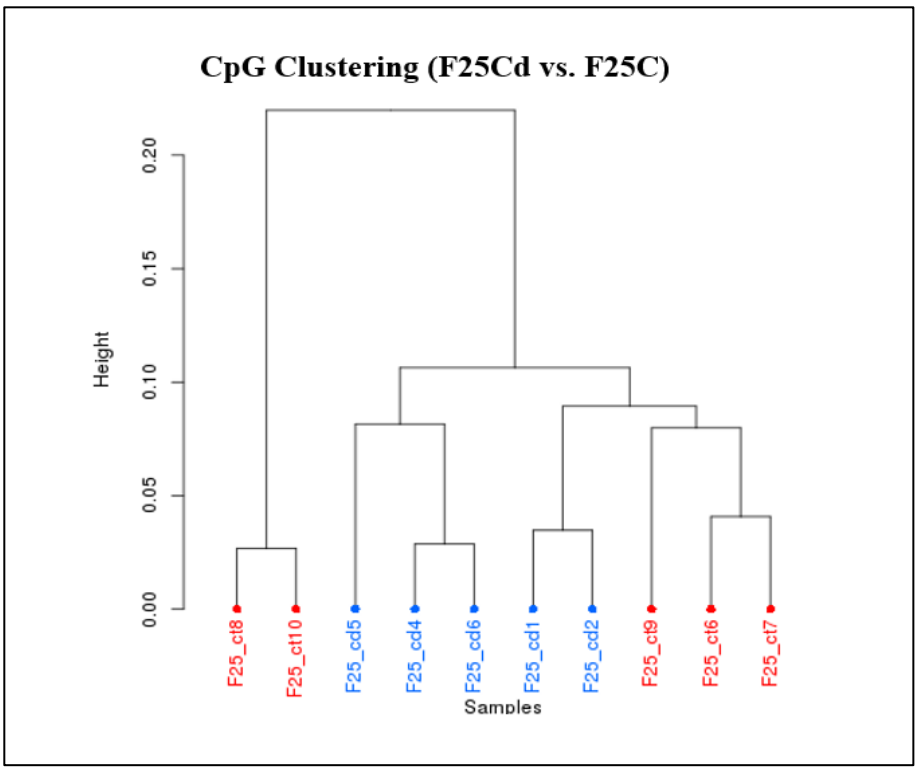


Figure 27. Global methylation clustering of DMRs in 100-bp windows in the CpG context (F25Cd vs. F25C).

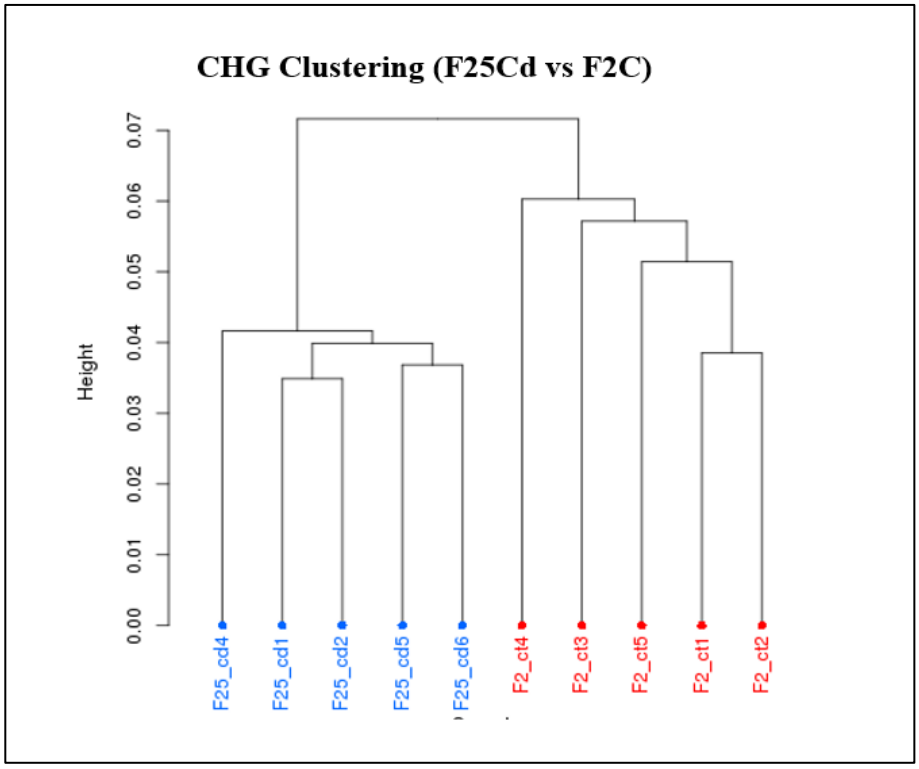


Figure 28. Global methylation clustering of DMRs in 100-bp windows in the CHG context (F25Cd vs. F2C).

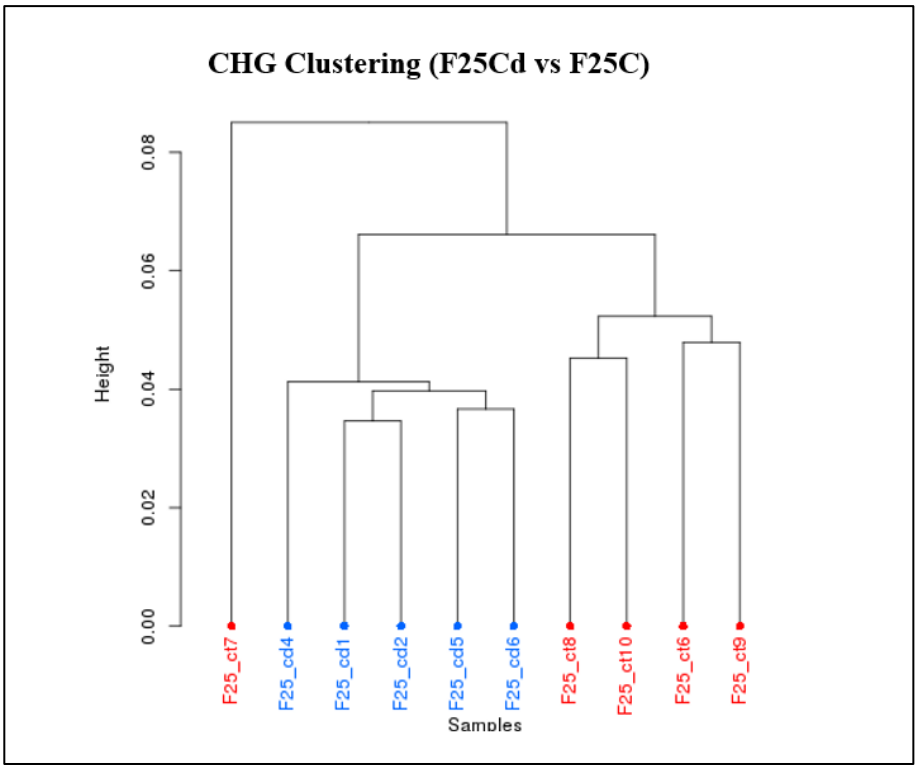


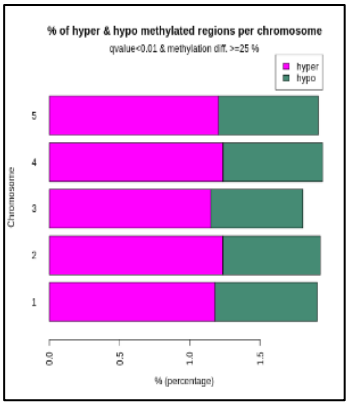
Figure 29. Global methylation clustering of DMRs in 100-bp windows in the CHG context (F25Cd vs. F25C).

4.3.7 The Distribution of Differentially Methylated Cytosines (DMCs) across the chromosomes

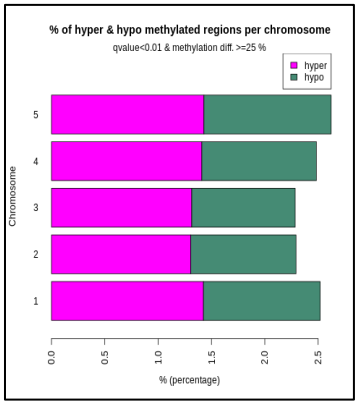
The analysis of changes in DMCs in CpG sites at the level of chromosome showed that there was a lower percentage (~2%) of methylated DMCs in F25Cd vs. F2C compared with F25Cd vs. F25C (~2.5%) or F25C vs. F2C (~2.3%) (figure 30A). The distribution of hypermethylated and hypomethylated regions was more or less equal in F25Cd vs. F25C or F25C vs. F2C comparison groups, while hypermethylated regions prevailed in F25Cd vs. F2C comparison group. In contrast, in CHG sites, the percentage of methylation was much higher in comparison groups involving F25Cd (~0.06% in F25Cd vs. F2C and ~0.05% F25Cd vs. F25C) compared with control groups F25C vs. F2C (~0.02%). In CHG sites, hypomethylation prevailed in F25Cd vs F2C comparison group compared with F25C vs. F2C group where hypermethylation prevailed (figure 30B). A similar picture was observed in CHH sites; there was a substantially higher percentage of DMCs in F25Cd vs. F2C (~0.013%) and F25Cd vs. F25C (~0.007%) compared with F25C vs. F2C group (~0.0012%). In the CHH context, hypomethylation prevailed dramatically in F25Cd vs. F2C and F25C vs. F2C groups compared with F25Cd vs. F25C where hypermethylation prevailed (figure 30C).

So, it appears that stress indeed causes more dramatic hypomethylation changes in CHG sites. These data are in agreement with the above-mentioned data on the number of DMCs found in different methylation contexts.

