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Enhanced Epigenetic Inheritance in *Arabidopsis thaliana*

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Enhanced Epigenetic Inheritance in *Arabidopsis thaliana*

ABSTRACT

Plants are sessile organisms and as such are frequently exposed to a wide range of environmental stress conditions. Exposure to such stresses, can affect plant growth and survival. Therefore plants have developed strategies to rapidly adapt to fluctuating environmental conditions which, in some cases, these adaptive responses can be passed on to the offspring. One mechanism implicated in this trans-generational “stress memory” involves changes in DNA methylation that are directed by external stress. However, it has previously found that this so-called “stress memory” is temporary, as in the absence of stress these epigenetic changes are rapidly erased. In this study, a new methodology was established to enhance the transmission of epigenetic changes directed by stress by exposing the stress during somatic embryogenesis by using overexpression of *AtRWP-RKD4* in *Arabidopsis thaliana*. The data show that the introduction of abiotic stress during somatic embryogenesis leads to partly heritable adaptation phenotype in the non-stress progenies. The phenotypical changes are followed by the changes in transcriptome and methylome in which these molecular changes are observed in tissue-specific manners. Based on these findings, this new methodology can be applied to develop stable stress-adaptation phenotypic in plants.

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List of Abbreviations

ABA	Abscisic Acid
ABRE	ABA-Responsive Element
ATP	Adenosine Triphosphate
BABA	β -aminobutyric acid
bp	base pair
CAM	Crassulacean Acid Metabolism
CDPK	Calcium-Dependent Protein Kinase
CIM	Callus Induction Media
cm	Centimeter
ddH ₂ O	Double Distilled Water
dsRNA	Double-Stranded RNA
DEG	Differentially Expressed Genes
DEX	Dexamethasone
DMG	Differentially Expressed Gene
DMR	Differentially Methylated Region
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
Et	Ethylene
FAO	Food and Agriculture Organisation
FDR	False Discovery Rate
GO	Gene Ontology
h	Hour
HAT	Histone Acetyltransferase
HD	Histone Deacetylase
JA	Jasmonic Acid
lincRNA	Long Intergenic Non Coding RNA
LB	Luria Bertani
LO	Leaf Origin
LRR-RK	Leucine-Rich Repeat Receptor Kinase
MAPK	Mitogen Activated Protein Kinase
mg	milligram
min	Minute

mL	Milliliter
mM	Milimolar
MR	Methylation Rate
mRNA	Messenger RNA
MS	Murashige and Skoog
NO	NitricOxide
nt	Nucleotide
OD	Optical Density
OFP	Ovate Family Protein
PAMP	Pathogen-Associated Molecular Pattern
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
<i>Pst</i> DC3000	<i>Pseudomonas syringae</i> pv <i>tomato</i> DC3000
PTGS	Post Transcriptional Gene Silencing
RdDM	RNA Directed DNA Methylation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotation per Minute
RO	Root Origin
SA	Salicylic Acid
SAR	System Acquired Resistance
SC	Sperm Cell
SDMR	Specific Differentially Methylated Region
sec	Second
siRNA	Small Interfering RNA
SOS	Salt Overly Sensitive
ssRNA	Single Stranded RNA
tbs	Tablespoon
TE	Transposable Element
TF	Transcription Factor
TGS	Transcriptional Gene Silencing
U	Unit
UVC	Ultra Violet C

VN	Vegetative Nucleus
μ L	Micro Litre
μ M	Micro Molar

Declarations

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. The work presented in this thesis is original, and has not been published or presented for any other degree. The work described in this thesis has been carried out by myself, with the exceptions described below:

Dr Emma Wallington (National Institute of Agricultural Botany, United Kingdom)

Assisting with *Oryza sativa* transformation with AtRKD4 pERV1 binary construct (Chapter 3).

Jonathan Price (University of Warwick, United Kingdom)

Assisting with differentially expressed genes analysis (Chapter 5).

Lesley Ward (University of Warwick, United Kingdom)

Produced RNAseq libraries.

Dr Claude Becker (Gregor Mendel Institute, Austria)

Produced bisulphite converted Illumina libraries and performed RNAseq and bisulphite sequencing.

Dr Julia Engelhorn (University of Warwick, United Kingdom) and Patrick Huether (Gregor Mendel Institute, Austria)

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1. General Introduction

1.1 General Overview

Since late 1700s the amount of carbon dioxide in the air has increased by 40 percent (EPA, 2016). The reading of atmospheric CO₂ exceeded the 400 ppmv benchmark in 2013 and is predicted to double or even treble by the end of the century (Váry et al., 2015). Increasing in atmospheric CO₂ is followed by increasing in temperature. This has influenced landscape water balance, thus it indirectly affects plants performance and survival (Duan et al., 2018). It is also reported that the elevated level of carbon dioxide linked to the severity of some stresses such as plants diseases (Váry et al., 2015).

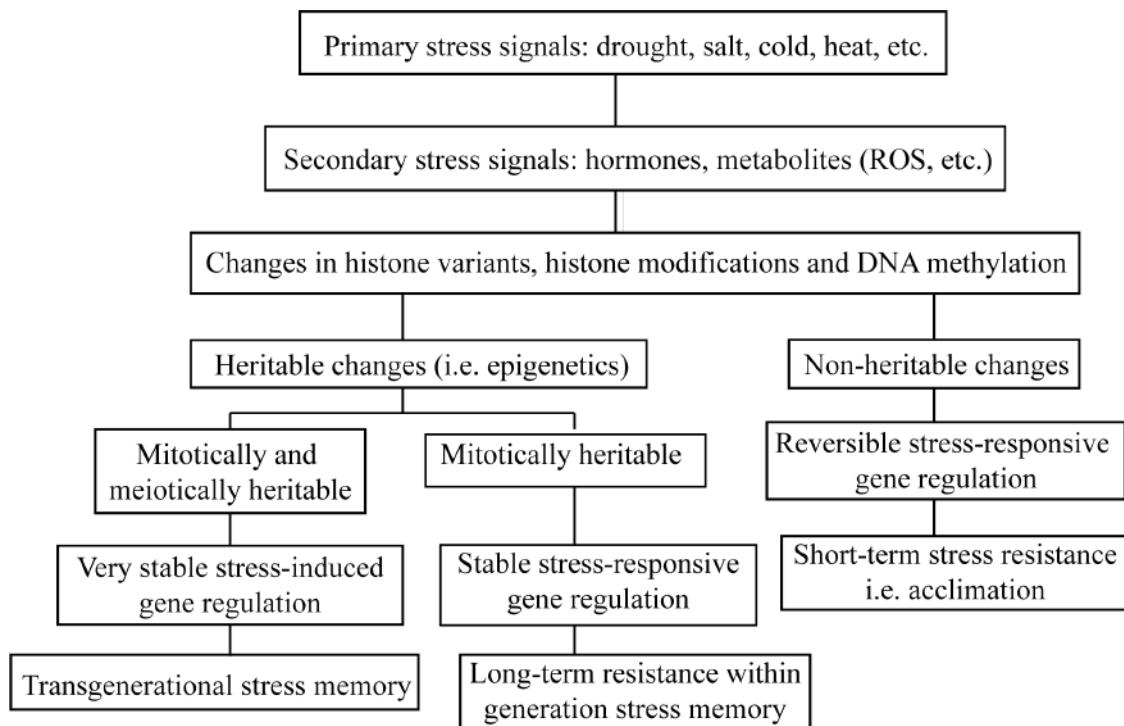


Figure 1.1 Epigenetic regulation of stress tolerance (modified from : Chinnusamy & Zhu, 2009)

Biotic and abiotic stress serve as a consistent treats to a plant's life cycle (Ghosh et al., 2017). Being sessile, plants do not have the ability to escape from stress and exposure to stress can lead to cell apoptosis and also to plant necrosis. Thus, plants need efficient short-term strategies to adapt to environmental stress. These strategies include changes on plant homeostasis during somatic growth and heritable transgenerational changes of gene expression (Boyko & Kovalchuk, 2008). These changes on gene expression can involve

changes on DNA methylation and histone modification, because these are typical epigenetic mechanisms associated with heritable changes in gene expression without changes in DNA sequence (Boyko & Kovalchuk, 2008). Several studies have been done in plants and it is found that the exposure to biotic and abiotic stress leads to epigenetic changes especially in DNA methylation (Boyko et al., 2010; Choi & Sano, 2007). In addition, recent research by Wibowo et al., (2016) also shows repetitive exposure to the stress can trigger changes in the patterns of DNA methylation that can persist transgenerationally. In general, when plants are exposed to stress such as drought, salt, cold, hormones or metabolites, unknown signals alter the expression of epigenetic regulators such as small RNAs, RNA directed DNA methylation (RdDM), histone variants, histone modification enzymes, or chromatin remodeling factors (Fig.1.1) (Chinnusamy & Zhu, 2009). These changes can be heritable or transient, but if heritable they produce stable epialleles, a form of long-term stress memory. However, if the changes are not heritable, the stress-responsive gene regulation is rapidly reversed and only give short-term stress memory (Chinnusamy & Zhu, 2009).

In this chapter, I will discuss the importance of epigenetic changes mediated by DNA methylation in shaping plant responses to the environmental stimuli, the inheritance of epigenetic marks, epigenetic reprogramming through plant development, and the importance of embryogenesis during epigenetic reprogramming.

1.2 DNA Methylation in Plants

DNA methylation is an important biological process that has been conserved across the six kingdoms. The first observation of DNA methylation was discovered in bacteria where the genome is methylated to differentiate the genomic DNA from invading phage DNA (Chen, & Zhu, 2011). In eukaryotes, DNA methylation serves as a mechanism to regulate gene expression (Watson et al., 2008). DNA is methylated by transferring a methyl group from the methyl donor S-adenosylmethionine to cytosine residues, at position 5 of the pyrimidine ring (Smith et al., 2010). The process of methylation can also occur at adenine, while guanine and thymine are not methylated. However, the function of adenine methylation in plants is not yet fully understood. In plants, the methylation occurs in the

context of CG, CHG and CHH ($H = A, C$ or T) (He et al., 2011). In the Arabidopsis genome, the methylation at CG sites is found to be around 24%, whereas CHG is 6.7% and 1.7% for CHH methylation (Cokus et al., 2008). The vast majority of DNA methylation is found in transposons (91% of transposons), 58% of pseudogenes are methylated and 20% of *bona fide* genes (expressed nonoverlapping genes free of known transposable elements) (Zilberman et al., 2007). While for transcribed genes, only one-third of expressed contains methylation within transcribed regions, and only ~5% of methylation found within promoter regions (Zhang et al., 2006a). The level DNA methylation is depleted at the 3' end of a gene, and the distribution of DNA methylation within genes does not depend on the length of the genes (Zilberman et al., 2007). The high level of DNA methylation found within transposons in Arabidopsis indicates that the role of DNA methylation is not limited to controlling gene expression, but it also regulates the expression and activation of transposable elements. In the Arabidopsis genome, a large number of transposons (~4000) are found to be inserted mostly near centromeres and in heterochromatic regions (Smith et al., 2010). The genetic changes caused by transposon insertions are rarely advantageous, and most of the time it is deleterious due to its ability to affect gene expression (Hollister & Gaut, 2009; Smith et al., 2010). If the activity of transposons is high, it may lead to gene mutation in plants. Thus, it is crucial for plants to tightly control the activity of transposons.

In Arabidopsis there are four classes of DNA methyltransferases: DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), METHYLTRANSFERASE 1 (MET1, and CHROMOMETHYLASE 3 (CMT3) (Chan, Henderson, & Jacobsen, 2005). Before the process of replication takes place, both strands of DNA are methylated; and after replication, a newly synthesised DNA strand consists of an old strand which is methylated and a new strand that is not methylated. MET1 uses the methylated DNA strand as the template for the methylation of the newly synthesised strand (Fig. 1.2) (Smith et al., 2010). During replication, the CHG methylation is also copied to the newly synthesised DNA strands. The maintenance of CHG methylation by CMT3 (Fig. 1.2). To perform methylation at CHG sites, CMT3 is fully dependent on H3K9 methylation, in which the 9th lysine residue of histone 3 is methylated by KRYPTONITE (KYP),

SU(VAR) HOMOLOGUE 4 (SUVH4) and SUVH6 (Stroud, Do, et al., 2013). Both H3K9me1 and H3K9me2 can initiate CMT3 activity to establish CHG methylation. However, only H3K9me2 can trigger the activity of CMT2 to initiate methylation at CHG sites (Stroud, Do, et al., 2013). In contrast, non-symmetric CHH methylation cannot be copied to the newly synthesised DNA strands, and so CHH methylation must be reestablished after each cell division (Smith et al., 2010). DRM2 regulates the *de novo* establishment of CHH methylation through the RNA-directed DNA methylation (RdDM) pathway (Matzke & Mosher, 2014). One of the RdDM pathways is fully dependent on the production of 21-22 nt small interfering RNAs (siRNAs) from newly transcribed transposons. These 21-22 nt siRNAs are loaded into ARGONAUTE 2 (AGO2) and forms complexes with DRM2 and NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD) to initiate CHH methylation (Matzke & Mosher, 2014).

The methylation marks established in the genome can also be removed through the process of DNA demethylation. In plants, the process of removing methylation marks involve DNA glycosylases. In plants, there are two DNA glycosylases, *REPRESSOR OF SILENCING 1 (ROS1)* and *DEMETER (DME)* (Chan et al., 2005). *ROS1* encodes a 5-methylcytosine DNA glycosylase/demethylase, which is a nuclear protein containing a C-terminal DNA glycosylase domain and an N-terminal histone H1-like basic region (He et al., 2011). During cytosine demethylation, ROS1 recognises methylated cytosine and removes the base from the DNA backbone by its glycosylase activity. The AP (apurinic) lyase activity cleaves the DNA backbone at the site of cytosine removal which is then repaired with unmethylated cytosine (Chan et al., 2005). *ROS1* is found to be active in the late embryogenesis, but *DME* is only expressed and functions during female gametogenesis (Kawakatsu et al., 2016).

DNA hypomethylation often causes developmental abnormalities. The homozygous mutant of *met1* shows late-flowering phenotype due to the ectopic expression of *FWA* genes (Saze et al., 2003). In addition, the *met1-6* mutation causes defects in suspensor and embryo development, where *met1-6* and *cmt3-7* double mutant shows a decrease in seed

viability and plant robustness (Xiao et al., 2006). It may indicate that DNA methylation is crucial for plant embryogenesis and seed viability.