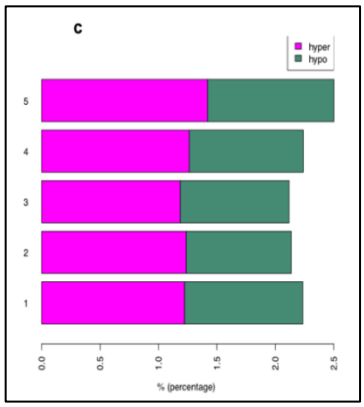
(A)



F2C_F25Cd (DMCs in CpG)

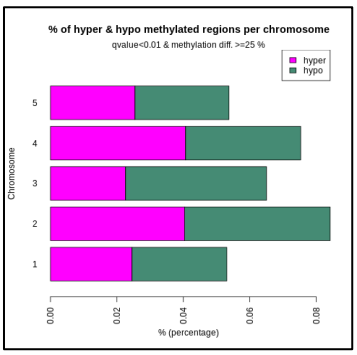


F25C_F25Cd (DMCs in CpG)

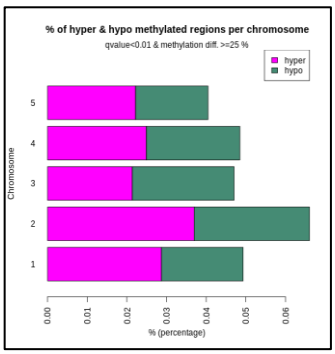


F2C_F25C (DMCs in CpG)

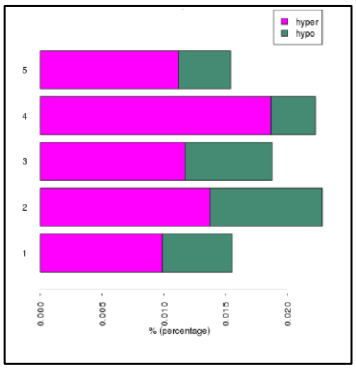
(B)



F2C_F25Cd (DMCs in CHG)

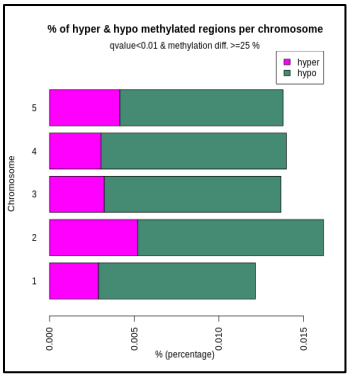


F25C_F25Cd (DMCs in CHG)

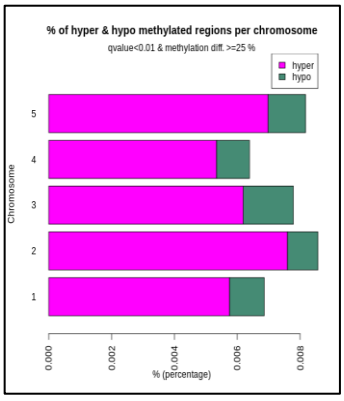


F25C_F2C (DMCs in CHG)

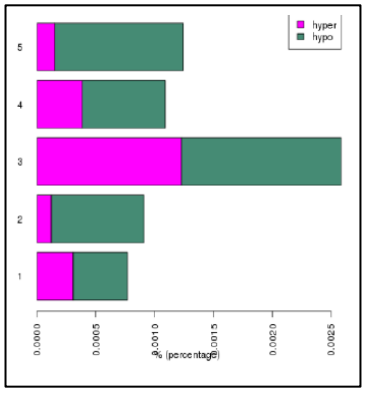
(C)



F2C_F25Cd (DMCs in CHH)



F25C_F25Cd (DMCs in CHH)



F25C_F2C (DMCs in CHH)

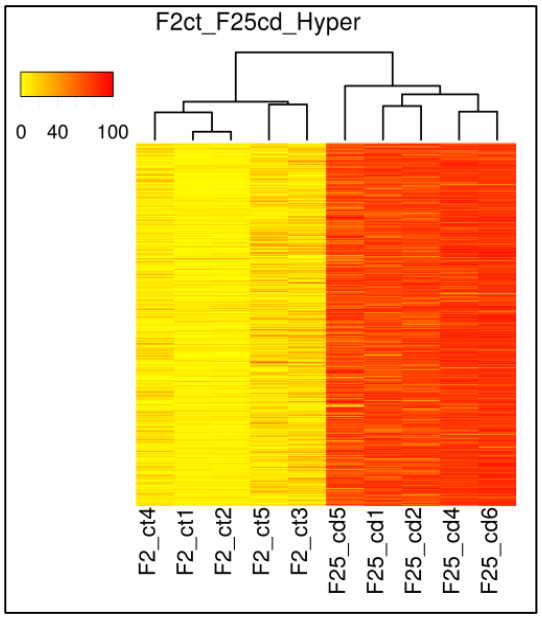
Figure 30. The distribution of Differentially Methylated Cytosines (DMCs) across the chromosomes. Distributions of differentially methylated bases are shown here in **(A)** the CpG context, **(B)** the CHG context, and **(C)** the CHH context, respectively; the context for each chromosome was identified in a 100-bp window. The horizontal bar plots show the number of methylated events per chromosome as a percent of sites with minimum coverage and differential. The pink section indicates the percentage of hypermethylation, and the green one indicates hypomethylation, q-value <0.01, and methylation difference > 25%.

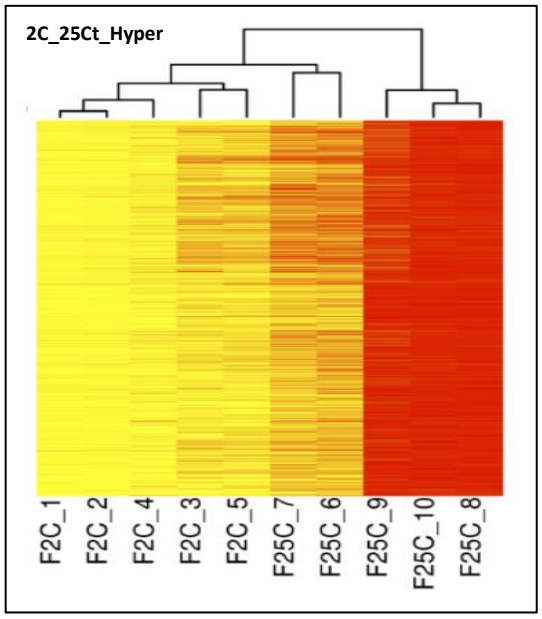
4.3.8 Heatmaps of Differentially Methylated Cytosines (DMCs)

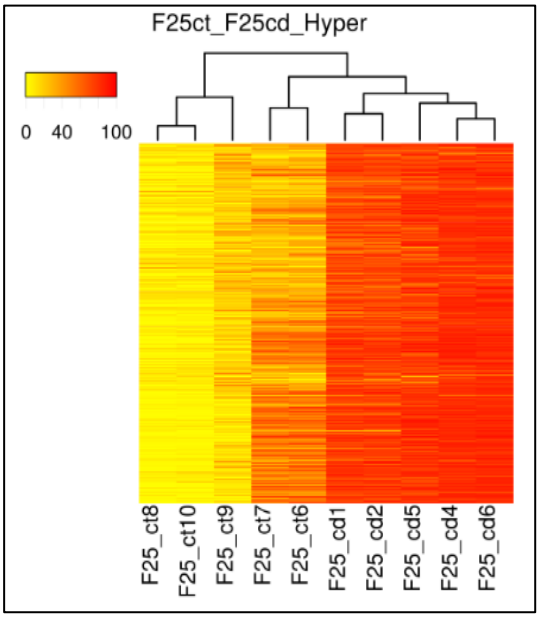
We also analyzed the relatedness of the samples using heatmaps and hierarchical clustering in the CpG, CHG and CHH sites. Plants were segregated by creating distinct groups and branching from their ancestral generations, as depicted in the heatmaps (figure 31).

When DMCs in CpGs were compared, clustering of hypo- and hypermethylated regions clearly separated F25Cd from F2C samples, whereas the separation of F25Cd samples from F25C samples and the separation of F25C from F2C samples were less clear (figure 31AB).

(A)


F2ct_F25cd_Hyper
0 40 100
F2_ct4
F2_ct1
F2_ct2
F2_ct5
F2_ct3
F25_cd5
F25_cd1
F25_cd2
F25_cd4
F25_cd6


2C_25Ct_Hyper
F2C_1
F2C_2
F2C_4
F2C_3
F2C_5
F25C_7
F25C_6
F25C_9
F25C_10
F25C_8


F25ct_F25cd_Hyper
0 40 100
F25_ct8
F25_ct10
F25_ct9
F25_ct7
F25_ct6
F25_cd1
F25_cd2
F25_cd5
F25_cd4
F25_cd6

(B)