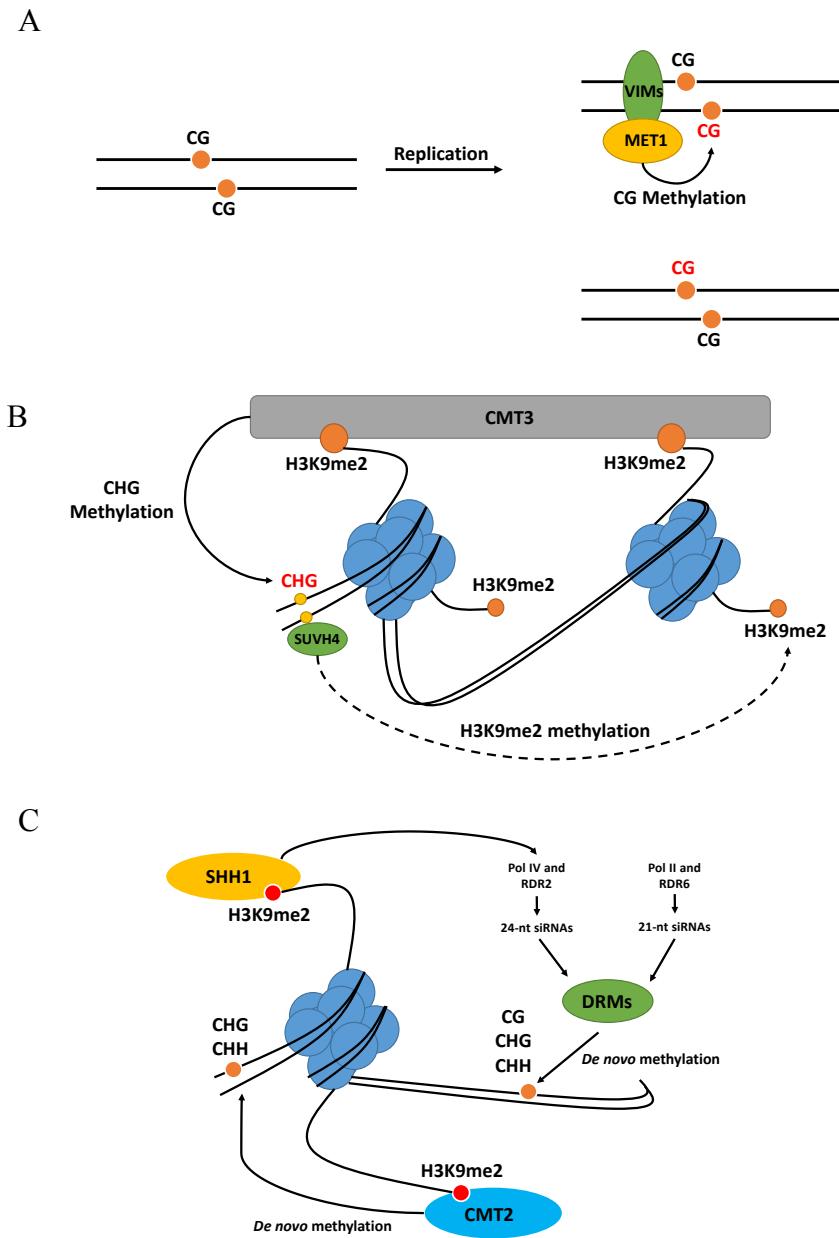


Figure 1.2 Models of DNA methylation maintenance at CG, CHG and CHH context.

(A) CG methylation is recognised by VARIANT IN METHYLATION (VIM) which it recruits DNA METHYLTRANSFERASE 1 (MET1) to maintain CG methylation after replication. (B) CHROMOMETHYLASE 3 (CMT3) binds to H3K9me2 and establishes CHG methylation. The CHG methylation recruits SU(VAR) HOMOLOGUE 4 (SUVH4). (C) SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1) binds to H3K9me2 and recruits RNA polymerase IV (Pol IV). Pol IV and RNA-DEPENDENT-RNA-POLYMERASE 2 (RDR2) with the others component of RNA-directed DNA methylation (RdDM) generated 24-nt siRNAs. These siRNAs are recognised by Argonaute proteins and recruits DOMAINES REARRANGED METHYLTRANSFERASE 1 (DRM1) and DRM2 for *de novo* DNA methylation in all sequence context (modified from: Kawashima & Berger, 2014).

1.3 Histone Modifications as Epigenetic Mechanisms

All higher organisms including mammals and plants have a large amount of genetic material. The DNA strands are tightly wrapped around a protein complex of eight histone molecules (two each of histones H2A, H2B, H3 and H4) to form a nucleosome (Smith et al., 2010). In assembling a nucleosome, the histone first bind to each other to establish H3-H4 and H2A-H2B dimers, and the H3-H4 dimers further form tetramers. This H3-H4 tetramer will combine with two H2A-H2B dimers to form a compact octamer core (Alberts et al., 2002). Each of the histones has a long N-terminal amino acid tail which extends out from the DNA-histone core that are highly conserved in their sequence, and are important for regulating chromatin structure (Alberts et al., 2002). The changes in chromatin structure affect the expression of genes by three processes. Firstly, histone modifiers covalently change amino acids in the N-terminal tails of histones which alters the histone-DNA interaction, opening or blocking protein-binding sites. Secondly, chromatin remodelling ATPases can utilise the energy produced from ATP hydrolysis to change the position or composition of nucleosomes. Finally, methylation in the cytosine of DNA can inhibit or promote protein binding, especially in the promoter regions of genes (Pfluger et al., 2007). In plants there are four histone modifications: ubiquitination on lysine (causing either transcription activation or repression), methylation on lysine and/or arginine (causing either transcription activation or repression), acetylation on lysine (causing transcription activation) and phosphorylation on serine and/or threonine (causing transcription activation) (Pfluger et al., 2007). Among the four histone modifications, histone acetylation and histone methylation have been most extensively studied.

The process of histone acetylation is catalysed by an enzyme called histone acetyltransferase (HAT) (Chen & Tian, 2007). There two classes of HATs which are HAT-A and HAT-B in which HAT-A is located in the nucleus and acetylates nucleosomal core histones whereas HAT-B is located in the cytoplasm and acetylates free histones specifically at H4 lysine 5 and lysine 12 (Boycheva, Vassileva, & Iantcheva, 2014). In *Arabidopsis*, the acetylation of N-terminal H3 histone at lysine 9, 14, 18, 23 and 27 and H4 histone at lysine 5, 8, 12, 16 and 20 are linked to the regulation of plant cell cycle and

epigenetic processes (Boycheva et al., 2014; Chen & Tian, 2007). Histone acetylation marks at H3K9 are correlated with euchromatic regions of chromosomes where actively transcribed genes are abundantly found (Benhamed et al., 2006; Boycheva et al., 2014; Chandrasekharan & Hall, 2006). Histone acetylation is a reversible process. The removal of acetyl groups from the N-terminal tail of core histones is catalyzed by histone deacetylase. In plants there are four classes of histone deacetylases characterised, RPD3, HDA1, SIR2 and plant-specific histone deacetylase HD2 families (Boycheva et al., 2014; Wu et al., 2003). The removal of an acetyl group from the N-terminal histone tail causes chromatin to condense and inhibit the transcription at both euchromatic and heterochromatic regions of the genome (Verdone et al., 2005).

Methylation of histone H3 and H4 takes place at arginine and lysine residues (Liu et al., 2010). In Arabidopsis, histone methylation mainly occurs at lysine 4, 9, 27 and 36 of histone H3, where H3K9 and H3K27 are associated with gene silencing, whereas H3K4 and H3K36 methylation are linked with active genes (Berger, 2007). SET domain proteins are responsible for catalysing histone methylation at lysine residues of the N-terminal tail (Liu et al., 2010). In plants, there are four groups of SET domain proteins, SUVH and SUVR, E(Z) (enhancer of zest) homologs, TRX (trithorax) group and ASH1 (absent, small, or homeotic discs 1) groups (Baumbusch et al., 2001; Liu et al., 2010). In addition, a polycomb group proteins, proteins that maintains the gene-expression pattern of different cells by regulating chromatin structure, POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) catalyses the methylation of histone H3 at lysine 27 (Margueron & Reinberg, 2011). Histone methylation is also a reversible process. Two histone demethylases remove methylation marks at lysine residues; they are lysine-specific demethylase 1 (LSD1) and Jumonji C (JmJC) proteins (Liu et al., 2010).

Histone modification is an important developmental pathway in plants. As a non-motile organism, plants exhibit extraordinary molecular adaptation mechanisms in response to environmental plasticity. Histone modifications also involve in regulation of plant adaptation to various abiotic stresses including drought, high salinity, cold and heat stress (Asensi-Fabado et al., 2017). Histone modifications are also found to affect DNA

methylation. Loss function in *DECREASE DNA METHYLATION 1 (DDMI)* (encodes chromatin remodelling factor SWI2/SNF2 family) causes the global reduction of DNA methylation and leads to reactivation of silent transposable elements (TEs) (Tariq & Paszkowski, 2004). Additionally, reduction of H3K9 methylation due to a mutation in the *KRYPTONITE (KYP)* gene is linked to the depletion of plant-specific CHG methylation in *Arabidopsis* (Tariq & Paszkowski, 2004). Therefore histone modifications potential to act as a mechanism to regulate gene expression, thereby allowing plants to rapidly adapt to changes in environmental conditions.

1.4 Epigenetic Responses to the Environmental Stimuli

Plants are sessile organisms that are constantly exposed to various environmental stresses that may have a negative impact on growth, development and reproduction (Boyko & Kovalchuk, 2011). The best known epigenetic change affected by signals from the environment is the activation of FLOWERING LOCUS C (*FLC*). *FLC* is a MADS-box transcriptional repressor involved in silencing the genes that are required for the transition to flowering (Song et al., 2012). In *Arabidopsis*, *FLC* acts as a repressor of flowering, and it is crucial for the vernalisation mechanism (Sheldon et al., 1999). The expression of *FLC* is affected by temperature. Before cold exposure, the expression of *FLC* is at its highest level. When plants are exposed to prolonged cold during the winter, the expression of *FLC* is repressed to allow the flowering during spring (Song et al., 2012). Histone methylation regulates *FLC* expression during cold. Exposure to cold causes the accumulation of POLYCOMB REPRESSIVE COMPLEX 2 (PRC2), which binds with PLANT HOMEODOMAIN (PHD) at intron 1 of *FLC* to form a PRC2-PHD complex (Song et al., 2012). The accumulation of PRC2-PHD complexes increased trimethylation of histone H3 lysine 27 (H3K27me3) at the nucleation region of *FLC* and inhibits its expression (Song et al., 2012). The repression of *FLC* expression by H3K27me3 causes the vernalized state that is maintained mitotically after returning to warmth in the spring (Tao et al., 2017a). *FLC* is found to be silenced in mature pollen and egg cells, and it is re-expressed during pro-embryo state within one day after pollination (Tao et al., 2017a). The absence of *FLC* activity in mature pollen and egg cells allow it to escape the process of reprogramming and pass the vernalized state to the next generation. Thus, to reset the

vernalis state in the next generation, H3K27 demethylation by the EARLY FLOWERING 6 (ELF6) is required where the depletion in H3K27 methylation allows the accumulation of active histone modifiers at the *FLC* locus such as H3K36 methyltransferase to promote H3K36me3 (Tao et al., 2017a). This indicates that histone modification is crucial for the reprogramming of *FLC* expression.

The exposure to both abiotic and biotic stresses also caused a high frequency of genome rearrangements that cannot be explained by genetic mutations due to the low frequency of genome mutation caused by stress exposure (Boyko & Kovalchuk, 2011). The study on genome stability in *Pinus silvestris* exposed to radioactive contamination after Chernobyl accident, it found that global genome methylation was increased (Kovalchuk et al., 2003). The genome hypermethylation is hypothesised to serve as a defence strategy to prevent genome instability and reshuffling of hereditary material, allowing the plants to survive in an extreme environment (Kovalchuk et al., 2003). Additionally, epigenetics has also been shown to regulate responses to biotic stress. The exposure of *Arabidopsis* to pathogenic bacteria and to salicylic acid (SA) induced genome-wide DNA demethylation, leading to transcriptional changes in affected TEs and proximal elements (Dowen et al., 2012). This is not the only example of epigenetically regulated TEs becoming activated after exposure to stress. Prolonged heat stress caused activation of repetitive elements of *A. thaliana* that, at ambient temperature, are regulated by epigenetic transcriptional gene silencing (Pecinka et al., 2010).

TEs are a major component of the genome, and represent 3 to 50% of the genome content, depending on the species (Capy et al., 2000). In *Arabidopsis*, 90% of DNA methylation is found to be within TEs (Zilberman et al., 2007). Even though random activation of TEs has the deleterious effect of plants growth and development (Smith et al., 2010), the activation of some TEs are found to be essential for stress responses and adaptation (Negi et al., 2016). In maize, biotic stress in the form of barley-stripe mosaic virus infection caused activation of Bs1 retrotransposon (Johns et al., 1985), and the activation of mPing transposable element in *Oryza sativa* was induced by exposure to low-temperature stress (Naito et al., 2009). In *Arabidopsis* the activation of the ONSEN transposon was linked to

heat stress (Pecinka et al., 2010), and this activation persists in the activation of TEs upon stress causes the production of TE-associated long intergenic noncoding RNAs (TE-lncRNAs) that play important roles in plant abiotic stress responses and also as an adaptive mechanism in eukaryotes (Wang et al., 2017). The regulation of stress responses through the activation of TEs is thought to be due to their ability to affect the regulatory regions of stress-responsive genes, by inserting into the promoter region of specific genes or through the production of small RNAs that are involved in the RdDM pathway (Negi et al., 2016). Recently, the activation of TEs also found to affect the expression of adjacent genes by the production of small antisense RNAs (Wibowo et al., 2016). TEs are important environmental rheostats to adjust to the changes in the environmental stimuli. The evidence also suggests the epigenetic regulation of these elements has been used by plants to regulate stress response genes.

1.5 Epigenetic Inheritance

All living organisms are exposed daily to the changes in environmental cues, many of which lead to heritable changes in gene expression. For example, when *Drosophila melanogaster* is treated with heat-shock or osmotic stress, white gene de-repression can be inherited both maternally and paternally over several generations (Heard & Martienssen, 2014; Seong et al., 2011). Furthermore in mice, the diet of *Agouti^{xy}* mothers can affect the coat colour phenotype of their progeny, however this trait is only transmitted over two generations (Daxinger & Whitelaw, 2012; Heard & Martienssen, 2014).