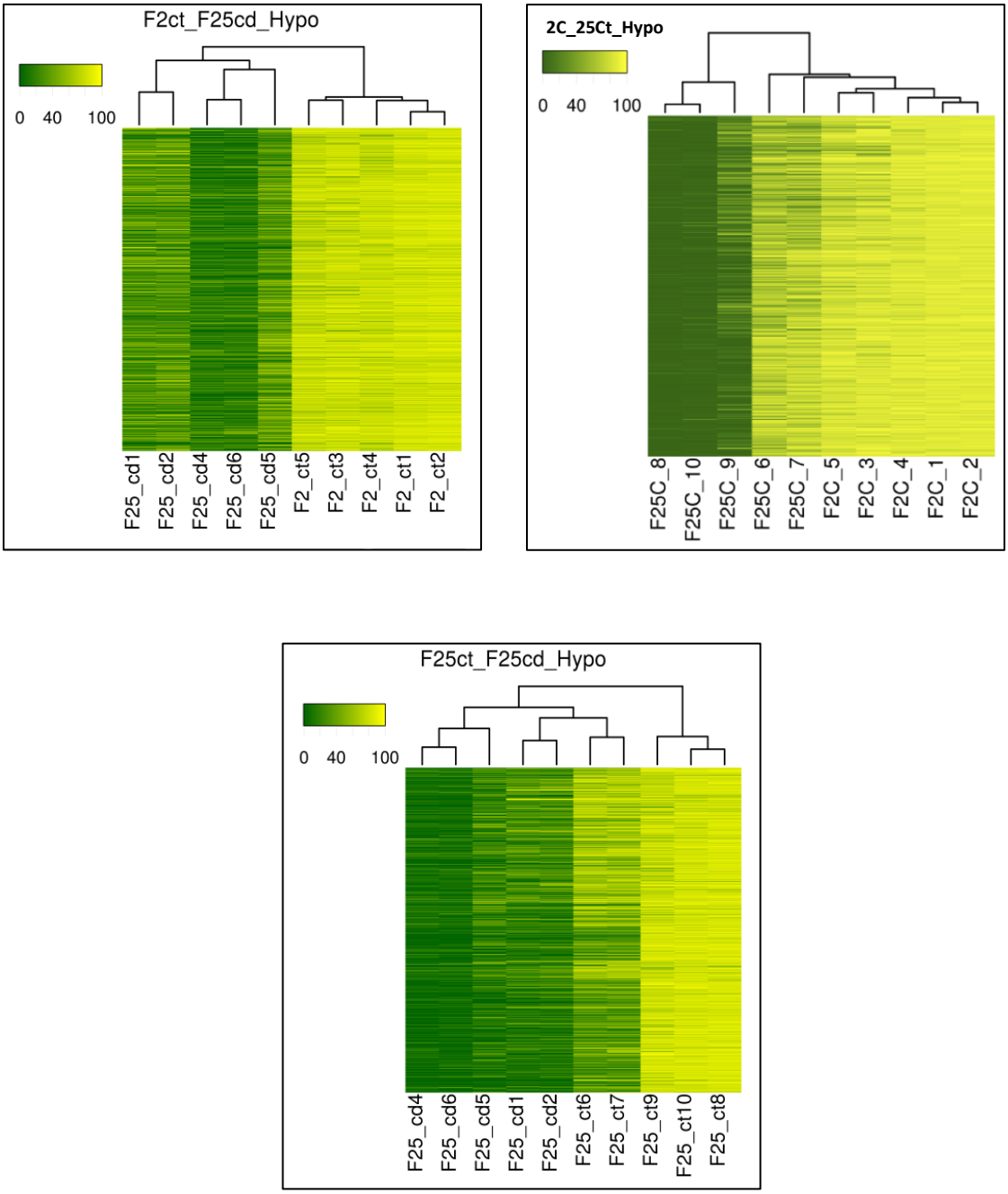


Figure 31. A comparison of heatmaps of differentially **(A)** hypermethylated and **(B)** hypomethylated cytosines in the CpG context; the comparison was done for the following groups: F2C vs. F25Cd, F2C vs. F25C and F25C vs. F25Cd (q-value <0.01).

4.3.9 Annotations of Differentially Methylated Cytosines (DMCs)

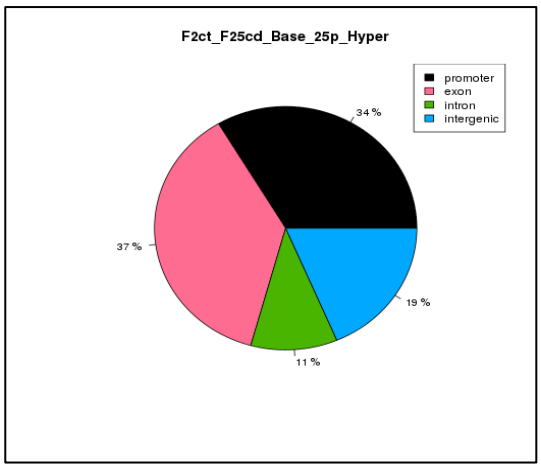
To decipher the impact of all observed differential methylation events, DMCs were further characterized to determine whether they were preferably located near genes. The location of hypo- or hypermethylated DMCs was compared to the annotated *Arabidopsis* genes using genomation.

The percentages of hypermethylated DMCs in all groups (F25Cd vs. F2C, F25Cd vs. F25C and F25C vs F2C) were the highest in the exon region (37%, 44% and 47%, respectively), then in the promoter region (34%, 26% and 30%, respectively), followed by the intron region (11%, 15% and 12%, respectively) and the intergenic region (19%, 13% and 11%, respectively) in CpG sites (figure 32A-C). Similarly, the percentages of hypomethylated DMCs sites were higher in gene bodies, especially in the exon regions followed by the promoter regions in CpG sites (Figure 32D-F). In the case of CHG sites, all groups (F25Cd vs. F2C, F25Cd vs. F25C and F25C vs F2C) showed the highest percentage of hypermethylated DMCs in the promoter (49%, 47% and 53%, respectively), then in intergenic regions (41%, 46% and 36%, respectively), followed by exon (8%, 5% and 7%, respectively) and intron (2%, 2% and 3%, respectively) regions (figure 33A-C). Interestingly, F25Cd vs. F2C and F25Cd vs. F25C groups showed the highest percentage of hypomethylated DMCs in intergenic regions (61% and 60%, respectively), followed by the promoter (35% and 34%, respectively) as compared with F25C vs F2C group where the highest percentage of DMCs was in the promoter (52%) followed by intergenic (44%) regions (figure 33D-F). In the CHH context, the percentage of both hyper- and hypo-DMCs was the higher in the promoter region then in the intergenic region followed by exon

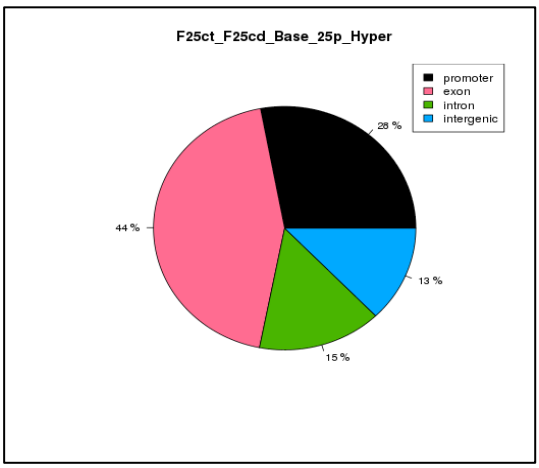
and intron regions in all comparison groups (figures 34A-F). Interestingly, the control group F25C vs. F2C showed the higher percentage of hyper- and hypomethylated DMCs (77% and 73%, respectively) in the promoter region compared with F25Cd vs. F2C (53% and 64%, respectively) and F25Cd vs. F25C (50% and 49%, respectively) groups in CHH sites.

Overall, in the case of the CpG context, the highest percentage of DMCs was observed in gene body, whereas in the case of the CHG and CHH contexts, it was in the highest in the promoter and intergenic regions.

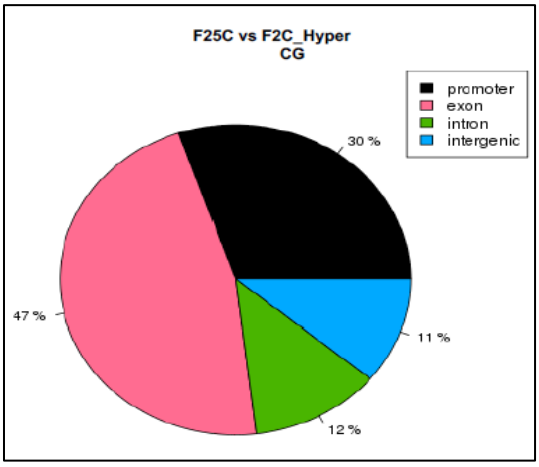
(A)



(B)



(C)