In plants, the events of epigenetic inheritance has been recorded. When plants are exposed to the environmental stress, the perceived environmental signals must be memorised and propagated through mitotic and meiotic division to form transgenerational adaptation to the stress (Mirouze & Paszkowski, 2011). In *Arabidopsis thaliana*, exposure to UVC stress increased the frequency of somatic homologous recombination for up to four non-stressed generations (Molinier et al., 2006). The stress signal is also perceived to specifically target certain parts of the genome. In plants, stress signals are known to affect and activate TEs (Mirouze & Paszkowski, 2011). Furthermore, TEs produce a large number of small RNAs that can regulate the expression of stress-related genes or play a

pivotal role as general transcriptional repressors upon stress (Hilbricht et al., 2008; Mariner et al., 2008). These small RNAs are mobile where the mobility has been observed in grafted Arabidopsis (Molnar et al., 2010), and these small RNAs could influence gene expression in different cells or even organs, which may be important for stress adaptation in plants (Mirouze & Paszkowski, 2011).

The evidences show that plants adapt to environmental stress by the activation of stress genes. The activation of stress-related genes is linked to the DNA methylation, either by the activation of suppressed TEs or suppression of TE (Mirouze & Paszkowski, 2011). As stress is transient, when plants are exposed to a type of stress, after the recovery period plants can prime and develop tolerance to the stress. This stress tolerance may become stress memory in plants that leads to the survival. The development of stress memory involves the maintenance of epigenetic marks, and in the absence of stress, these epigenetic marks are erased and reprogrammed to ensure the correct development (Crisp *et al.*, 2016).

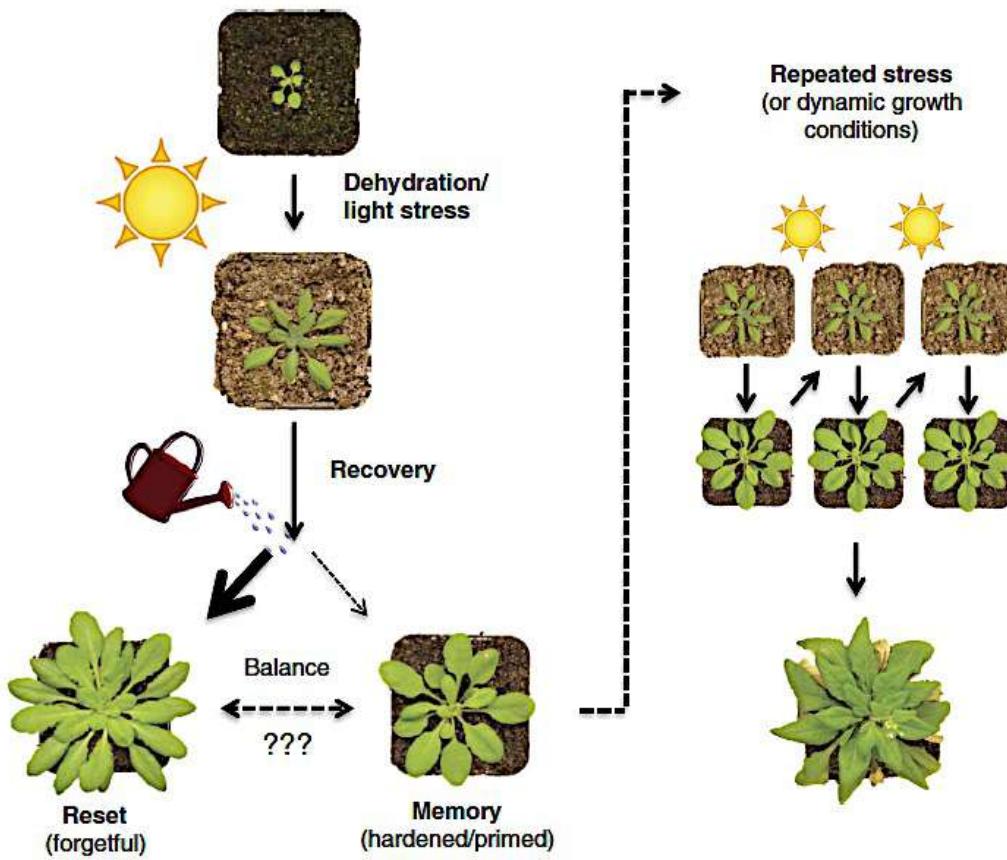


Figure 1.3 Hypothesis for development of stress memory in plants. Stress is transient and is followed by a period for plants to recover, in which the plants must strike a balance between priming and resetting. It is proposed that the major response is resetting. This recovery time is a crucial evolutionary strategy. However, the memory level is also important in a dynamic environment distinguished by repetitive stress exposure. Thus, plants need to balance the stress defence mechanism by forming stress memories with the potential growth and yield advantages of resetting if favourable conditions occur (modified from: Crisp et al., 2016).

1.6 Epigenetic Reprogramming

Epigenetic information encoded as DNA methylation can be transmitted through several generations. Research in both *Arabidopsis thaliana* and *Nicotiana tabacum* has shown transgenerational homologous recombination frequency (HRF) in response to stress (Alex et al., 2010). However, in the absence of stress, the epigenetic changes are erased and rapidly reprogrammed. One classic example of epigenetic reprogramming is the resetting the floral repressor locus *FLC* in *A.thaliana* (Alex et al., 2010; Crevillén et al., 2014); and

in recent published paper by Wibowo et al. (2016) shows that plants exposed to the similar stress can reset their epigenetic marks if they find themselves growing and reproducing under non-stress conditions. The reprogramming of chromatin during development and in response to external stimuli is crucial to ensure correct development in plants to reduce the risk of perpetuating dangerous epigenetic alleles (Crevillén et al., 2014).

Unlike mammals, the germlines in higher plants are defined late in their life cycle when plants are producing flowers (Kawashima & Berger, 2014). In higher plants, male and female gametophytes come from different somatic cells where female gametophyte comes from megasporangium mother cell and male gametophyte is from pollen mother cells. During sporogenesis, pollen mother cells enter meiosis to produce a haploid microspore cell, where the microspores will proceed through gametogenesis to produce bicellular pollen containing a vegetative cell and a generative cell and finally develop into mature pollen consisting one vegetative cells and two sperm cells. On the other hand, the megasporangium mother cell enters meiosis to produce the megasporangium, which undergoes nuclear division to produce a syncytial female gametophyte with eight nuclei. The eight nuclei are partitioned by cytokinesis to generate the mature embryo sac that comprises the egg cell, the central cell and accessory cells (antipodal and synergids) (Kawashima & Berger, 2014).

The process of epigenetic reprogramming in plants occurs in both male and female gametogenesis in addition to embryogenesis (Kawashima & Berger, 2014). During the production of microspores from pollen mother cells, there are slight increases in the transcription of TEs in microspore cells, however the TE transcripts are not over-represented in the microspore transcriptome (Fig. 1.5). This indicates that the epigenetic silencing pathway, most probably involving RdDM, is quickly restored after meiosis (Gutierrez-Marcos & Dickinson, 2012). This may explain the loss of CHH methylation in microspores (Kawashima & Berger, 2014). The TE transcripts further accumulate in vegetative cells followed by the increase of CHH methylation, while the levels of TE transcripts decreases in sperm cells followed by the loss of CHH methylation. The involvement of *CMT2* in regulating CHH methylation in both sperm and the vegetative

cell remains unknown, but the RdDM pathways are involved in regulating CHH methylation in vegetative cells by upregulation of *DRM2* (Kawashima & Berger, 2014). Interestingly, the level of CG methylation remains constant in both microspores and sperm cells due to the expression of *MET1* in both cells. The loss of *MET1* expression in the vegetative cell causes CG hypomethylation in the vegetative cell (Kawashima & Berger, 2014). Maintenance of DNA methylation by MET1 is also essential for epigenetic inheritance. Saze et al. (2003) showed that the *A. thaliana* *MET1* mutant displays an immense epigenetic diversification of gametes, mainly due to post-meiotic demethylation. Furthermore, Calarco et al., (2012), also showed that microspore and sperm cell pericentromeric DNA demethylation at retrotransposons and satellite repeats is mediated by the downregulation of *DRM2*. Their work suggests that epigenetic inheritance is regulated by the presence of 24nt small RNAs. (Calarco et al., 2012), indicated that these small RNAs might be involved in the silencing of TEs in the sperm cells and may be involved in epigenetic inheritance (Kema et al., 2018; Martínez et al., 2016).

Similar to male gametogenesis, the female megagametophyte also undergoes epigenetic reprogramming during gametogenesis, shortly before entering nuclear division to produce the megasporangium. AGO5 acts independently of AGO9 indicating that RdDM plays an important role during megagametophyte development (Gutierrez-Marcos & Dickinson, 2012). After meiosis, the central cell loses methylation in all sequence contexts as a result of *MET1* repression and DME activation (Fig. 1.6) (Kawashima & Berger, 2014). This leads to transcriptional activation of any locus that is controlled by the methylation of *cis*-elements, including TEs and imprinted genes (Kawashima & Berger, 2014). In the egg cell the expression of both the CG and CHG maintenance DNA methyltransferases, *MET1* and *CMT3* are barely detected (Kawashima & Berger, 2014). Expression of DME is not detected, but *DRM1* and *DRM2* are highly expressed, indicating that the RdDM pathways are still active in the egg cell which play a role in non-CG methylation for TE silencing (Kawashima & Berger, 2014; Do et al., 2013). This is not the only difference between different cell types in female gametophyte; chromatin state is also affected. It has been found that the chromatin organisation of the egg cell and central cell differs from that of the accessory cells. This indicates that megagametophyte cells have distinct epigenetic

machinery that are established before or during female gametogenesis (Gutierrez-Marcos & Dickinson, 2012). The differences in chromatin state and asymmetry in DNA methylation between cells within the female gametophyte is because of the expression of DME in central cell and repression of MET1 during the maturation of female gametes (Gutierrez-Marcos & Dickinson, 2012).

After fertilisation in early embryogenesis, both CG and non-CG methylation are reestablished by the expression of *de novo* DNA methyltransferases; the re-methylation process is likely to compensate for the loss of CG methylation in the egg cell and CHH methylation in sperm cells (Kawashima & Berger, 2014). However, CHH methylation decreases during early embryogenesis, and is re-established at the globular stage, only reaching the parental state of CHH methylation at heart stage embryo (Kawashima & Berger, 2014). The establishment of CHH methylation after the globular stage of embryogenesis is thought to be due to the transfer of small RNAs from endosperm (presumably from the TEs activity) to the embryo which then initiate the RdDM pathways (Ibarra et al., 2012; Kawashima & Berger, 2014).

These findings indicated that the changes in DNA methylation primarily occur during germline development to reprogram epigenetic information before fertilisation and the development of the embryo.

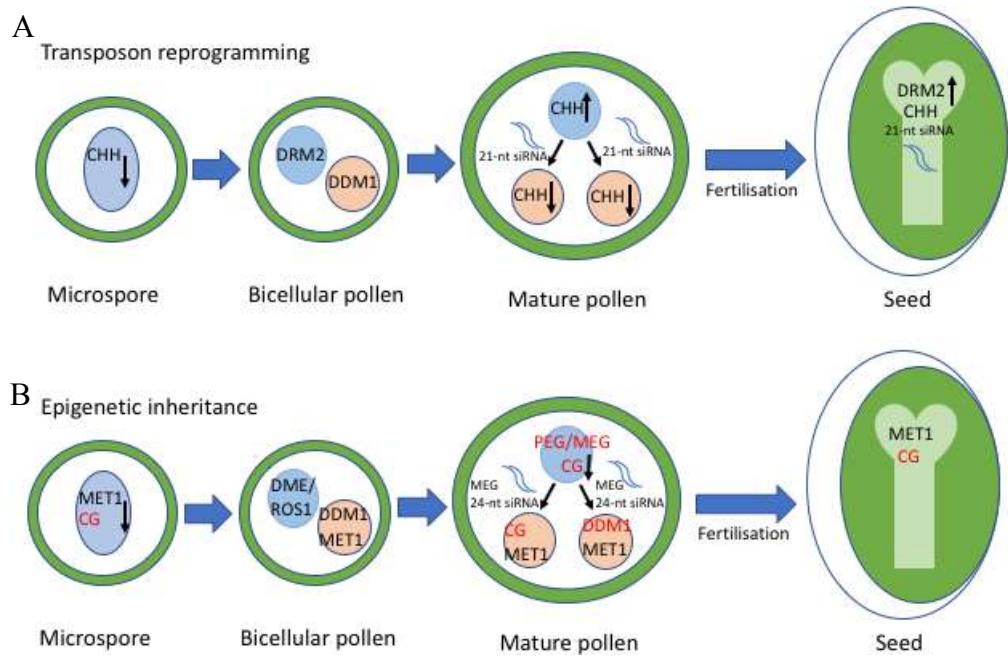


Figure 1.4 Genome reprogramming during pollen development. (A) CHH methylation is lost followed by the downregulation of DRM2 in the microspore and sperm cells, and it is re-methylated after fertilization in the embryo (green), guided in part by maternal 24nt siRNA. DRM2 restores CHH methylation in the VN, guided by pollen 24nt siRNAs. In the vegetative cell, the loss of DDM1 expression and reactivation of transposons generates 21nt siRNA that accumulate in sperm cells. (B) In the VN, DME and ROS1 demethylate specific transposons, including imprinted genes. In SC, CG methylation is maintained by MET1, and 24nt siRNA accumulate specifically from transposons. Vegetative Nucleus (VN), Sperm Cell (SC) (modified from: Calarco et al., 2012).

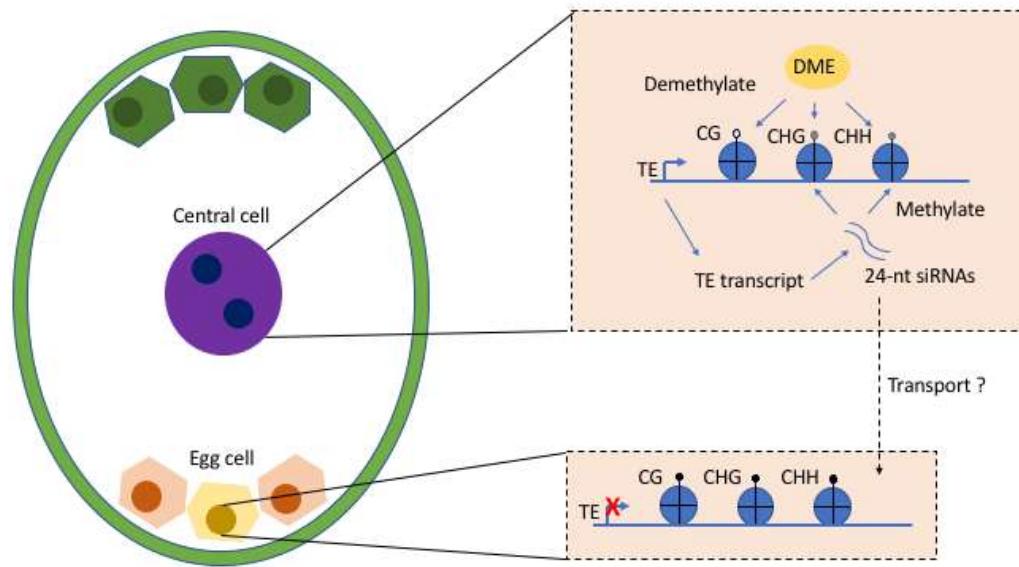


Figure 1.5 Model of epigenetic reprogramming in female gametophyte. In female gametophyte, the central cell is demethylated by DEMETER (DME). This activates TEs, and TEs transcripts produce 24-nt siRNAs. The siRNAs further activates RNA-directed DNA methylation (RdDM) to methylate at CHG and CHH context. The siRNAs produced by TEs in central cell may also travel to egg cell and enhance TEs silencing in egg cell (modified from: Feng et al., 2010)

1.7 Asexual Reproduction and Embryogenesis in Plants

Reproduction is an important aspect of all living organisms. Through reproduction, the genetic variation of a specific organism can be maintained. Two forms of reproduction are known. Sexual reproduction is a process of fusion between male and female gametes to produce a zygote, where the offspring inherits the genetic identity from both the paternal and maternal genome. The other mode of reproduction is through asexual reproduction where the embryos do not form from a process of fusing male and female gametes, and instead only one parental genome information is inherited. Both animals and plants can undergo asexual reproduction, but the event is common to plants due to the presence of meristematic tissue, which is undifferentiated cells that are actively dividing (Campbell et al., 2008). This makes plants high totipotent organisms with the ability to regenerate from any vegetative segments. In the vascular plants, the asexual reproduction can occur by forming either budding, branching, or tillering (vegetative reproduction) or by producing spores or seed genetically identical to the sporophytes that generated them (agamospermy in angiosperm and apogamy in pteridophytes) (Holsinger, 2000). Asexual reproduction results in more homozygous progenies with fewer genotypes per population than outcrossers, they also have fewer polymorphic loci and fewer alleles per polymorphic locus than closely related outcrossers (Holsinger, 2000). Asexual reproduction does not reduce genetic variation in a population of organisms, neither does it affect the equilibrium genotype frequencies of neutral alleles in an organism where least some of them outbreed sexually (Bengtsson, 2003).

The possession of a high degree of totipotency gives plants high developmental plasticity in comparison to animals. The ability of plants to regenerate has been used for clonal propagation in the form cutting and grafting for many years (Ikeuchi et al., 2016). In the early 20th century, Haberlandt introduced a concept of tissue culture when he tried to develop a methodology to regenerate whole individual plants from small tissues or single cells *in vitro* (Ikeuchi et al., 2016). The capacity of plants to regenerate in *in vitro* conditions can be improved by the application of plant growth hormones and culturing on nutrient rich media (Ikeuchi et al., 2016; Murashige, 1974). *In vitro* regeneration in plants can be induced from various organs: haploid plants can be formed through embryogenesis

using pollen (Maraschin et al., 2005), shoot segments can be used to form multiple shoots (Ikeuchi et al., 2016), *de novo* organogenesis can be initiated from somatic tissues and protoplast can undergo regeneration to form fully functional plants (Chupeau et al., 2013; Ikeuchi et al., 2016; Takebe et al., 1971).

To initiate regeneration, plants utilise two cellular strategies. One is through reactivation of undifferentiated cells, and the other one is through a reprogramming of differentiated somatic cells (Ikeuchi et al., 2016). Wounding stimuli serve as a primary induction for regeneration to take place at the wound site (Sugiyama, 2015). The wound signal activates the expression of WOUND-INDUCED DEDIFFERENTIATION 1 (WIND1) which promotes callus formation at the wounding site (Iwase et al., 2011). The overexpression of *WIND1* also causes shoot and root regeneration; this indicates that WIND promotes regeneration by reprogramming somatic cells and confers pluripotency (Ikeuchi et al., 2016; Iwase et al., 2013).

The exposure to stress can also induce the process of somatic embryogenesis in plants. In *Arabidopsis*, the exposure to different abiotic stresses such as osmotic, heavy metal ion, and drought stress can induce somatic embryogenesis from the shoot-apical-tip and floral-bud explants (Ikeda-Iwai et al, 2003). Somatic embryogenesis is a unique morphological and developmental process within the plant kingdom in which somatic cells undergo developmental reprogramming to have embryological characteristics that are capable of developing into a mature plant (Rose, 2004; Zimmerman, 1993). The process of formatting somatic embryos is divided into two main stages, the induction stage and developmental stage (Magnani et al., 2017). During the induction stage, somatic cells are exposed to the conditions that promote cells division and dedifferentiation. During developmental stage of somatic embryogenesis, the cultured cells start differentiating into somatic embryos (Magnani et al., 2017). The main factors that control somatic embryogenesis in plants involve cell signalling (Hecht et al., 2001), cell wall modifications (Majewska-Sawka & Nothnagel, 2000) and hormonal alterations (Su et al., 2009). Recently, it has also been found that during embryogenesis, the cells shut down

metabolic processes including carbohydrate and lipid metabolism, and the transcriptional machinery is highly activated (Magnani et al., 2017).

In addition, epigenetics regulation also plays a crucial role in regulating the process of somatic embryos formation and regeneration. The repression of POLYCOMB REPRESSIVE COMPLEX2 (PRC2) is essential to initiate the process of embryogenesis (Ikeuchi et al., 2016). PRC2 maintains transcriptional repression through the accumulation of H3K27me3 marks (Holec & Berger, 2012). Loss function of PRC2 reduces the accumulation of H3K27me3 and allows the expression of the reprogramming regulator *WIND3* and embryonic regulator *LEC2* (Mozgová et al., 2017). Somatic embryogenesis is also under direct control of histone deacetylation, the loss function of two histone deacetylases, HDA19 and HDA6, in Arabidopsis leads to the formation of embryo-like structures in shoots (Tanaka et al., 2008). While *met1* mutation improves shoot regeneration by re-activation of MET1 target genes including *WUS* (Ikeuchi et al., 2016; Li et al., 2011).

In summary, epigenetics marks are crucial methods for plants adaptation to environmental stresses. Maintaining stress-induced epigenetic changes sometimes negatively affect plants growth and development. Thus to ensure correct development, these stress-induced epigenetic marks need to be erased and reprogrammed during male and female gametogenesis. I hypothesise that if the stress marks are introduced during the process of the somatic embryogenesis; the epigenetic changes can be propagated to the non-stress progenies without undergone the process of epigenetic reprogramming.

1.8 Project Aim

This project aims to investigate whether the stress introduced during somatic embryogenesis can develop the stress adaptation phenotype to the non-stress progenies, and to assess the phenotype changes are due to the changes and molecular level, in particular to transcriptomic and methylome changes.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

A. thaliana plants (Col-0) carrying an inducible construct for the expression of a synthetic RWP-RKD4 (At5g53040) gene was used in which the seed were provided by Waki et al. (2011). The seeds were sterilized with 5% bleach for 5 minutes then washed five times with sterile double distilled water. Few drops of 0.1% agarose was added after the final washing. The seeds then sowed into MS media (Sigma Aldrich) containing 2% of sucrose (Sigma Aldrich) and 8 g/L of phyto agar (Duchefa Biochemie) (pH 5.7-5.8 with 1M KOH). The seeds were stratified for four days at 4°C to break dormancy. After four days, the plates were moved into control environment with following conditions; 10 kLux light for 16 h, dark for 8 h; 22°C/18°C day/night temperature; 50% / 60% relative humidity at day/night. The plates were incubated to grow for six days.

2.2 Stress Treatment

For stress treatment, plants were grown for six days in MS media (Sigma Aldrich), and plantlets were moved into new MS media containing 30 mM of dexamethasone (DEX) (Sigma Aldrich). The stress treatment was performed as in the Table 2.1.

For control experiment, the plantlets were transferred into new MS media (Sigma Aldrich) containing 30 mM of DEX (Sigma Aldrich) without exposure to any stress and incubated for seven days before transferring to the media without DEX.

For both stress-treated and control plants after seven days in normal media without DEX, the somatic embryos formed in the root tips and young leaf primordial. The somatic embryos were cut into smaller pieces by using a sterile needle and transferred into new MS plates with growth condition similar on section 2.1. Each plant that originates from root's somatic embryo will be labeled RO (root origin) and leaf origin will be labeled as LO. After the RO and LO plants were fully regenerated into new plants, the plants were transferred into soil. Ten plants per experiment were grown with growth condition as mentioned on section 2.1. This generation was named as F0.

Table 2.1 Stress treatment during somatic embryogenesis induction.

Stress Type	Stress Used	Conditions
Cold		Cold stress induced when the plants were moved into media with DEX. The stress was induced for 7 days with 2 days in 4°C and 1 day in 22°C and it repeated until it reached 7 days.
Abiotic	Salt	Salt stress induced when the plants were moved into media with DEX for 7 days. There were two salt concentration used, 25 mM NaCl and 75 mM NaCl.
	flg22	The 1µM of flg22 was applied directly into the 5 days old seedlings when the seedlings were moved into media with DEX and let for 7 days before moved into new media without DEX.
Biotic		The 1µM of pep1 was applied directly into the 5 days old seedlings when the seedlings were moved into media with DEX and let for 7 days before moved into new media without DEX.
	pep1	

2.3 Salt Survival Assay

Seeds for each lines RO and LO were sterilised with 5% bleach for 5 minutes and washed 5 times with sterile double distilled water. Around 50 seeds per line per plate were sown in MS media (Sigma Aldrich) with 175 mM NaCl (Sigma Aldrich) (1% Sucrose, 4.5 g/L MS basal medial, 8 g/L phyto agar (Duchefa Biochemie), pH 5.7-5.8 using 1M KOH) with total of three replicates for each line. Plates were stratified for 2 days. The plates were put in a control environment for two weeks before the survival data were collected by scoring the seedlings that formed cotyledon.

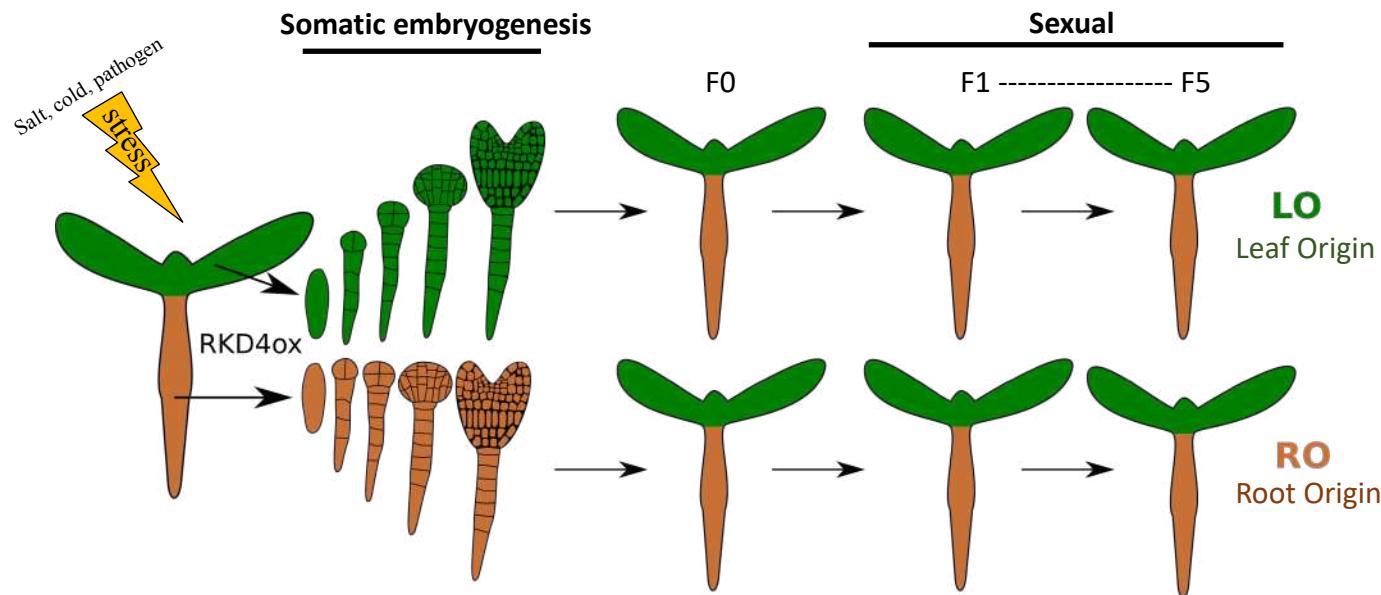


Figure 2.1. Experiment design. The *Arabidopsis* with inducible synthetic RWP-RKD4 gene were used to generate leaf and root origin control and stressed plants. After the seedlings were germinated in MS media, the seedlings were moved into new media containing dexamethasone. It allowed the formation of somatic embryo in young leaf primordial and root meristem. Each somatic embryo was moved into new MS media to regenerate into leaf and root origin plants. For stress treatment, the stress was introduced to the plants when the seedlings were moved into media containing dexamethasone, thus the stress was introduced during somatic embryogenesis. The F0 plants were grown in non-stress condition and allowed to produce seeds. The seeds from F0 were used to establish F1, and F1 seeds were used to produce F2 generations. The plants were grown until F5 generation under non-stress condition. For each line, the seeds were pooled from 10 plants.

Table 2.2 Seeds origin from each sample use in the experiment

Sample Name	Seeds Origin		
	F1	F2	F3
Control RO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Control LO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Salt RO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Salt LO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Cold RO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Cold LO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Flg22 RO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant
Flg22 LO (line 1, 2, 3, 4, and 5)	Each line comes from pooled of 10 F0 plant	Each line comes from pooled of 10 F1 plant	Each line comes from pooled of 10 F2 plant

2.4 Freezing Tolerance Assay

The method was developed by (Shi et al., 2012). Seeds for each lines RO and LO were sterilised with 5% bleach for 5 minutes and washed 5 times with sterile double distilled water. Around 50 seeds per line per plate were sown in MS media (1% sucrose, 4.5 g/L MS basal medial (Sigma Aldrich), 8 g/L phyto agar (Duchefa Biochemie), pH 5.7-5.8 with 1M KOH) with total of three replicates for each line. The plates then transferred into growth chamber at 22°C for two weeks. The two-week old plants were placed into freezing chamber (LMS Ltd) with the lids opened, and acclimatised at 0°C for 1 hour before the temperature was decreased by -1°C per hour until the temperature reached -5°C. After freezing treatment at -5°C for an hour, the plants were incubated at 4°C in the dark for 12 h, then transferred to light at 22°C. The survival rates of the seedlings were scored visually after 2 d by looking at the number of green seedlings.