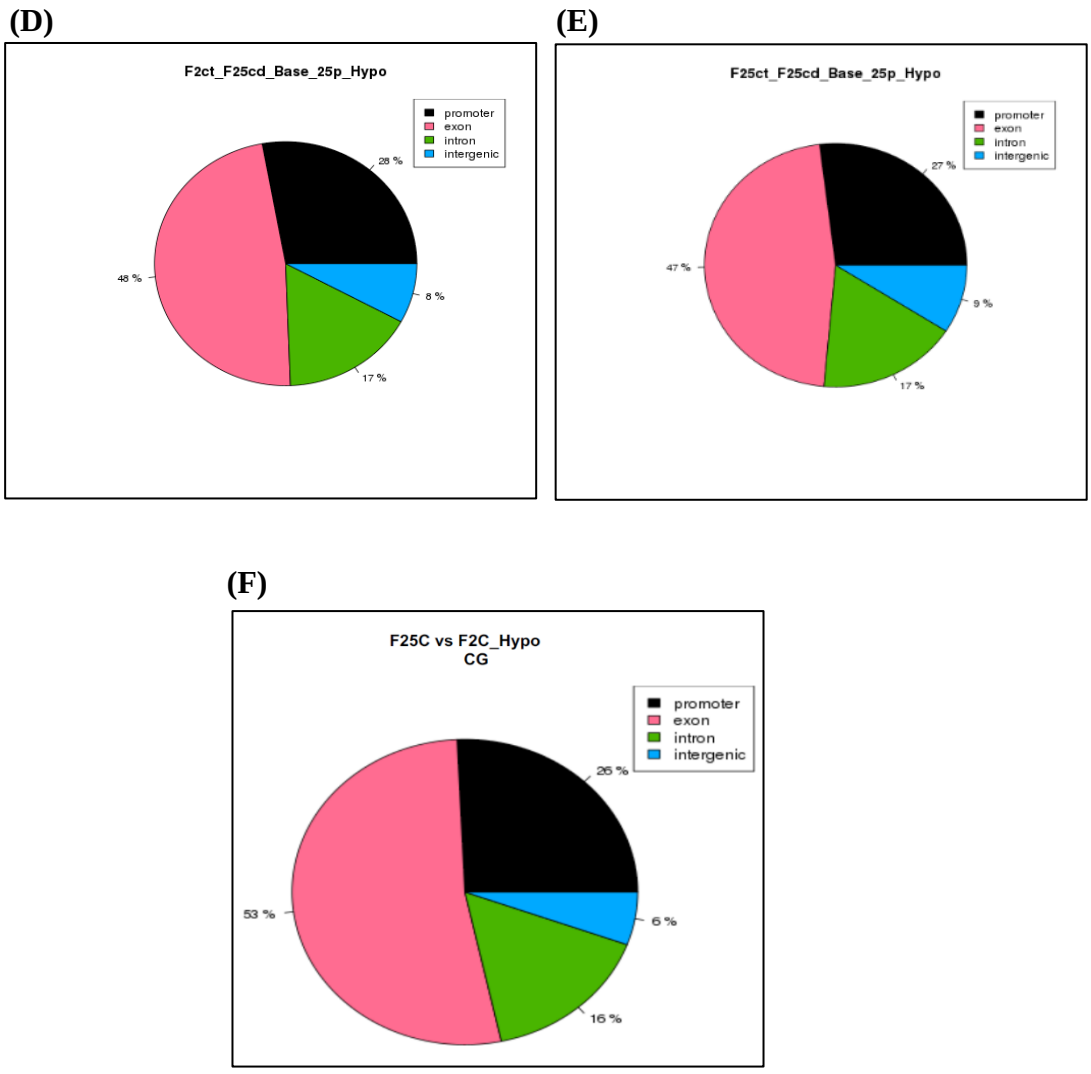
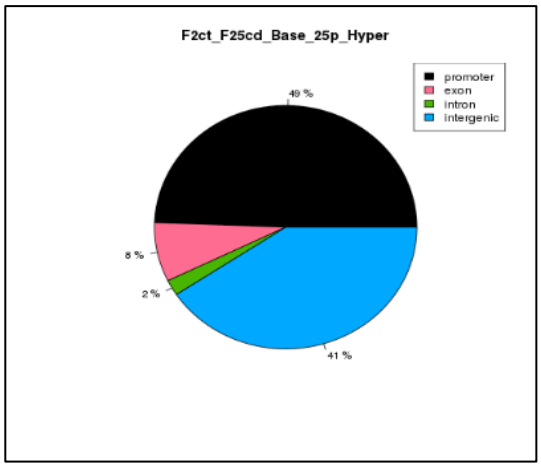
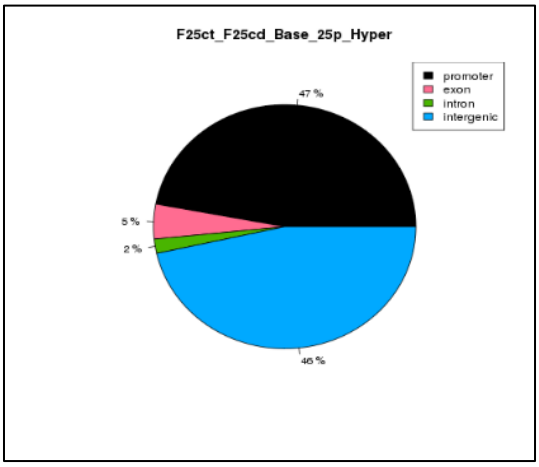


Figure 32. The percentages of differentially hypermethylated and hypomethylated DMCs in CpGs in different genomic regions in F2C vs. F25Cd, F25C vs. F25Cd, and F2C vs. F25C comparison groups. The percentages plotted are the average percentages of DMCs overlapping various genomics regions, including promoters, exons, introns and intergenic regions where DMCs were considered as regions with > 25% difference in methylation with the coverage of at least 10 sequence reads per DMC.

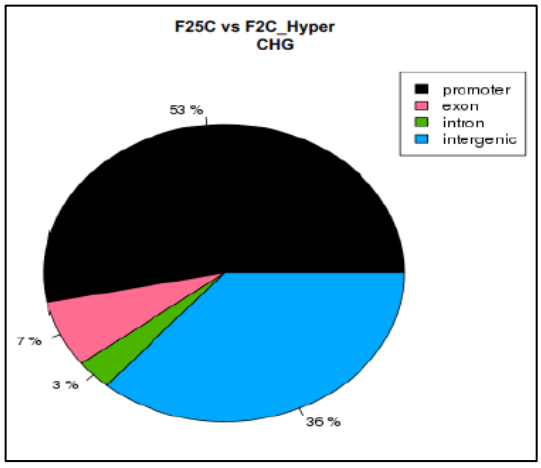
(A)


F2ct_F25cd_Base_25p_Hyper
promoter
exon
intron
intergenic
49 %
8 %
2 %
41 %

(B)


F25ct_F25cd_Base_25p_Hyper
promoter
exon
intron
intergenic
47 %
5 %
2 %
46 %

(C)


F25C vs F2C_Hyper
CHG
promoter
exon
intron
intergenic
53 %
7 %
3 %
36 %

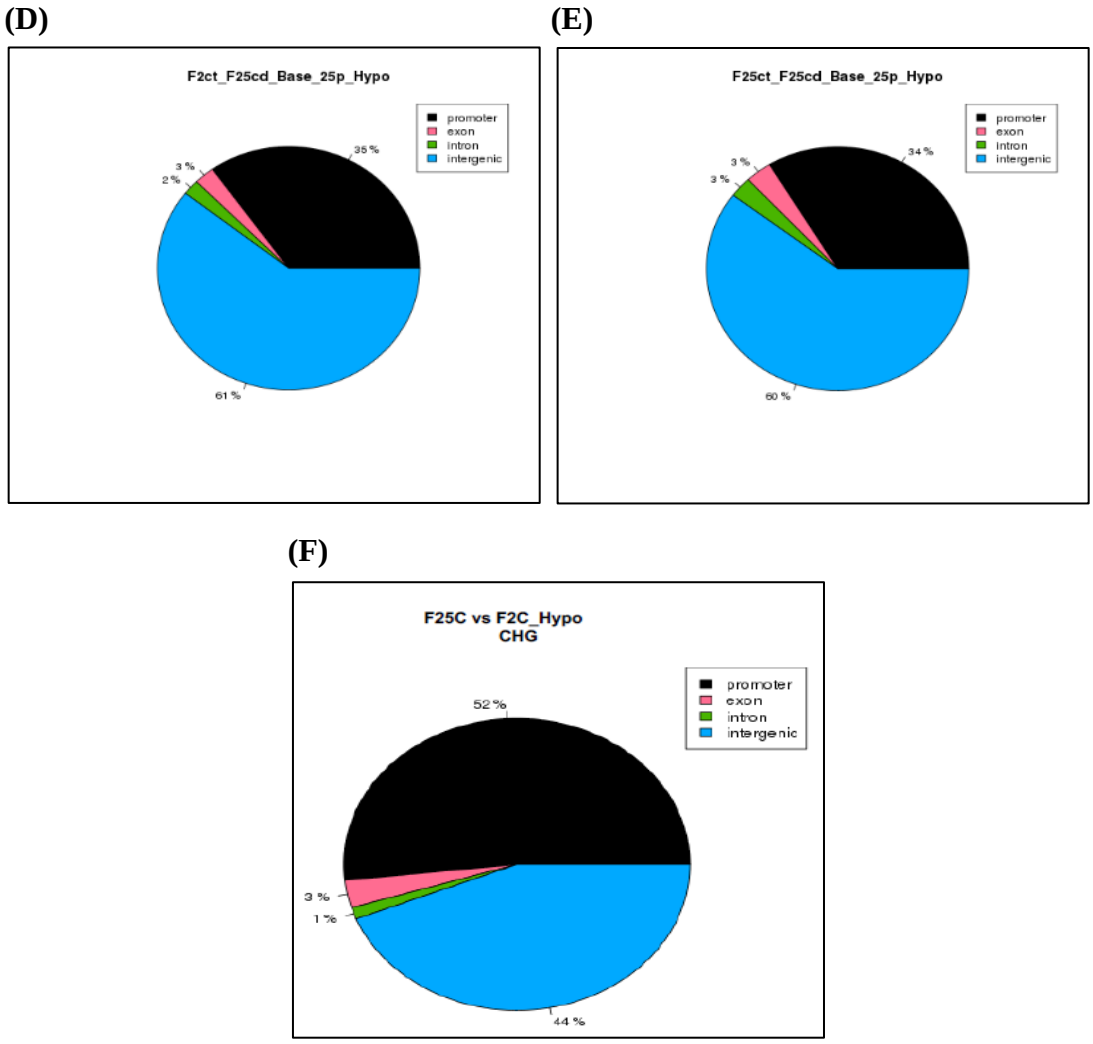
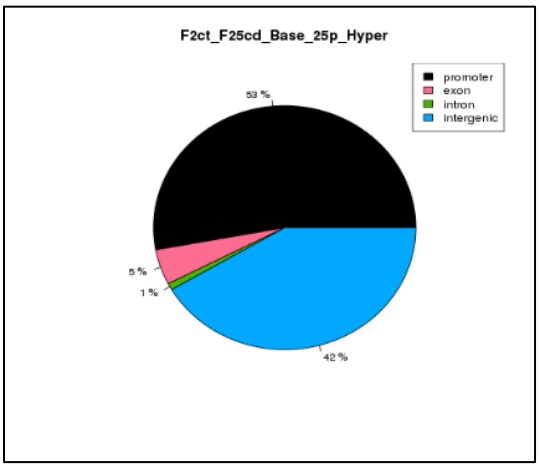
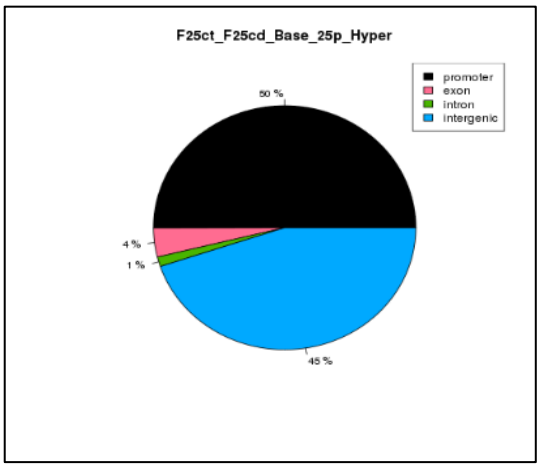