2.5 Pst-DC3000 Pathogenesis Assay

Four weeks old plants grown under a short-day condition (8 hours day and 16 hours night) were used for this assay. The *Pseudomonas syringae* pv *tomato* DC3000 single colony was inoculated into 10 mL of King B liquid media (Sigma Aldrich) at 28°C overnight. The liquid culture was centrifuged under 2700 rpm, the supernatant was discarded and the pellet was washed with 5 mL of 10 mM MgCl₂ (Sigma Aldrich). This step was repeated twice. After final wash, the pellet was resuspended in 10 mL of 10 mM MgCl₂. The concentration of bacteria was checked using spectrophotometry and it was diluted until the OD 0.15 was achieved.

A fully expanded leaf was infiltrated with the diluted *Pst* DC3000 until the liquid cover the whole surface of the plants. The plants were put under the Fluorimager Chlorophyll Fluorescence Imaging (Technologica, United Kingdom) for 24 hours. The fv/fm value of the infiltrated leaf was quantified using the software provided by the manufacturer.

2.6 Nucleic Acid Extraction

Leaf samples were collected from pooled ten individual plants of five-weeks-old plants. The leaf samples were inserted into 1.5 mL Eppendorf tubes and flash-frozen in liquid nitrogen and stored under -80°C until further use. The samples were pulverized in a mortar with the addition of liquid nitrogen to prevent sample from thawing. After the samples were completely pulverised, the genomic DNA was extracted using Qiagen Plant DNesay kit (Qiagen). The quality and quantity of genomic DNA was checked using agarose gel electrophoresis and NanoDrop (Thermo Scientific).

Leaf samples from pooled ten individual plants of five-weeks-old plants were collected and pulverised. The total RNA sample was extracted using a Qiagen Plant RNesay kit (Qiagen) following the manufacturers manual. The quality and quantity of total RNA was analyzed using agarose gel electrophoresis and NanoDrop (Thermo Scientific).

2.7 Next Generation Sequencing and Library Preparation

2.7.1 Library Preparation for Bisulfite Sequencing

DNA from leaf material of ten individual plants was used to generate DNA libraries for bisulfite sequencing. The library was generated using the Illumina TruSeq Nano kit (Illumina, CA, U.S.A) according to the manufacturers manual. The DNA was sheared by using Q800R2 Sonicator (Qsonica) to the exact size of 350 bp before the adapter was ligated into the sheared-DNA. The adapter-ligated DNA was undergone bisulfite treatment by using Epitect Plus DNA Bisulfite Conversion Kit (Qiagen, Hilden, Germany) according to the manufacturers manual. The treated DNA was cleaned-up before the library was enriched using Kapa Hifi Uracil+ DNA polymerase (Kapa Biosystem, MA, U.S.A) according to the manufacturers manual to read through uracil residues while retaining high yields, low-bias, and uniform sequencing coverage.

2.7.2 Bisulfite Sequencing

Bisulphite sequencing was performed on an Illumina HiSeq2000 instrument. Bisulphite-converted libraries were sequenced with 2×101 -bp paired-end reads. For bisulphite sequencing, conventional *A. thaliana* DNA genomic libraries were analysed in control lanes. Seven to eight libraries with different indexing adapters were pooled in one lane. For image analysis, the Illumina Real Time Analysis 1.13.48. software was used. The library QC was assessed using Bioanalyser (Agilent) to measure the size distribution and concentration.

2.7.3 Library Preparation for RNA Sequencing

RNA from leaf material of ten individual plants was used to generate RNA libraries for RNA sequencing. The library was generated using Illumina TruSeq RNA Sample Preparation Kit V.2 (Illumina, CA, U.S.A) based on the Low Sample standard manufacturers manual. The library QC was assessed using Bioanalyser (Agilent) to measure the size distribution and concentration.

2.7.3 RNA Sequencing

RNA sequencing was performed on an Illumina HiSeq2000 instrument. The RNA libraries were sequenced with 100bp single-end. The quality of the reads was assessed using FastQC and trimming of low quality bases at the 3' end of the reads and adapter removal was done using Trimmomatic. The final reads were then aligned to the TAIR10 reference genome.

2.8 Processing and Alignment of Bisulphite-Treated Reads

The method was adapted from Becker et al., (2011), the SHORE pipeline v0.9.0 was used to trim and filter the reads. Reads with more than 2 bases in the first 12 positions with a base quality score of less than 5 were discarded. All the trimmed reads shorter than 40 bases were deleted, and all the high quality reads (on average 82% of raw reads across the sequenced strain) were aligned against TAIR 10 reference genome using SHORE.

GenomeMapper reported all alignments with the least amount of mismatches for each read, but only reads mapping unique to a single position were used for this study. A paired-end correction method was used to discard repetitive reads by comparing the distance between reads and their partner to the average distance between all read pairs. Reads with abnormal distances were removed if there was at least one other alignment of this read in a concordant distance to its pairs (differing by more than two standard deviations). Finally, read counts on all cytosine sites were obtained with SHORE. The ‘scoring matrix approach’ of SHORE assigns a score to each site by testing against different sequence and alignment related features. For comparisons across lines, cytosines were accepted if at most one intermediate penalty on its score matrix was applicable to at least one strain (score matrix ≥ 32).

2.9 Identification of Methylated Regions (MRs)

The MRs analysis was done similar to Wibowo et al. (2016). Briefly, MRs was determined using a Hidden Markov Model as mentioned by Hagmann et al. (2015). MRs of replicates were merged into a common set of MRs. Whenever different samples were treated as a replicate group (e.g. control RO, control LO, salt-treated RO, salt-treated LO samples),

their MRs were merged into a common set. Regions that showed statistically significant methylation differences between at least two sets of strains were identified as DMRs (Hagmann et al., 2015). Segmentations across the genomes of every sample served to set breakpoints of start and end coordinates of all predicted MRs. Each combination of coordinates in this set defined a segment to perform the test for differential methylation in all pairwise comparisons of the strains, if at least one strain was in a high methylation state throughout this whole segment (Hagmann et al., 2015). Per pairwise comparison, between 30,000 and 50,000 segments were tested (Hagmann et al., 2015).

2.10 Identification of Differentially Methylated Regions (DMRs)

This was carried out according to Hagmann et al. (2015). The regions different or highly methylated between strains and statistically tested using Fisher's exact test for differentially methylation were selected. The epiallele frequencies were obtained by clustering the strains into several groups based on their pairwise comparison and statistically tested using Fisher's exact test. The regions that exhibited statistically significant methylation differences between at least two sets of strains were identified as DMRs. For test in the second generation (F2), salt RO from line 1, 2 and 3 were grouped as salt RO, and salt LO from line 1, 2, and 3 were grouped as salt LO. Similar grouping was carried out for cold LO, cold RO, control RO and control LO. Both salt RO and cold RO were tested against control RO, and salt LO and cold LO were tested against control LO.

2.11 RNAseq Analysis and Heatmap

The RNAseq reads that passed the quality control (on average 82% of raw reads across the sequenced strain) were aligned and mapped to *Arabidopsis* TAIR10 reference genome using TopHat 2 (Parameters –i 20 –l 30000) in combination with Bowtie to identify splice junctions between exons. The files were converted into Sequence Alignment Map (SAM) format. The transcript then annotated to count how many read map to each gene by using HTSeq ver. 0.9.1.

R package DeSEQ2 (version 1.10.1) was used to analyse differential gene expression. Genes were categorized as significantly differentially expressed at an False Discovery Rate (FDR) < 0.01 and log₂ fold change < -1.5 or log₂ fold change > 1.5.

2.12 Gene Ontology Analysis

Gene ontology analysis was carried out in agriGO v2.0 (Tian et al., 2017) by using the differentially expressed gene list generated by DESeq. The background used for identifying enrichment was the suggested background provided by AgriGO, which all contains all GO annotated genes in Arabidopsis.

2.13 Gene Network Analysis

The network analysis was carried out using GeneMania (Warde-Farley et al., 2010) in Cytoscape. All differentially expressed genes were loaded into GeneMania. The database of genes that had protein-protein interaction were generated using GeneMania databases. The gene network was created by intersected the differentially expressed genes to the protein-protein interaction database of GeneMania. The edges were removed unless they were sourced from physical interaction database, and nodes were removed if they had no connections with the main network.

2.14 RT-qPCR

cDNA used for RT-qPCR was synthesised using 500 ng of total RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturers manual. RT-qPCR experiments were performed in control ROF2 and salt ROF2 line 1, 2 and 3, with a final volume of 25 µl containing 5 µl of cDNA template (diluted beforehand 1:10), 0.2 µM of each primer (forward and reverse), and 12.5 µl of 2×MESA Blue qPCR MasterMix (Eurogentec Headquarters). The following cycling profile was used: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The melting curve was determined in the range of 60–95°C, with a temperature increment of 0.01°C/sec. Each reaction was run in triplicate (technical replicates). Negative controls included in each run were a reaction without reverse transcriptase and one without template (2 µL of nuclease-free water instead of 2 µL of cDNA). Raw Ct data were

analysed using qbase+ v3.0 (Biogazelle). Analysis of *MPK3* expression data was performed according to the $\Delta\Delta CT$ method (Schmittgen & Livak, 2008) using GADPH (At1g13440), PP2AA3 (At1g13320) and for normalization (Table 2.3) (Lippold et al., 2009).

Table 2.3 List of RT-qPCR primers used in the experiment.

Primers Name	Sequence (5'-3')	Comment
PP2AA3.FP	TAACGTGGCCAAAATGATGC	
PP2AA3.RP	GTTCTCCACAACCGCTTGGT	Housekeeping
GAPDH Fw	TTGGTGACAACAGGTCCAAGCA	
GAPDH Rev	AAACTTGTGCTCAATGCAATC	Housekeeping
MPK3_FP	CTTGCTAGACCTACTTCAGAGAATG	
MPK3_RP	TCCATAAAGATAACAACCAACAGACC	Target

2.15 Formation of Transgenic *Oryza sativa Japonica* cv. *Nipponbare*

2.15.1 Nucleic Acid Extraction

Genomic DNA was extracted from *A.thaliana* leaf containing an inducible RWP-RK gene by using Qiagen Plant DNesay kit (Qiagen) according to the manufacturers manual. The DNA quality and quantity were checked using agarose gel electrophoresis and NanoDrop (Thermo Scientific) by looking at the DNA integrity and A260/A230 ration.

2.15.2 RWP-RK Construct Preparation

Two steps of PCR were used to amplify RWP-RK gene by using Pyrobest DNA polymerase (Takara). The following composition and condition was used:

First step PCR was conducted by adding 38.25 μ L of ddH₂O, 5 μ L of 10x PCR buffer, 4 μ L of pyrobest dNTP mix, 0.25 μ L of pyrobest DNA polymerase (5U/ μ L), 1 μ L of DNA template, 0.5 μ L (AtRKD4_F) of forward primer (10 μ M) (Table 2.4) and 0.5 μ L (AtRKD4_R) of reverse primer (10 μ M) (Table 2.4). The following PCR program was used for first step PCR: 95°C for 30 sec, then 25 cycles of 95°C for 30 sec, 58°C for 1 min and 72°C for 2 min. The program was ended with 72°C for 10 min.

Second step PCR was done by using the following composition: 77.5 µL of ddH₂O, 10 µL of 10x PCR Buffer, 8 µL of pyrobest dNTP mix, .5 µL of pyrobest DNA polymerase (5U/µL), 2 µL of Template (from first step PCR), 1 µL (AtRKD4_Gateway_F) of forward primer (10 µM) (Table 2.4) and 1 µL (AtRKD4_Gateway_R) of reverse primer (10 µM) (Table 2.4). The following PCR program was used for the second step PCR: 95°C for 30 sec followed by 4 cycles of 95°C for 30 sec, 68°C for 30 sec and 72°C for 90 sec. Another 20 cycles of 95°C for 30 sec, 70°C for 30 sec and 72°C for 90 sec were added to the program. The program finished with 72°C for 10 min.

Table 2.4 List of primers used for the formation of transgenic *O.sativa*. (red: Attb site of gateway cassette).

Primers Name	Sequence (5'-3')	Product size
AtRKD4_Gateway_F	GGACAAGTTGTACAAAAAAGCAGGCT ATGAGTTCGTAAACATTCCCTTG	982 bp
AtRKD4_Gateway_R	ACCACTTGTACAAGAAAGCTGGGT TCAATAATAATCATCACCAAGTG	
AtRKD4_F	GTTCATTCATTGGAGAGGACG	1101 bp
AtRKD4_R	ATTGACCAATTGGGTCAACAAAG	
pERV1_RB_Fw	GGTTTACCCGCCAATATATCCTG	
T3A_Fw	GTCAAATCGTGGCCTCTAATGAC	
OsAct-I_Fw	TTCGTCAGGCTTAGATGTGCTAGATC	
pERV1_LB_Rev	GTGCACATGGCTCAGTTCTC	
OsRKD4_Syn_Fw	TCAGTTCGTGTCAACCTTGC	1388 bp
OsRKD4_Syn_Rev	ATTGCAGCGTCAAGGGTATG	
NOS3SacI.Rev	GAGCTCCGATCTAGAACATAGATGACACC	
AtRKD4_Seq_Fw	GAAGCGATGCAGGGAATTAG	

The PCR was run into agarose then the expected band was extracted by using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers manual. The extraction product was ligated into pGEMT-easy by using T4 ligation system (NEB) according to the manufacturers manual.

The ligated product was transformed into *E. coli* strain Top10 by using heat shock. The positive transformants were screened by using ampicillin with IPTG and X-Gal. BP-LR cloning (Invitrogen) was performed with the construct according to the manufacturers

manual with pERV1 (pERV1 vector was obtained from Valdivia et al. (2013)) as final destination vector. The pERV1 with insert was confirmed by using sequencing using the following primers pERV1_RB_Fw, T3A_Fw, OsAct-I_Fw, pERV1_LB_Rev, OsRKD4_Syn_Fw, AtRKD4_Seq_Fw and NOS3SacI.REV. The sequencing result was aligned against the pERV1 sequence and for the insert, it was BLASTed in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the gene sequence.

2.15.3 Agrobacterium Transformation

The electrocompetent *Agrobacterium tumifaciens* strain EHA105 was used. Before the transformation was conducted, the pERV1 with RWP-RK gene as insert was diluted 1:100. To transform the cells, 25 µL of competent cells was mixed with 1 µL of diluted plasmid. Then it was incubated on ice for approximately 10 mins. The cells were put into the electrophoresis cuvette and put into Gene Pulser Xcell Electroporation system (Biorad). The Agr program was chosen in the Gene Pulser Xcell Electroporation before the pulse was applied. The cells were collected and incubated at 1 hour in LB low salt media at 28°C. The cells were centrifuged at 5000 rpm for 5 mins, and the media was removed. The cells were resuspended using 150 µL of LB low salt media and streaked on a LB low salt plate containing rifampicin and spectinomycin.

2.15.4 Callus Induction

The method is adopted from Main et al. (2015). The seeds of *O.sativa Japonica* cv. *Nipponbare* were de-husked and pre-rinse in 10 mL of 70% Ethanol (Fisher Scientific) for one minutes and then washed with sterile water. The seeds then washed with 50% of commercial bleach for 30 minutes. The seeds were washed five times with 10 mL sterile water. The seeds then poured into filter paper for drying. The seeds were transferred into callus induction media adopted from Hiei et al. (1994) containing N6 salts and vitamins (Sigma Aldrich), 300 mg/L casamino acids (Sigma Aldrich), 2.8 g/L L-proline (Sigma Aldrich), 30 g/L sucrose (Sigma Aldrich) and 4 g/L gelrite (Duchefa Biochemie) (pH 5.8). The plates were put at 29°C with 16 hour : 8 hour photoperiod, 80-100 µmol/m²/s light intensity.

2.15.5 Agrobacterium Infection

The method is adopted from Main et al. (2015). A small amount of bacteria culture from plate was scraped and suspended in 15 mL of liquid infection medium (N6 salts and vitamins (Sigma Aldrich), 1.5 mg/L 2,4-D, 0.7 g/L L-proline (Sigma Aldrich), 68.4 g/L sucrose (Sigma Aldrich) and 36 g/L glucose (Sigma Aldrich) (pH 5.2) supplemented with 100 µM Acetosyringone (AS) in a conical flask. The optical density was adjusted to <0.1 ($OD_{550} = 0.06-0.08$). The rice calli were placed into bacteria-free infection medium + AS. The bacteria-free infection medium was then removed and replaced with 10 mL of *Agrobacterium* suspension. The conical flask was put into a shaker at low setting for two minutes at 28°C. The calli was dried using sterile filter paper and moved into co-cultivation media (N6 salts and vitamins, 300 mg/L casamino acids, 30 g/L sucrose, 10 g/L glucose and 4 g/L gelrite (pH 5.8) with the addition of 100µM AS and 2 mg/L 2,4-D) for three days in the dark at 25°C.

2.15.6 Selection for Transformants

The method is adopted from Main et al. (2015). After three days in co-cultivation media, the calli were washed five times with 5 mL liquid infection medium without AS but with addition of carbenicillin (500 mg/L) and vancomycin (100 mg/L). The calli were dried on sterile filter paper and then transferred into selection medium (N6 salts and vitamins (Sigma Aldrich), 300 mg/L casamino acids (Sigma Aldrich), 2.8 g/L L-proline (Sigma Aldrich), 30 g/L sucrose and 4 g/L gelrite (Duchefa Biochemie) (pH 5.8 with 1M KOH) with the addition of 2 mg/L bialaphos, 2 mg/L 2,4-D and 500 mg/L carbenicillin). The plates were placed in the light at 29°C with 16 hour : 8 hour photoperiod, 80-100 µmol/m²/s light intensity. The calli were sub-cultured every two weeks and the putative clones were observed after six to eight weeks on selection media.

2.15.7 Regeneration of Transgenic Plants

The method is adopted from Main et al. (2015). To regenerate the callus into plant, new lobes of growth was selected from callus and transferred it into regeneration medium 1 (MS salts and vitamins (Sigma-Aldrich), 2 g/L casamino acids (Sigma Aldrich), 30 g/L sucrose (Sigma Aldrich), 30 g/L sorbitol (Sigma Aldrich), and 4 g/L gelrite (Duchefa

Biochemie) (pH 5.8 with 1M KOH), 100 mg/L cefatoxime, 100 mg/L vancomycin, 0.02 mg/L NAA, 2 mg/L kinetin and 2 mg/L bialaphos) and placed in light at 25°C. After three weeks the mature tissues were transferred to regeneration medium II (MS salts and vitamins (Sigma Aldrich), 100 mg/L myo-inositol (Sigma Aldrich), 30 g/L sucrose (Sigma Aldrich), 3 g/L gelrite (Duchefa Biochemie) (pH 5.8 with 1M KOH)) for germination and placed in light at 25°C with 16 hour : 8 hour photoperiod, 80-100 $\mu\text{mol}/\text{m}^2/\text{s}$ light intensity.

2.15.8 Growth Condition for Transgenic Rice

The transgenic rice was transferred into soil (M2 compost) and planted into 3 $\frac{1}{2}''$ x 3 $\frac{1}{2}''$ pot containing 1 tbs osmocote 17-7-2 and 1 tbs sprint 330 (BASF) chelated iron. The soil was soaked in water to make it moist. After the plants were moved into the post and covered with clear plastic bag with one hole open. The plants were put at 12:12 photoperiod with temperature setting 28°C/25°C (day/night) and light intensity of 350 $\mu\text{E}/\text{m}^2/\text{s}$. After three days, another hole was made on the plastic bag cover. The cover was removed a week after planting and the water level maintained $\frac{3}{4}$ full.

2.15.9 Transgene Induction

To induce the expression of *AtRKD* and *OsRKD4* transgene in *O.sativa* cv. *Nipponbare*, approximately 10 cm leaf from transgenic *O.sativa* cv. *Nipponbare* was collected. The leaf then soaked into 50 mL of sterile ddH₂O containing 20 μM of β -estradiol (Sigma-Aldrich), 0.02% Silwet L-77 and kept at room temperature for 24 hours before the total RNA was extracted.

2.15.10 RT-PCR

cDNA was synthesised using 500 ng of total RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturers manual. The cDNA then diluted 1:100 using sterile ddH₂O before further used. The PCR was performed by using primers in the Table 2.5 with the following conditions: 19.55 μL of ddH₂O, 2.5 μL of 10x PCR buffer, 0.75 μL of 10mM dNTP mix, 0.2 μL of DNA polymerase (5U/ μL), 0.5 μL of cDNA template, 0.75 μL of forward primer (20 μM) and 0.75 μL of reverse primer (20

μM). The following PCR program was used: 95°C for 30 sec followed by 25 cycles of 95°C for 30 sec, 58°C for 1 min and 72°C for 2 min. The program was finished at 72°C for 10 min.

Table 2.5 Primers list for RT-PCR.

Primers Name	Sequence (5'-3')	Product Size
AtRKD4_RT.Fw	ACGACGGTCTCATTCCAAC	
AtRKD4_RT.Rev	CTTCTTCCATTCCAACATTCTTGAG	270 bp
OsRKD4_RT1 Fw	TCGAGATGGAAATGCACGAG	
OsRKD4_RT1 Rev	TCACCACCAGATCAGCAGTC	228 bp
TaRKD2_RT1 Fw	GATGTCTACTCTCCTACGCACCGA	
TaRKD2_RT1 Rev	CATCAACAGAGCGTCGTCAAGTCC	84 bp
OsActin_Fw	TCCATCTTGGCATCTCTCAG	
OsActin_Rev	GTACCCGCATCAGGCATCTG	337 bp
TaGAPDH_Fw	TTAGACTTGCGAAGCCAGCA	
TaGAPDH_Rev	AAATGCCCTTGAGGTTCCC	81 bp

2.16 Wheat Transformation

The method was adopted from Ishida et al. (2015). Briefly spring wheat cultivars Fielder (*Triticum aestivum* cv. Fielder) was cultivated in Levingtons M2 compost with growing condition 20/15°C day/night temperature, 16 hour day and 8 hour night cycle, 350-500 $\mu\text{Em}^{-2}\text{s}^{-1}$ light intensity. Immature seeds were collected from panicles about 2 weeks after anthesis, sterilised with 70 % ethanol and 1 % sodium hypochlorite and then washed three times with sterilised distilled water. Immature embryos were isolated from the seeds under stereoscopic microscope.

Agrobacterium tumefaciens strain EHA105, and the vectors pERV1 (Valdivia et al., 2013) containing synthetic TaRKD2 was used. The TaRKD2 was designed using gBlock® service by IDT (www.idtdna.com). The TaRKD2 then cloned into pERV1 vector using gateway LR clonase kit (Thermo Fisher) according to the manufacturer manual.

Isolated immature embryos were treated with centrifuging at various strength in the liquid medium and then inoculated with Agrobacterium. The embryos were placed on co-cultivation medium (co-cultivation medium was based on (Ishida et al., 2007)) that contained 5 μ M of AgNO₃ and/or CuSO₄ with the scutellum-side up and incubated at 23°C in the dark for 2 days. Embryo axis was excised and the embryos were transferred to resting medium (resting medium was based on (Ishida et al., 2007)) and incubated at 25°C in the dark for 5 days. The embryos were placed on the selection medium (selection medium was based on (Ishida et al., 2007)) that contained 15 mg/L of hygromycin and incubated for 2 weeks. Each of embryos was cut into two pieces, which were then transferred to the second selection medium (second selection medium was based on (Ishida et al., 2007)) that contained 30 mg/L of hygromycin and incubated for 3 weeks. The cell clumps proliferated from the pieces were placed on regeneration medium including 30 mg/L of hygromycin and incubated at 25°C under continuous illumination (35 μ mol m⁻²s⁻¹) for 2 weeks. Regenerated shoots were transferred to regeneration medium (regeneration medium was based on (Ishida et al., 2007)) that contained 15 mg/L of hygromycin and incubated for 2 weeks. Regenerated plants were transferred to soil in pots and grown in a greenhouse.

3. Plant Somatic Regeneration Through Overexpression of *RWP-RKD4*

3.1 Introduction

3.1.1 Plant Regeneration Through Tissue Culture

Unlike animals, plants have a high level of totipotency which allow the plant to regenerate itself upon damage or wounds. The first concept of regeneration in plants was mentioned by Goebel (1903) where he tried to regenerate parts of plants that have been wounded, and he found the ability of plants to regenerate from embryonic tissue is faster than from a fully differentiated organ.

The understanding of plant regeneration and the discovery of plant hormones, auxin and cytokinin, by F. Skoog and C. O. Miller in 1957 opened a wide opportunity for the advancement of plant tissue culture. Gamborg et al. (1976) are the first scientist that formulated a suitable composition for tissue culture media. Since then, the techniques in tissue culture have varied and developed in faster pace rapidly, starting from callus culture (Street, 1973), segments of shoot, leaf and root culture (Murashige, 1974), meristem culture (Quak, 1972) to the development of haploid culture from reproducing organs such as anther and pollen (Forster et al., 2007).

The first evidence of asexual embryogenesis was reported by Leeuwenhoek when he observed the occurrence of polyembryony in seeds from orange plants in 1719 (Tisserat et al., 1979). In plants, the initial somatic embryogenesis was through protoplast culture (Cocking, 1972). Gamborg et al. (1974) also develop a technique to perform somatic embryo culture by somatic hybridisation. This technique involves the production of plant protoplast through enzymatic processes and protoplast fusion to generate hybrid cells. They also perform plant transformation in protoplast by utilising different type of plants growth regulators.

Unfortunately, the techniques currently used for the establishment of somatic embryos still rely on different plant growth regulators, mainly cytokinin and auxin. It is argued that somaclonal propagation associated with mutagenesis and aberrant DNA methylation changes in cell and tissue cultures due to the influenced by oxidative stress at excision of

the tissue, media and *in vitro* environmental factors (Joyce et al., 2006). However, there are several transcription factors known to play a role in inducing the formation of somatic embryos in plants in the absence of these growth regulators. These transcription factors are haem activator protein 3 (HAP3), B3-domain proteins, Agamous-like 15 (AGL15), APETALA 2/ ETHYLENE RESPONSIVE FACTOR (AP2/ERF) domain proteins, homeodomain and RWP-RK domain proteins (Smertenko & Bozhkov, 2014).

HAP is a multimeric transcriptional activators complex that recognises the CCAAT box, and it consists of three subunits. At early embryogenesis, HAP3 helps to specify cotyledon cell identity and maintains the fate of suspensor cells by controlling the expression of cruciferin and oleosin genes (West et al., 1994). In later stages, it plays a role in controlling initiation and maintenance of embryo maturity and suppressing germination (Edwards et al., 1998; Smertenko & Bozhkov, 2014).

The B3-domain family is a plant-specific family of transcription factors with seven β -strands and two α -helices, which recognise the major groove of DNA helices. The function of B3-domain transcription factors overlaps with HAP3 transcription factor (Smertenko & Bozhkov, 2014).

AGL15 transcription factors contain a conserved MADS-box (MCM1, AG, DEFA, SRF) motif within their DNA binding domain. The expression of *AGL15* starts at globular stage during embryogenesis and it is transiently expressed in the young shoot apical meristem and floral buds. The overexpression of the *AGL15* transcription factor promotes direct and indirect somatic embryogenesis in both *A. thaliana* and soybean (Harding et al., 2003; Smertenko & Bozhkov, 2014).

LEAFY COTELYDON1 (LEC1) and LEAFY COTYLODEN2 (LEC2) belong to the HAP3 domain transcription factor. These proteins and AGL15 are known to be able to induce the formation of somatic embryogenesis by refinement of auxin production and signaling genes such as *YUCCA10*, *YUCCA2*, *YUCCA4* and *INDOLE ACETIC ACID IDUCIBLE30 (IAA30)* (Ikeuchi et al., 2016).