Figure 33. The percentages of differentially hypermethylated and hypomethylated DMCs in CHG in different genomic regions in F2C vs. F25Cd, F25C vs. F25Cd, and F2C vs. F25C comparison groups. The percentages plotted are the average percentages of DMCs overlapping various genomics regions, including promoters, exons, introns and intergenic regions where DMCs were considered as regions with > 25% difference in methylation with the coverage of at least 10 sequence reads per DMC.

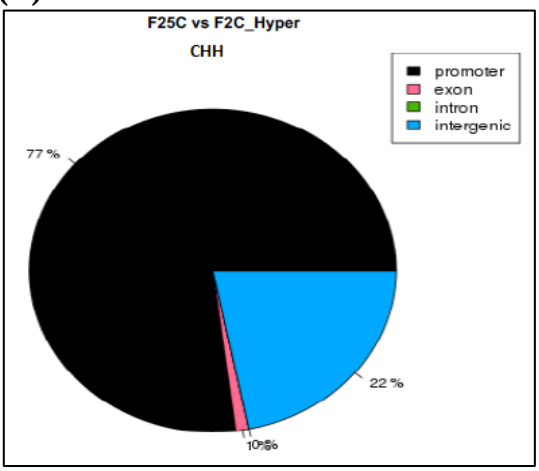
(A)


F2ct_F25cd_Base_25p_Hyper
promoter
exon
intron
intergenic
53 %
5 %
1 %
42 %

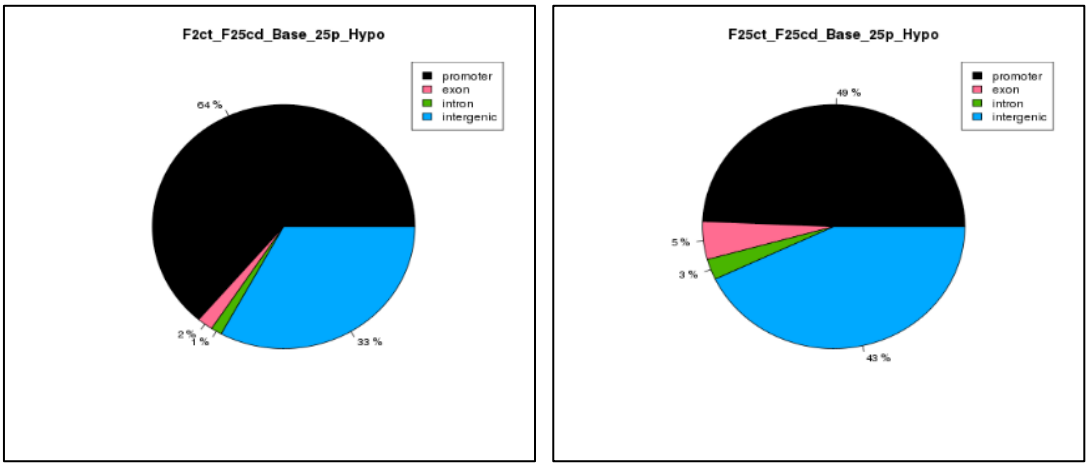
(B)


F25ct_F25cd_Base_25p_Hyper
promoter
exon
intron
intergenic
50 %
4 %
1 %
45 %

(C)


F25C vs F2C_Hyper
CHH
promoter
exon
intron
intergenic
77 %
22 %

(D) **(E)**



(F)

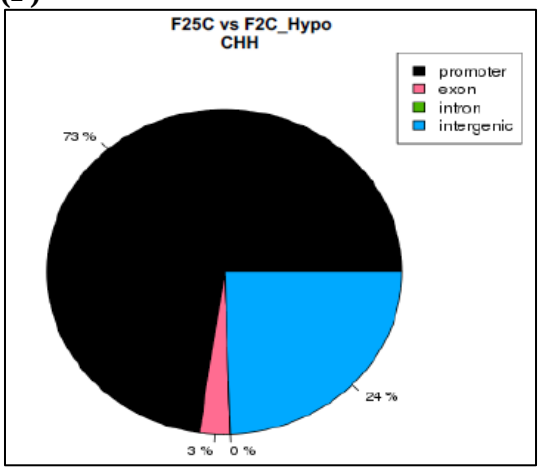


Figure 34. The percentages of differentially hypermethylated and hypomethylated DMCs in CHH in different genomic regions in F2C vs. F25Cd, F25C vs. F25Cd, and F2C vs. F25C comparison groups. The percentages plotted are the average percentages of DMCs overlapping various genomics regions, including promoters, exons, introns and intergenic regions where DMCs were considered as regions with > 25% difference in methylation with the coverage of at least 10 sequence reads per DMC.

4.3.10 Biological Enrichment Analysis

The functional classification of variants, DMRs and DMCs unique to each test group, was interpreted using SuperViewer to identify regions with the statistically over-represented numbers of genes and genomic features. The biological processes that might be enriched or under-represented within and between generations were assessed. All values were normalized by bootstrap x100, and p-values < 0.05 only were retrieved as significant. The x-axis is the normalized frequency with binomial coefficients as calculated by SuperViewer. The biological enrichment analysis of hyper- or hypomethylated DMRs in 100-bp windows in the CpG, CHG, and CHH contexts and their functional classification were analyzed based on the biological processes.

The biological enrichment analysis revealed that CpG hyper- and hypomethylation in DMRs sites were enriched in many general and specific biological processes. More specifically, when F25Cd were compared with F2C and F25C, CpG hypermethylation sites in F25Cd were statistically significant over-represented in the biological processes such as responses to abiotic and biotic stimuli, cell organization and biogenesis, responses to stresses, and transport processes. (figure 35).

On the other hand, interestingly, when control groups (F25C vs. F2C) were compared using the biological enrichment analysis of DMRs CpG hypermethylation, several biological processes showed a statistically significant over-representation in the functions such as DNA or RNA metabolisms, transcription and DNA dependent and development processes (figure 35).

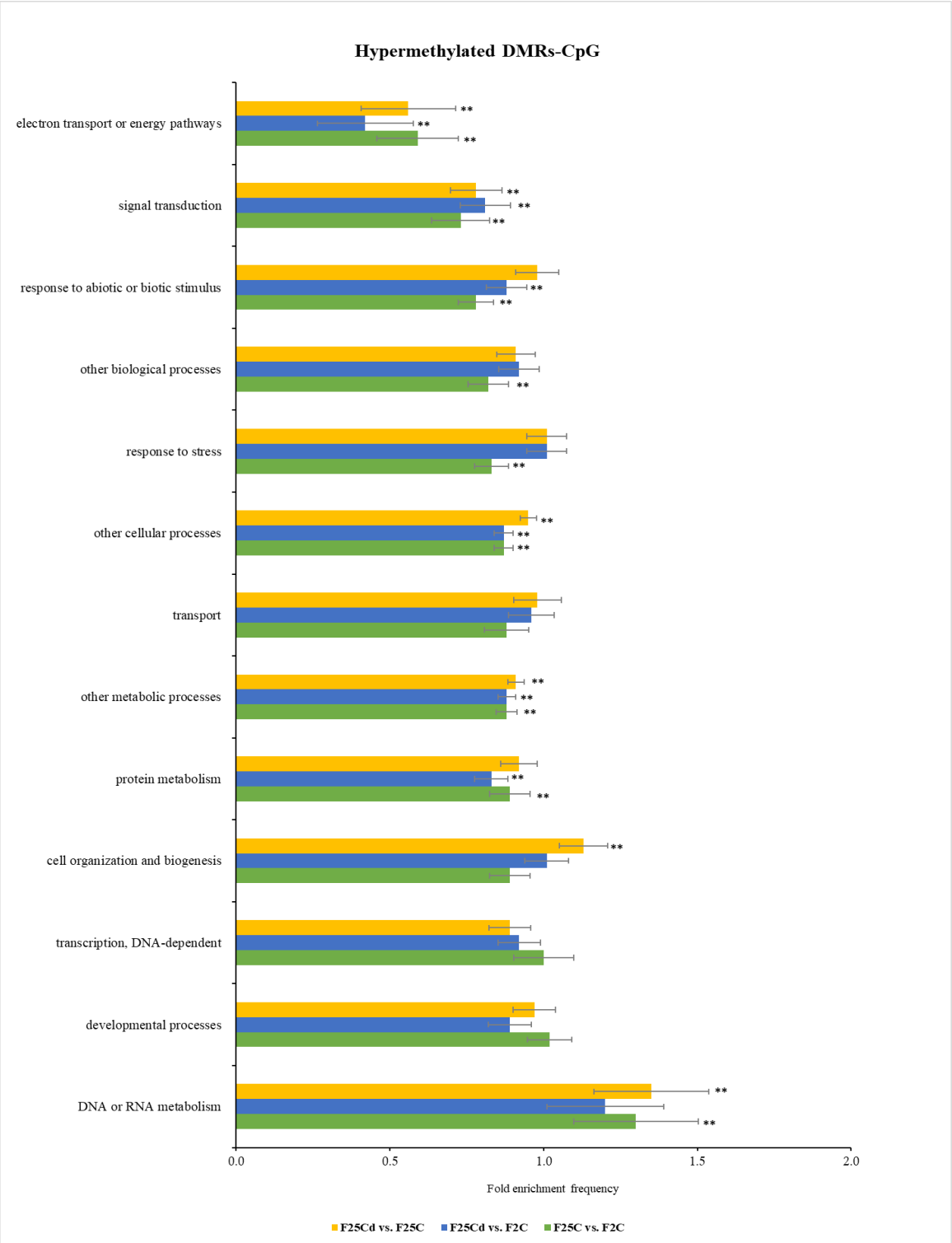


Figure 35. The enrichment analysis of hypermethylated DMRs in CpG sites and their classification based on the biological processes. The X-axis is the normalized frequency

with binomial coefficients as calculated by SuperViewer. Genes are classed with p-values < 0.05, ±bootstrap StdDev.