AP2/ERF proteins play a role in regulation of a plethora of developmental processes, for instance, floral meristem identity, lateral root morphogenesis, and response to external stimuli such as abiotic and biotic stresses. The overexpression of AP2/ERF transcription factors resulted in the induction of indirect somatic embryogenesis. Well-studied genes encoding AP2/ERF transcription factors are *WOUND-INDUCED DIFFERENTIATION*

Table 3.1 Regulation of somatic embryogenesis (modified from : Smertenko & Bozhkov, (2014))

Name	Functions	Species	Overexpression phenotype
Agamous-like 15 (AGL15)	MADS-box TF	<i>A.thaliana</i>	Primary somatic embryogenesis from zygotic embryos and also in the shoot apical meristem (Perry et al., 1999)
		<i>Glycine max</i>	Enhanced indirect somatic embryogenesis (Thakare et al., 2008)
Baby Boom (BBM)	AP2/ERF family TF	<i>B. napus</i> ,	Direct somatic embryogenesis on seedlings (Ouakfaoui et al., 2010)
		<i>A.thaliana</i>	
		<i>N. tabacum</i>	Sterility if constitutive; spontaneous organogenesis if inducible (Srinivasan et al., 2006)
		<i>C. annuum</i>	Indirect somatic embryogenesis in otherwise recalcitrant plants (Heidmann et al., 2011)
		<i>P. tomentosa</i>	Indirect somatic embryogenesis (Deng et al., 2009)
Embryo maker	AP2/ERF family TF	<i>A.thaliana</i>	Enhanced direct and indirect somatic embryogenesis (Tsuwamoto et al., 2010)
Leafy Cotyledon 1 (LEC1)	HAP3 domain TF	<i>A.thaliana</i>	Induction of direct somatic embryogenesis (Lotan et al., 1998)
Leafy Cotyledon 2 (LEC2)	B3 domain TF	<i>A.thaliana</i>	Induction of direct somatic embryogenesis without auxin (Stone et al., 2001)
Shootmeristemless (STM)	Homeobox domain TF	<i>B.oleraceae</i>	Enhanced efficiency of indirect somatic embryogenesis (Elhiti et al., 2010)
Wuschel (WUS)	Homeobox domain TF	<i>A.thaliana</i>	Induction of direct somatic embryogenesis without auxin (Zuo et al., 2002)
		<i>C. canephora</i>	Enhanced efficiency of indirect somatic embryogenesis (Arroyo-Herrera et al., 2008)
HBK3	Homeobox domain TF	<i>P.abies</i>	Increased yield of somatic embryogenesis (Belmonte et al., 2007)
RKD4	RWP-RK domain TF	<i>A.thaliana</i>	Short-term expression promotes somatic embryogenesis without auxin (Waki et al., 2011)

1 (WIND1) and its homologs *WIND2*, *WIND3* and *WIND4*. These genes are induced during wounding and promote callus formation at cut sites. Also overexpression of

WIND1 can regenerate roots and shoots when moved into non-inducible media. This indicates that *WIND1* can reprogram somatic cells into pluripotent cells (Ikeuchi et al., 2016a). The genes *PLETHORA3* (*PLT3*), *PLT5* and *PLT7* also belong to AP2/ERF group. These transcription factor-encoding genes are among the earliest responses induced by callus induction media (CIM) and lead to the activation of the key root meristem regulators to establish a pluripotent callus (Ikeuchi et al., 2016). The best-studied homeodomain transcription factor involved in embryogenesis is *WUSCHEL* (*WUS*) (Mayer et al., 1998). It helps cells to maintain a state of proliferation and responsiveness to other developmental cues. The *WUS* activity maintains shoot and flower apical meristems during embryonic and post-embryonic development (Smertenko & Bozhkov, 2014).

The RWP-RK transcription factors also play a role in embryogenesis, as it has been shown that they act as key regulators of egg cell gene expression required for embryo specification and differentiation (Koszegi et al., 2011). Mutations in RWP-RK proteins causes smaller embryos and seed germination defects as a consequence of the loss of suspensor cell identity. Additionally, the induction of ectopic expression of RWP-RK proteins activates the expression of embryogenesis-related genes and promotes somatic embryogenesis but with low efficiency (Smertenko & Bozhkov, 2014; Waki et al., 2011). However, regeneration by using somatic embryos developed from over expression of RWP-RK transcription factor is remained unknown.

Therefore, it is possible to perform tissue culture by using hormones and zygotic factors manipulation. However, these methodologies are inefficient, time-consuming and can lead to genomic mutation such as the activation of TEs or deletion. Thus it is important to develop a more efficient clonal propagation methodology for plants, especially for commercial corps such as rice, wheat and maize.

Rice is a major food crop and contributes to more than one fifth of all calories consumed by the world's population (Stroud et al., 2013). As consequences, it has become one of the most investigated crop plants (Satish, 1989) with rice tissue culture being highly studied and applied in Japan (Satish, 1989). However the conventional method of rice

tissue culture is time-consuming with low recovery of plants per unit culture (Ali et al., 2004), and rice regeneration from the overexpression of zygotic factors such as RWP-RK transcription factors never been recorded.

In summary, the development of a new methodology for plants regeneration is highly valuable. The overexpression of embryonic transcription factors would serve as a new method of propagation through somatic regeneration. In which this method can be applied for faster propagation of commercial crops and endangered plants.

3.1.2 Chapter Aim

This chapter aims to assess the effectiveness of plants regeneration from somatic embryogenesis by the overexpression of *RWP-RKD4* in *Arabidopsis thaliana* and *Oryza sativa* cv. *Nipponbare* by using a binary construct containing the *Arabidopsis RWP-RKD4* gene in Arabidopsis in which the expression of the transgene is controlled by DEX. Whereas in rice the expression of transgene is controlled by β -estradiol.

3.2 Results

3.2.1 Overexpression of *RWP-RKD4* is Effective Technique to Regenerate Plants from Somatic Cells

The ability of plants to produce somatic embryogenesis by overexpressing a transcription factor has previously been observed by others. The ectopic expression of LEAFY COTYLEDON2 (LEC2) leads to the formation of somatic embryos in hypocotyls (Sandra L Stone et al., 2008; Wójcikowska et al., 2013). Overexpression of BABY BOOM (BBM) was able to induce the formation of somatic embryos in the cotyledon (Horstman et al., 2017). However, the induction of somatic embryos by overexpression of LEC2 and BBM is limited to specific tissue, the response rate is relatively slow, and the response is dose-dependent. Thus the somatic regeneration using the overexpression of LEC2 and BBM is never reported. The overexpression of *RWP-RKD4* was used in the current study. Waki et al. (2011) showed in their study that the overexpression of *RKD4* in *Arabidopsis* can efficiently form somatic embryos in root meristem and leaf primordia. However, the complete regeneration using overexpression of *RKD4* is remained unknown. The *RWP-RKD4* system in *Arabidopsis* was obtained from Waki et al. (2011) while the overexpression of *AtRKD4* in *O.sativa* and *Triticum aestivum* were developed by using pERV1 binary construct from Valdivia et al. (2013). When five-day-old *Arabidopsis thaliana* seedlings were transferred into media with dexamethasone, the root tips area started to swell, and the cotyledon started to turn white (Fig. 3.1. A and F). The overexpression of *RWP-RKD4* stopped the growth of the seedlings and started the process of generating somatic embryos structure in leaf primordia and root meristems. This response was observed under 20 μ M of dexamethasone. An increase in dexamethasone concentration did not affect the formation of somatic embryos (data not shown). When the seedlings with somatic embryo swellings were transferred to normal MS media without dexamethasone, a callus structure formed in the root's swelling area and cotyledons (Fig. 3.1 B, C and H), and after seven days clumps of embryos formed (Fig. 3.1 I). These embryo clumps needed to be isolated into individual embryos (Fig. 3.1 D and J), and moved onto new MS medium for full regeneration (Fig. 3.1 E and K).

Overexpression of *AtRWP-RKD4* was also tested in *Oryza sativa* cv. *Nipponbare* using β -estradiol inducible binary construct containing *AtRWP-RKD4*. To reconfirm whether the overexpression of *AtRKD4* in pERV1 construct working in rice, the expression of transgene upon the induction using β -estradiol, RT-PCR was used (Fig. 3.2). The results indicated that the transgene was highly expressed when induced with β -estradiol. However, some of the transgenic lines failed to express the transgene even after the induction with β -estradiol. Some lines also had leaky expression of the transgene, as indicated by the highly detectable expression of the transgene when the plants were treated with water. Similar to Arabidopsis, the swelling of primordial leaf was observed when five-day-old seedlings were transferred to media with β -estradiol. However, in this system, a higher concentration of β -estradiol was needed due to the nature of β -estradiol which is degraded by light over time (data not shown). After two days in media with β -estradiol, the seedling growth halted, and the cotyledons started to turn into transparent and white structures, much like in the Arabidopsis system (Fig. 3.1 M, P and Q). The somatic embryo swelling occurred after four days in β -estradiol media (Fig. 3.1 N and O). However, when a seedling with this somatic embryo structure was moved into medium without β -estradiol, the somatic embryo structures failed to form a callus structure. Therefore, there were no somatic embryos rescued from the overexpression of *AtRWP-RKD4* in *O. sativa*.

The pERV1 induction system also tested in *T. aestivum* with the overexpression of *TaRKD2* (Fig. 3.2). To understand whether the overexpression system in *T. aestivum* working, the RT-PCR was done by cut a small proportion of the leaf and incubated it into water solution containing 60 μ M of β -estradiol. The result showed that some lines contained leaky expression of *TaRKD2* (2.20 and 2.23). However line 2.24 is a good line indicated by highly expression of the transgene when induced by β -estradiol.

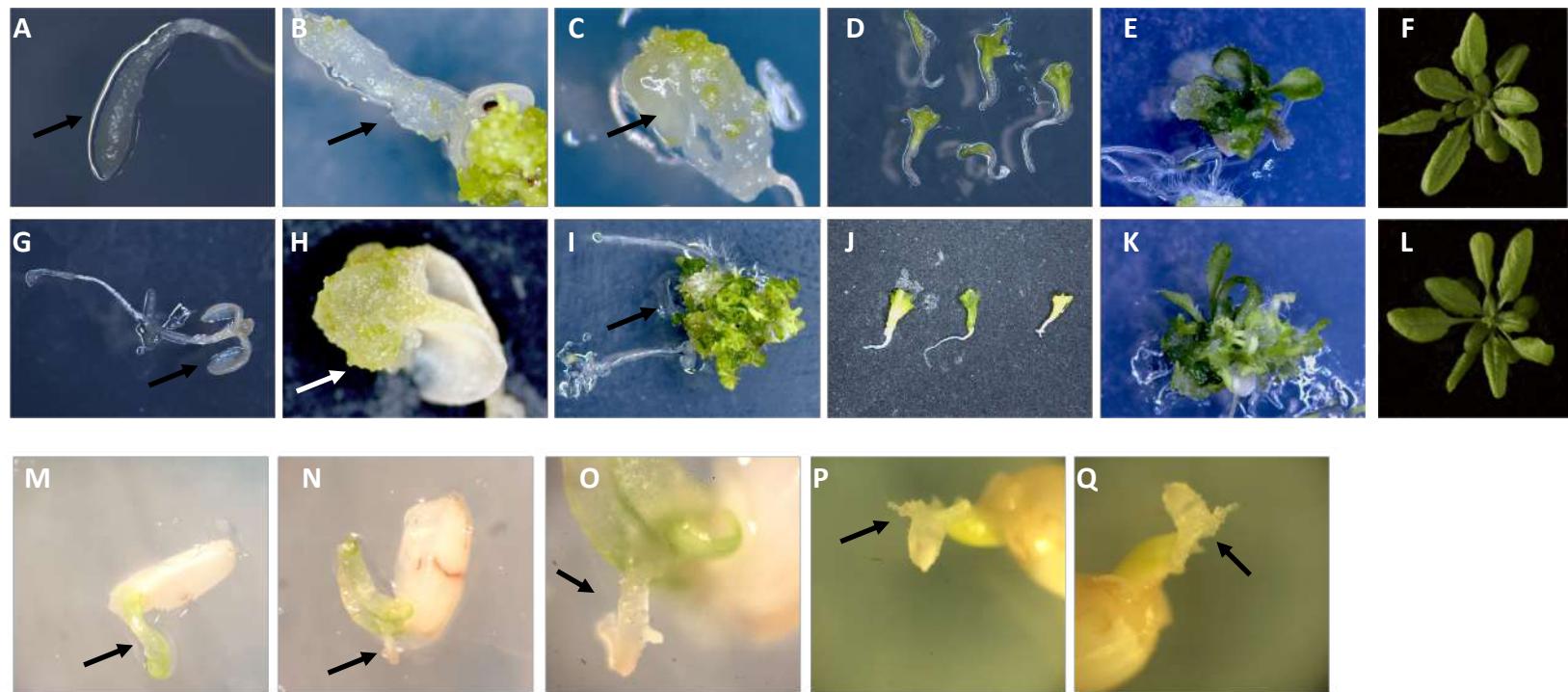


Figure 3.1 Somatic regeneration by the overexpression of *AtRWP-RKD4* in *A.thaliana* and *O.sativa* cv. *Nipponbare*. The overexpression of *AtRWP-RKD4* in 5 days old seedlings induced somatic embryogenesis in leaf primordia and the root meristem. After two days of induction using dexamethasone, the callus-like structure started to develop in the root (A) and leaf (F). After 5 days of induction, the plantlets were moved into MS-only media and the somatic embryos formed from the callus-like structure (B, C, G and H). These somatic embryos were isolated (D and I), allowed for full regeneration (E and J) and grown into a fully functional plant (K and L). In 5 days old rice seedlings containing β -estradiol inducible *AtRWP-RKD4* construct, the overexpression of *AtRWP-RKD4* was induced using β -estradiol, and a swelling structure was observed after 5-days of induction in the shooting area (M, N, O, P and Q), but the somatic embryos failed to form from a swelling structure after the seedling transferred to MS-only medium. (K: RO plant; L: RO plant)

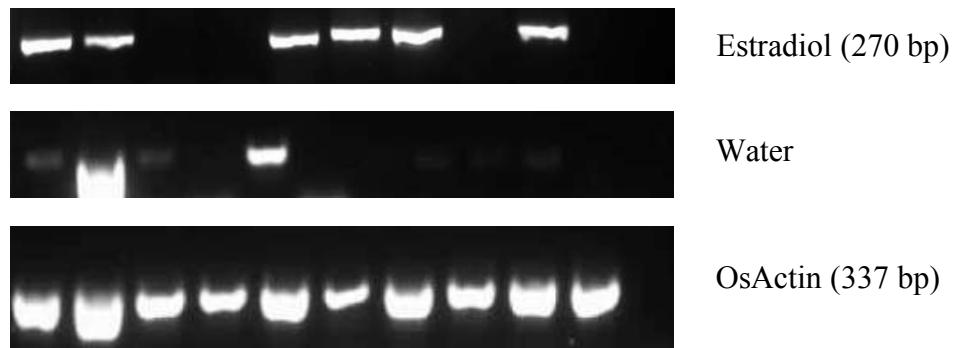
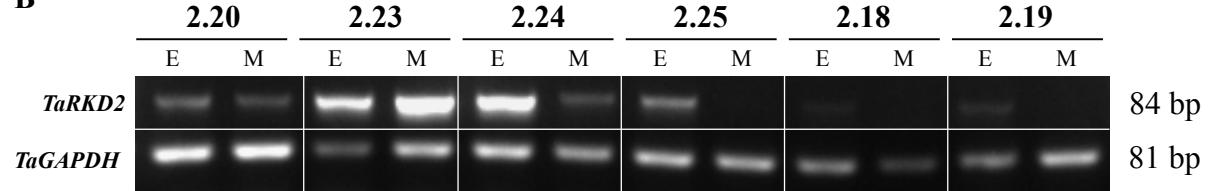
A**B**

Figure 3.2 The expression of inducible *AtRKD4* in transgenic *O. sativa* and *TaRKD2* in transgenic *T. aestivum*. (A) The expression of *AtRKD4* transgene was induced in the leaf of transgenic *O. sativa* using β -estradiol, and water was used as negative control in the experiment while *OsActin* was used as a control gene expression. (B) The expression of *TaRKD2* transgene was induced in the leaf of transgenic *T. aestivum* using β -estradiol (E), and water (M) was used as negative control in the experiment while *TaGAPDH* was used as a control gene expression (the experiment was helped by Yang Seok-Lee).