Similarly, when F25Cd was compared with F2C and F25C, the CpG hypomethylation sites in F25Cd were over-represented in the biological processes such as DNA or RNA metabolism, responses to stress, and transport processes. In addition, when F25Cd was compared with the control groups (F2C vs. F25C) using the biological enrichment analysis of CpG hypomethylation, F25Cd also showed the higher over-representations in the biological processes like other biological processes, transcription, DNA-dependent and developmental processes, and responses to stress (figure 36).

On the other hand, when control groups (F25C vs. F2C) were considered for a comparison using the biological enrichment analysis of CpG hypomethylation, several biological processes also showed over-representation such as in the electron transport or energy, signal transduction, responses to abiotic or biotic stimuli, protein metabolism, cell organization and biogenesis, other cellular processes, and other metabolic processes (figure 36).

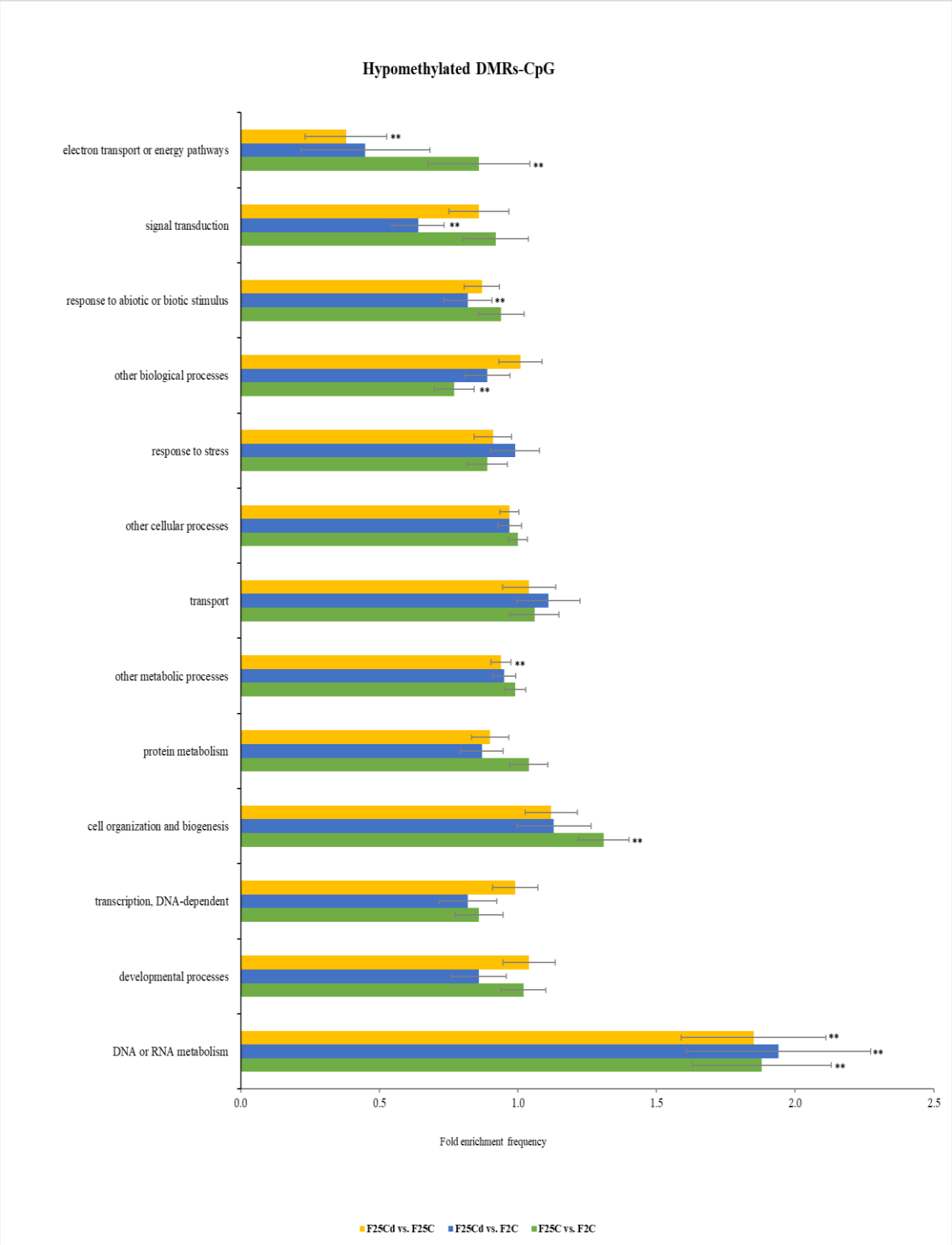


Figure 36. The enrichment analysis of hypomethylated DMRs in CpG sites and their classification based on biological processes. The X-axis is the normalized frequency with

binomial coefficients as calculated by SuperViewer. Genes are classed with p-values < 0.05, ±bootstrap StdDev.

The biological enrichment analysis revealed that CHG hypermethylated sites in DMRs were the only sites in F25Cd that showed the biologically enriched functions compared with controls (F2C and F25C). In the case of DMRs CHG hypermethylated regions, biological processes such as other cellular processes and other biological processes showed a statistically significant ($p<0.05$) over-representation in F25Cd.

On the other hand, interestingly, when control groups (F2C vs. F25C) were compared by the biological enrichment analysis of DMRs CHG hypermethylation, only a signal transduction process showed a statistically significant ($p<0.05$) over-representation among the control groups. In the case of DMRs hypomethylation in the CHG context in the controls group, F2C vs. F25C, several biological processes showed a statistically significant over-representation ($p<0.05$) such as other metabolic processes, transport, and other cellular processes (figure 37).

There was no significant biological enrichment observed in the CHH contexts.

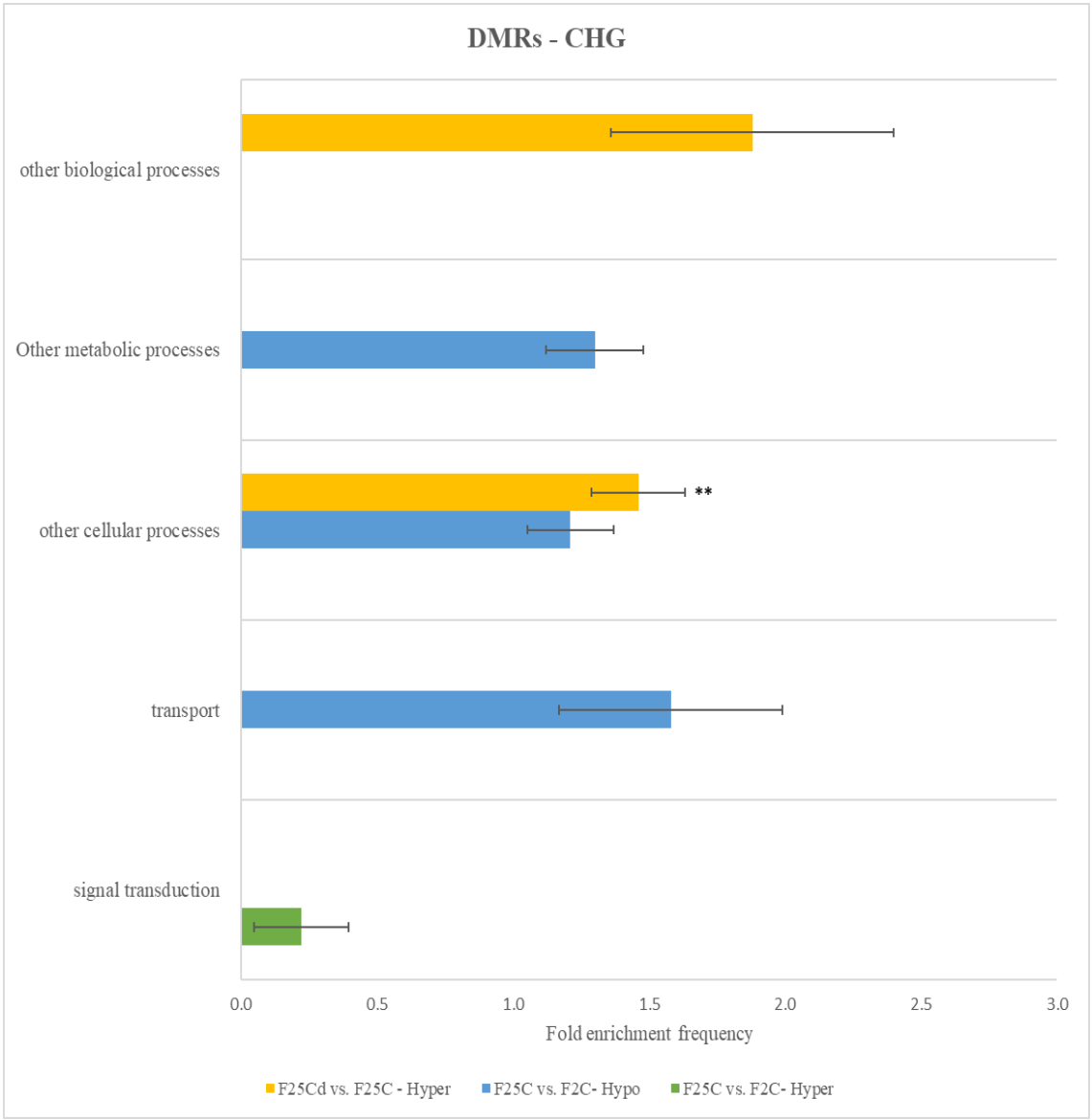


Figure 37. The enrichment analysis of hypermethylated and hypomethylated DMRs in CHG sites and their classification based on biological processes. The X-axis is normalized frequency with binomial coefficients as calculated by SuperViewer. Genes are classed with p-values < 0.05, ±bootstrap StdDev.

CHAPTER 5: DISCUSSION

Being sessile, plants are more vulnerable to environmental changes. Plants face several external stresses such as abiotic stresses, including chemical and physical changes, changes in light intensity, temperature fluctuations, nutrients and water availability, wind and other mechanical stimuli, and biotic stresses, including various pathogens (Madlung & Comai, 2004). Environmental stresses have an impact on the directly exposed generations as well as on their progeny (Herman and Sultan 2011). This study aimed to understand the multigenerational cold-stress effects on plant phenotypes, genotypes, and epigenotypes. Specifically, we compared the progeny of plants exposed to cold stress over 25 consecutive generations with their counterparts (control progenies). We have carried out our experiments under both control and cold stress conditions over multiple generations. We studied the genetic inheritance by means of the whole genome sequencing analysis as well as the epigenetic inheritance by the whole genome bilsufite sequencing analysis of both control and cold-stressed progenies. Overall, this study has deciphered the phenotypic, genomic and epigenomic resilience in the cold-stressed progeny in response to cold stress.

Phenotype Resilience:

We have carried out the phenotypic analysis to decipher effects of multigenerational cold stress exposure in the stressed progenies. For phenotypic profiling, we analyzed a number of true leaves, their bolting and flowering time, and measured the seed size.

Changes in the leaf number

The number of leaves was significantly higher in the advanced progenies (F25C and F25Cd) compared with the parental progenies regardless of propagation conditions. This might be due to the slow germination; F1C seeds were much older than the advanced progenies. Interestingly, progenies of the cold-stressed plants (F25Cd) showed a significantly lower number of leaves compared with the parallel control progeny (F25C) under both control and cold stress conditions. Previously Chinnusamy et al. (2007) demonstrated that cold stress led to a decrease in leaf numbers. Zoë & Kovalchuk (2015) also showed that the progeny of cold-stressed plants had fewer leaves when propagated under cold-stress and normal growth conditions. Similar to the cold- stress progeny, the heat-stressed progeny showed a lower number of leaves under normal conditions (Migicovsky, Yao et al. 2014). They suggested that the heat-induced modifications of the gene expression could cause phenotypic changes such as leaf number and flowering time. The epigenetic memory of the parental cold stress slows down the growth rate of plants and can affect the rate of the growth of the new leaves in the progenies, under both stressed and normal conditions. In slower growth conditions, plants can utilize less energy in the metabolic processes. Since energy is crucial, a plant might develop tolerance mechanisms that can help the progenies to cope up with the stress conditions and thus influence the leave number.

The Flowering and Bolting Phenotypes

We found that all plants propagated under non-stress conditions showed an early flowering phenotype compared with plants propagated under cold stress conditions. Plants

propagated under cold stress conditions showed the delayed flowering responses in general but varied from generation to generation in both the control and cold stressed progenies. Seo et al. (2009) reported the flowering time delays because of the activation of cold-responsive genes under cold stress which might modify the flowering time regulation. A similar phenomenon occurred in the *Arabidopsis thaliana* plants that also showed the delayed flowering responses after salt stress exposure (Kim, Kim et al. 2007, Suter and Widmer 2013). In contrast, Brachi et al. (2012) reported early bolting in the progeny of the water-stressed plants. Furthermore, parental exposure to heat stress was reported as a crucial factor for increasing the time of bolting when progeny plants were propagated under the normal growth conditions (Cicchino, Edreira et al. 2010).

Changes in the average size of seeds

Next, we noticed that when plants were propagated under cold stress conditions, the average height and width of seeds decreased in both the control and cold-stressed progenies. The progenies of cold-stressed plants had smaller seeds when propagated under cold stress conditions. However, interestingly, when plants were propagated under non-stress conditions, the average height and width of seeds increased in almost all tested cold-stressed progenies. As reported previously, a larger seed reserves the energy, whereas a decreased seed size can be the indication of the being less sensitive to the environmental conditions (Hu, Zhang et al. 2017). Since we found that cold stress resulted in smaller seeds in the progenies of cold-stressed plants, it is plausible to think that the reduction in seed size in response to the multigenerational cold stress is an adaptive trait. Cold stress was shown to cause pollen sterility and reduce seed length (Zinn, Tunc-Ozdemir et al. 2010).

Likewise, heat stress also caused the reduced seed size in the heat-stressed progenies (Morrison and Stewart 2002, Sadras 2007, Prasad, Staggenborg et al. 2008). Another report in soybean also reported that seed sizes decreased due to water stress and high-temperature stress (Dornbos Jr and Mullen 1991).

Genomic Analysis:

Environmental stresses can be mutagenic and capable of causing genome instability (Boyko, Golubov et al. 2010, Boyko and Kovalchuk 2011, Gill, Anjum et al. 2015). Environmental changes may also increase homologous recombination events or can facilitate the mobilization of transposable elements (Long, Ou et al. 2009, Boyko, Blevins et al. 2010, Migicovsky and Kovalchuk 2013). Eventually, environmental conditions may induce changes in the genetic material and increase the chances of genomic diversity leading to adaptation to fighting back against ever-changing environmental conditions (Filichkin, Priest et al. 2010).

Single Nucleotide Polymorphisms (SNPs)

SNPs are vital genetic variations that can directly disrupt gene function and affect plant adaptability in the changing environment (Shastry 2009). For instance, SNPs can affect light response and flowering time by changing amino acids in phytochromes A and B (Filiault, Wessinger et al. 2008); (Maloof, Borevitz et al. 2001). In our study, genomic analysis revealed that the number of SNPs variants were significantly higher in the cold-stressed progeny. More specifically, we found that the progeny of cold-stressed plants, F25Cd, showed a significantly higher number of SNPs compared with the control

progenies, F2C or F25C. Zhang et al. (2013) found a frequent occurrence of SNPs in drought-resistance genes in common wheat. They also found that SNPs were associated with the genes responsible for the developmental processes and abiotic stress resistance in wheat (Zhang, Mao et al. 2013). SNPs can also create new splice sites and alter gene functions (Guyon-Debast, Lécureuil et al. 2010). Therefore, a higher number of SNPs in the cold-stressed progeny may be an indication of the adaptive processes occurring in the progeny of stressed plants.

Insertions and Deletions (INDELs)

In our study, we have found that the number of INDELs is higher in the cold-stressed progeny (F25Cd) as compared with the control progenies, F2C or F25C. INDELs variants could impose potential effects on the genome. It has been reported that INDELs can cause changes in gene expression. Jain et al. (2014) discussed that positions of INDELs within the genome could affect the function and expression of genes. They indicated that INDELs were detected in the coding and regulatory regions (Jain, Moharana et al. 2014). Moreover, it has been reported that small and large INDELs can cause pathogen sensitivity (Mindrinos, Katagiri et al. 1994, Kroymann, Donnerhacke et al. 2003). Therefore, higher numbers of INDELs in the stressed progeny can be an indicator of genomic diversity since INDELs can be considered as a crucial factor shaping the evolution of the genomes and species.

SNPs and INDELs associated with transposons

We have also noticed that the average number of transposons associated with SNPs and INDELs revealed that the cold-stressed progeny (F25Cd) showed a significantly higher number them compared with the control progenies. It has been reported that transposable elements can act as stress-responsive regulators by controlling gene expression (Wheeler 2013). Moreover, as we discussed earlier that SNPs and INDELs could affect the genomic function in general, the higher number of transposons associated with SNPs and INDELs in the cold-stressed progeny might eventually result in phenotypic changes.

The functional classification of SNPs and INDELs

An enrichment analysis of SNPs revealed the over-representation of protein metabolism processes only in the cold-stressed progeny. In the case of INDELs, the cold-stressed progenies (F25Cd) showed the over-representation of other biological processes such as cell organization and biogenesis, and responses to stress. Similar to our study, Wang et al. (2017) reported a total of 211 differentially expressed proteins due to cold stress response where the over-representation was observed in protein metabolism and translation, stress responses, the membrane, and transport processes. Liu et al. (2018) also showed that SNPs and INDELs were overrepresented in many biological processes.

An Epigenomic Analysis:

Finally, we have carried out whole-genome bisulfite sequencing (WGBS) of the control (F2C and F25C) and cold-stressed (F25Cd) plants to decipher epigenomic variations among the tested generations. DNA methylation is the most studied and best-narrated

epigenetic modification for decoding the mechanisms of gene expression and the status of the chromatin structure (Pikaard and Mittelsten Scheid 2014). It has been reported that abiotic stresses can cause hyper or hypomethylation in the genome after either short-term or long-term exposure (Uthup, Ravindran et al. 2011). Several studies suggested an idea that multigenerational exposure compared with exposure of a single generation could cause substantially higher heritable epigenetic variations (Remy 2010, Groot, Kooke et al. 2016, Zheng, Chen et al. 2017). This phenomenon also focused on a gradual acclimatization of epigenetic effects (Groot, Kooke et al. 2016). Besides, it has been reported that plants that have experienced multiple exposures to drought stress within the same generation display an enhanced ability to fight against future stresses compared with plants that have no such previous experience (Ding, Fromm et al. 2012).

The Percentage of Global DNA Methylation

We analyzed the epigenome of plants in the control and cold-stressed progeny in the light of previous studies that reported hyper- and hypomethylation in plants under stress (Uthup, Ravindran et al. 2011, Suter and Widmer 2013, Migicovsky, Yao et al. 2014). The methylome analysis revealed that the average percentage of global genome methylation in the CpG and CHG contexts was higher in the cold-stressed progeny, F25Cd. But in the CHH context, F25Cd showed the lower global methylation. Similar to the results of our study, Jiang et al. (2014) showed that soil salinity stressed lineages accumulate more methylation at the CpG sites than the control progenies. It has been well documented that epigenomic alterations can occur due to different types of stresses (Thiebaut, Hemerly et al. 2019). For example, epigenomic changes have been observed in response to salt

treatment in the offspring of the exposed plants in rice and *Arabidopsis thaliana* (Bilichak, Ilnystkyy et al. 2012, Karan, DeLeon et al. 2012). Likewise, in response to environmental signals such as heat and salt, distinct DNA methylation patterns have been observed (Yao, Bilichak et al. 2012, Popova, Dinh et al. 2013).

The Distribution Frequency of Methylation Percentages

We observed that the cytosine methylation frequency was higher in CpG but lower in CHG and CHH in all of the tested groups. The methylation percentages of the tested progenies, F2C, F25C, and F25Cd, were similar context-wise. The percentages of the frequency distribution of cytosine methylation were found mostly in the cytosines in the symmetrical context, which is commonly found in the plants' genome (Finnegan, Genger, Peacock, & Dennis, 1998; Law & Jacobsen, 2010; Niederhuth et al., 2016). These results are in agreement with most of the existing literature since it is widely accepted that DNA methylation is commonly found non-random, and it clusters in specific segments of the genome (Tran, Henikoff et al. 2005, Vaughn, Tanurdzić et al. 2007).

The Total Number of Differentially Methylated Cytosines (DMCs) and Differentially Methylated Regions (DMRs)

We also observed that the total number of differentially methylated cytosine (DMCs) was higher in the control group F25C vs. F2C (80,464) as compared with the stressed progeny groups F25Cd vs. F2C (63,524) and F25Cd vs. F25C (77,648). The total number of differentially methylated regions (DMRs) showed an almost similar pattern in the stressed and control groups in the case of the 100 bp window. This was somewhat surprising as we

hypothesized that there would be more epigenetic changes in the progeny of stressed plants (F25Cd) as compared with the parallel and parental control progenies. Our study suggests that epigenetic variations are more spontaneous in nature, so it is plausible to think that fewer DMRs can be an adaptation trait in response to cold stress where stressor might reduce the rate of spontaneous epimutations. It has been shown that differently from genetic mutations, epimutations are dynamic in nature and can be maintained by the forward-backward dynamics (van der Graaf, Wardenaar et al. 2015). Environmental stimuli can cause changes in DNA methylation in specific loci or in the entire genome (Bartels, Han et al. 2018). The DNA methylation status can be changed during the regular cell division when cells fail to maintain the same methylation levels. Therefore, it is plausible to think that epimutations are spontaneous in plants (Johannes and Schmitz 2019).

Hierarchical Clustering in DMCs

The clustering analysis of DMCs among the progenies of cold-stressed plants and the progenies of control plants showed a clear separation in the clusters, when we compared the cold-stressed progenies (F25Cd samples) with the parental progenies (F2C samples). Moreover, heatmaps of DMCs showed a clear separation among the cold-stressed and control progenies. Ganguly et al. (2017) also found similar clustering patterns in response to heat. They showed that the progeny of stressed plants clustered more separately than the progeny of non-stressed plants due to the treatment with heat stress in *Arabidopsis* (Ganguly, Crisp et al. 2017).

The Distribution of Differentially Methylated Cytosines (DMCs) Across the Chromosomes

The distribution analysis of changes in DMCs at the CpG sites at the level of chromosomes showed that there was the lower percentage of DMCs in F25Cd compared with the controls (F2C and F25C); but the comparisons between controls showed the higher percentages of DMCs at the CpG sites. Although the distribution of hypermethylated and hypomethylated regions was equal when the comparison was made among controls, the hypermethylated regions prevailed in F25Cd vs. F2C groups. In contrast, at the CHG sites, the percentage of DMCs methylation was much higher in comparison groups involving F25Cd compared with the control group F25C vs. F2C. At the CHG sites, hypomethylation prevailed in F25Cd vs F2C groups as compared with F25C vs. F2C groups where hypermethylation prevailed. So, it appeared that stress indeed causes more dramatic hypomethylation changes at the CHG sites. A similar phenomenon has been narrated in response to heavy metal stresses where it has been reported that hypomethylation prevails in several loci in hemp and clover (Aina, Sgorbati et al. 2004).

Likewise, a similar picture was observed at the CHH sites; however, hypomethylation prevailed dramatically in F25Cd vs. F2C and F25C vs. F2C groups compared with F25Cd vs. F25C where hypermethylation prevailed. In support of this concept, several reports actually suggested that it is common for plants to methylate cytosines in the sequence contexts of CHG and CHH, but mechanisms are generally needed for the ongoing reestablishment of epigenetic modifications mostly guided by small RNAs or the

heterochromatin-directed methylation pathways (Feng, Jacobsen et al. 2010, Stroud, Greenberg et al. 2013).

Annotations of Differentially Methylated Cytosines (DMCs)

The annotated DMCs revealed that in the case of the CpG context, the highest number of DMCs occurred in the gene body, whereas in the cases of CHG and CHH context, it was in the promoter and intergenic regions. It was reported before that promoters of stress-responsive genes may be hypomethylated under stress conditions (Yao and Kovalchuk 2011, Bilichak, Ilnystkyy et al. 2012), but other genomic positions may not be changed.

Biological Enrichment Analysis

In our study, the Gene Ontology analysis of differentially methylated regions revealed the over-representation of DNA or RNA metabolism, responses to abiotic and biotic stresses, transport, responses to stimulus and cell organization and biogenesis processes in the cold-stressed progeny compared with the control progenies. These functions and pathways are related to a normal plant development and associated with the stress response (Cramer, Urano et al. 2011). As a result, the possibility of the activation and deactivation of the stress-responsive gene can vary in the stressed and control progenies. Hence, it can be inferred that cold stress has a substantial effect on the *Arabidopsis* epigenome (Banerjee, Wani et al. 2017).

Transgenerational Inheritance:

Transgenerational stress memory is often connected with alterations of DNA sequence, chromatin structures and modifications of DNA such as DNA methylation. These could be potential underlying mechanisms associated with plant stress responses and perhaps the mechanisms of passing the memory of stress on to subsequent generations (Iwasaki and Paszkowski 2014). Our genomic study revealed higher numbers of SNPs and INDELs in the cold-stressed progenies. Similarly, epigenomic studies showed that the average percentage of global genome methylation was higher in the cold-stressed progenies in the CpG and CHG contexts. Baulcombe & Dean (2014) reported that methylome variations in plants could be a potential cause of phenotypic differences to mitigate the environmental stresses where heritable differentially methylated cytosines might be responsible for phenotypic differences. To clearly understand the inheritance processes, the interpretation of the epigenetic inheritance system of epialleles in plants and their effects on the nearby genes is crucial (Hauser, Aufsatz et al. 2011). It is well documented that the methylated regions are found in the contexts of CpG, CHG, or CHH regions of the plants' genome (Tirado-Magallanes, Rebbani et al. 2017). The inheritance of epimutations could be observed in these regions in the subsequent progeny generations. Eichten & Springer (2015) suggested that the separate hierarchical clustering of epimutations was associated with cold stress treatment. In our study, we evidently observed that the progenies of cold-stressed plants and the progenies of control plants showed a clear separation in the clustering analysis.

Conclusion:

In conclusion, global analysis of the genome and epigenome showed many changes in the cold-stressed progenies, including the higher number of SNPs, INDELs, and changes in the percentage of global DNA methylation in the CpG and CHG contexts. Likewise, several reports also suggested that epigenomic modifications could alter the DNA methylation status which could induce a stable inheritance of phenotypic traits (Song, Angel et al. 2012, Thiebaut, Hemerly et al. 2019). So, cold stress might have a substantial effect on the methylome of the *Arabidopsis* epigenome, which subsequently leads to phenotypic resilience (Chinnusamy, Zhu et al. 2007). The accumulation of the stress responsiveness over multiple generations due to the consecutive cold-stress could be guided by the cold-stress responsiveness. A similar observation has been reported in the case of rice exposed to drought stress over eleven successive generations (Zheng, Chen et al. 2017). Similarly, to their study, we also observed hierarchical clustering differences between the control and cold-stressed progenies, which indicated that the methylation pattern was common in all individuals of the same group. Cumulatively, these changes could be due to the cold-included stress responses of transgenerational inheritance in *Arabidopsis.* Our study highlights that multigenerational exposure to cold stress has a substantial effect on the cold-stressed progenies in shaping their phenotypes, the genome and epigenome, which potentially suggests driving force of creating the variations across progeny generations.

CHAPTER 5 - BIBLIOGRAPHY

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