3.3 Discussion

The mechanism of how RWP-RKD4 initiates the formation of somatic embryos in *Arabidopsis thaliana* is still poorly understood. However, Waki et al. (2011) have extensively studied the involvement of RKD4 in embryogenesis. They found that the *RKD4* is transcribed in early embryogenesis. Loss of RKD4 function causes embryonic defects during zygotic cell elongation as well as cell division patterns. They found that overexpression of *RKD4* initiates somatic embryogenesis in young leaf primordia and in the root, where cell proliferation occurs in most cells. However, Waki et al. (2011) only showed the overexpression of RKD4 capable to develop somatic embryos in root meristem and leaf primordial, but they did not show the ability of somatic embryos generated from overexpression of RKD4 to regenerate into fully functional plants.

When *RKD4* is overexpressed, it stops plants growth while initiating the formation of somatic embryos. This halting of growth during a somatic embryogenesis phase may be related to the auxin response. *rkd4* mutants displayed root growth defects, which indicates impairment in auxin distribution; it was also found that PIN1, an auxin efflux facilitator is misregulated in *rkd4* embryos (Waki et al., 2011). In addition, the previous study on LEC2, a zygotic transcription factor, also shows the involvement of YUCCA, an enzyme involved in auxin biosynthesis, during LEC2 induced somatic embryogenesis (Wójcikowska et al., 2013). However, somatic embryogenesis through RWP-RKD may not only involve auxin. Instead, it acts together with *WOX* homeodomain proteins to initiate embryonic polarity during the first cells division. *YODA* dependent signalling also requires *RWP-RKD*, but it does not regulate YODA MAPK cascades (Jeong et al., 2011).

The somatic regeneration induced by overexpression *AtRKD4* is relatively effective in the induction of somatic embryogenesis with over 100% efficiency (data not shown). Somatic regeneration using the overexpression of *LEC2* requires 25 days to form somatic embryos under 30 μ M DEX (Guo et al., 2013), while overexpression *BBM1* effectively induced somatic embryos in the cotyledon or leaf (Boutilier et al., 2002). The ectopic expression of *AtRKD4* induced the formation of somatic embryos in young leaf primordial and root meristem after 7-days of induction with 20 μ M of DEX. When rescued, the somatic

embryos from the overexpression of *AtRKD4* can regenerate into fully functional plants within 7-days after rescue. However, there is no evidence on full-regeneration from somatic embryos developed by the overexpression of either *BBM1* or *LEC2*.

The failure of swelling-structure upon the overexpression of *AtRKD4* *O.sativa* may indicate that the AtRKD4 protein is lethal in rice. Thus, finding the putative *RWP-RK* genes that have a similar biological function with *AtRKD4* in rice is crucial to elucidate further whether the overexpression of rice endogenous *RWP-RKD* can induce the formation of somatic embryos.

The ability of AtRKD4 to form a somatic embryo-like structure in *O.sativa* cv. *Nipponbare* indicates that the *Arabidopsis thaliana* RKD4 has a conserved function in rice. The presence of RKD proteins, a subfamily of plant RWP-RK, is widespread in angiosperms, and it has been reportedly involved in female gametogenesis in wheat and *Arabidopsis* (Koi et al., 2016). Based on evolutionary relationships of RWP-RK protein across seven plant species, AtRKD4 has an RWP-RK motif and motif #12 downstream of this RWP-RK motif. The motifs found in *AtRKD4* are similar to the four *Oryza sativa* RWP-RK genes, *OsRKD3*, *OsRKD4*, *OsRKD5* and *OsRKD6*, and these genes, including *AtRKD4*, belong to the same RKD subfamily which is RKD(A) subfamily (Chardin et al., 2014). This may explain the ability of AtRKD4 to induce somatic embryo-like structures in *O.sativa* cv. *Nipponbare*, and it is the reason for selecting *AtRKD4* to be used in rice. However, there is no information regarding the putative function of *O.sativa* endogenous RWP-RK genes in embryogenesis or female gametogenesis. The study of RKD protein in *Chlamydomonas* and *Pledoraina starii* indicated their function in gametogenesis during nitrogen starvation due to the RWP-RK protein only present in the gametes during nitrogen starvation. This function is similar in *A. thaliana* and *T. aestivum* (Chardin et al., 2014). Since *T. aestivum* and *O. sativa* belong to the same family of *Poaceae*, the function of RKD protein in *O.sativa* maybe similar to the one in *T.aestivum*, which is expressed in egg cells, and may indicate their function during female gametogenesis.

A higher concentration of β-estradiol has been linked to inhibition primary root growth (Siligato et al., 2016) and may be problematic in the formation of somatic embryos from

root meristem. This explain the failure of swelling structures to form in the root meristem in rice after the induction of the *AtRKD4*. Thus, using different induction system may necessary effectively generate somatic embryos in rice in both root and shoot tissue.

3.4 Summary

The overexpression of *AtRKD4* is an effective way to regenerate plants through somatic embryogenesis from young leaf primordia and root meristems in Arabidopsis. The ability of overexpression of *AtRKD4* in *O.sativa* to induce somatic-embryo-like structures may indicate that AtRKD4 has a conserved function in *O.sativa*. The function of endogenous RWP-RKD proteins in *O.sativa* remains unknown, but it may be similar to their relatives in *T.aestivum* in which it involves during female gametogenesis. Thus further investigation is required to elucidate the endogenous RWP-RKD in *O.sativa* with similar biological function.

4. Transgenerational Stress Adaptation in *Arabidopsis thaliana* Regenerated from Leaf and Root tissues

4.1 Introduction

4.1.1 Plant Responses to Environmental Stress

Environmental stress has detrimental effects on plant growth and development. A report published by Cramer et al. (2011) stated that environmental stress could decrease crop production by up to 70%. FAO also reports that beyond 2030, the devastating impact of climate change on agricultural yields will become significantly severe in all regions; and by 2100, it will contribute to the decreasing yields of several major crops such as up to 45% for maize, 50% for wheat, 30% for rice and 60% for soybean (FAO, 2016).

As sessile organisms, plants have complex adaptation mechanisms to acclimatise towards environmental stress. In the presence of stress, either abiotic or biotic, plants will perceive the stress signals by cellular sensing mechanisms. The recognition of stress signals is crucial for plants as it forms the basal defense mechanism towards abiotic and biotic stimuli (Rejeb et al., 2014). These signals activate signaling cascades which regulate stress-related molecules such as reactive oxygen species (ROS), abscisic acid (ABA), salicylic acid (SA) and ethylene (ET) (Rejeb et al., 2014). It means that plants possess unique molecular changes to the specific types of stress. Thus, there are possibly no universal stress responses to deal with various stress signals (Rejeb et al., 2014). There are major environmental stresses that plants deal on a daily basis such as high salinity, freezing temperature and not limited to the pathogenic infection.

Approximately one-third of the agricultural land is affected by high salinity (Smith et al., 2010). Plants exhibit unique pathway to deal with a specific stress, and there is a complex mechanism where plants tolerant towards that stress. Salt in the form of sodium chloride (NaCl) is toxic to the plants. However, halophyte plants are naturally resistant to a saline environment, while glycophyte plants such as *A. thaliana* are susceptible to the high concentration of NaCl (Smith et al., 2010). Studies show halophytes maintain salt tolerance by controlling the ion homeostasis pathways; research in Arabidopsis using mutants that are overly sensitive to salt found a similar conclusion; that ion homeostasis regulates salt stress tolerance in Arabidopsis (Smith et al., 2010). One mechanism that

controls ion homeostasis in plants is through the Salt Overly Sensitive (SOS) pathway. As mentioned by Ji et al. (2013), in the presence of high concentrations of sodium chloride in the soil, the sodium ion (Na^+) will be taken up by root cells and will bind to a Na^+ sensor. The binding of Na^+ to the sensor initiates the accumulation of intercellular calcium ions (Ca^{2+}). This Ca^{2+} binds to the SOS3 protein leading to a change in the conformational structure of SOS3. Activated SOS3 binds to SOS2 to create an SOS3-SOS2 complex. This complex phosphorylates SOS1, a Na^+/H^+ antiporter of the plasma membrane. Increased levels of active SOS1 will reduce the level of cytosolic Na^+ . Thus SOS is a type of salinity protectant in plant.

While salt stress responses correlate with the response to water deficiency, exposure to low temperature also inhibits the same water deficiency responses. Temperature is a crucial component that influences plant growth and metabolism. The stress caused by low temperature can be grouped as: chilling when some plants are exposed to temperatures below 20°C , and freezing when the temperature drops below 0°C (Chinnusamy et al., 2010). Low temperature raises critical issues for plants that live outside the equator (Smith et al., 2010), and the cold adaptation process in plants depends on the plasma membrane. When plants are exposed to cold temperature, it induces plasma membrane rigidification which leads to actin rearrangement and accumulation of cytosolic Ca^{2+} (Chinnusamy et al., 2010). The accumulation of Ca^{2+} in the cytosol acts as the second messenger of the cold stress signal that induces the expression *C-REPEAT BINDING FACTOR (CBF)* (Chinnusamy et al., 2007). CBFs are CRT/DRE binding proteins that belong to the ethylene-responsive element binding factor/APETALA2 (ERF/AP2)-type transcription factor family (Chinnusamy et al., 2010). In *Arabidopsis*, there are three *CBF* genes: *CBF1*, *CBF2*, and *CBF3*. When low temperature induces the CBF expression, it binds to a CRT/DRE motif at the promoter of *cold-regulated genes (COR)* (Smith et al., 2010). The ectopic expression of CBFs in *Arabidopsis* leads to increase tolerance towards cold stress (Chinnusamy et al., 2007a).

4.1.2 Plant Responses to Biotic Stress

Climate change will not only impact the effects of salinity and low temperature on plants,

but it will also alter the disease risk of several pathogenic microorganisms due to changes in the rates of replication, development, and transmission of the pathogenic microorganisms (Altizer et al., 2013). Pathogenic bacteria and other microorganisms use plants as their carbon source and they have diverse mechanisms for attacking plants. Fortunately, like animals, plants also have a defense mechanism to protect from the infection of pathogenic organisms. The cell wall is the first line of defense in plants, but when the pathogens successfully penetrate to the intercellular spaces, then this will trigger the activation of the innate immunity in plants. When pathogens enter the intercellular spaces, they release pathogen-associated molecular patterns (PAMPs) such as chitin or flagellin that will be recognised by transmembrane pattern recognition receptors (PRRs) (Jones & Dangl, 2006). Different PAMPs are recognised by different PRRs, for instance, flagellin produced by motile bacteria such as *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) is recognised by FLS2, a leucine-rich repeat receptor kinase (LRR-RK) (Belkhadir et al., 2014). When flagellin binds to FLS2, this complex phosphorylates and initiates MAP Kinase cascades that activate different transcription factors (e.g. *WRKY22* and *WRKY29*); that will further activate the expression of immunity genes (Asai et al., 2002).

4.1.3 Stress Memory and Active Reprogramming in Plants

As a non-motile organism, plants lack a mechanism to avoid the exposure to environmental stress. However, plants are capable of altering their transcriptome allowing them to adjust to the environmental changes. Stress can last for as little as weeks or as long as months promoting evolutionary adaptation through resilience over subsequent generations (Crisp et al., 2016). Priming, a process in which plants are exposed to mild stress, is a crucial step for acquiring stress memory in plants (Crisp et al., 2016; Slaughter et al., 2011). Research conducted in *Arabidopsis thaliana* by Slaughter et al. (2011) showed that when *Arabidopsis thaliana* was primed either with β-aminobutyric acid (BABA) or with avirulent *Pseudomonas syringae* pv *tomato* (*PstavrRpt2*); the progeny of primed plants had a faster and higher accumulation of defense-related transcripts and enhanced disease resistance when challenged with a virulent *Pseudomonas syringae* (*Pst*). Furthermore, when they further primed the transgenerationally primed plants, they found

the offspring displayed a stronger resistance phenotype. Another experiment conducted by Molinier et al. (2006) revealed that *Arabidopsis thaliana* exposed to ultraviolet-C radiation had elevated levels of somatic homologous recombination of a transgenic reporter, and these homologous recombination was inherited to untreated further generations. This implies that plants are capable of inheriting the information that they receive during priming.

Unlike mammals, plants do not have a central nervous system to help in the process of memorisation. Thus, complex molecular mechanisms are underlying the process of formatting stress memory in plants. One possible mechanism is by maintaining the changes in signaling metabolites or transcription factors (Bruce et al., 2007; Crisp et al., 2016). A good example of this mechanism is in the study conducted by Ton et al. (2005) where they found that *Arabidopsis thaliana* primed with BABA shows enhanced tolerance towards biotic and abiotic stress. This indicates that when plants are exposed to a broad range of abiotic or biotic stresses, they will produce BABA as a priming response towards stress, and this response will form an adaptation mechanism to stress within generations. Yamaguchi & Shinozaki (2006) studied transcription factors that are essential in the process of retaining stress memory; they found that during cold adaptation, *HOS10* is expressed which encodes for a putative R2R3-type MYB transcription factor that controls stress-induced ABA biosynthesis.

Another mechanism of retaining stress memory is through epigenetic mechanisms. Epigenetics is termed as the alteration of gene function without any changes in the underlying gene sequence (Smith et al., 2010). These changes include the modification of DNA activity by methylation, histone modification or chromatin remodeling (Bruce et al., 2007; Madlung & Comai, 2004). It has been well documented that environmental stresses can alter the chromatin state and epigenetic marks within the genome (Crisp et al., 2016; Eichten et al., 2014a). A well-understood mechanism on the regulation of stress memory by epigenetics is in the case of *FLOWERING LOCUS C (FLC)*. A study in *Arabidopsis* indicated that the expression of *FLC* is repressed during exposure to cold temperature, allowing the transition to the reproductive state in plants (Bastow et al., 2004; Berry &

Dean, 2015). Epigenetic regulation has been found to play a pivotal role in regulating the expression of *FLC*. A genome-wide study revealed that, during the exposure to cold temperatures, the histone marks across the *FLC* locus are actively switched from H3K36me3 to H3K27me3 by the activity of EARLY FLOWERING 6 (ELF6) (Tao et al., 2017b); the accumulation of H3K27me3 histone mark recruits the polycomb repressive protein complexes that change the state of euchromatin to the compact heterochromatin that prevents transcription of *FLC* from taking place (Berry & Dean, 2015). The repression of *FLC* is maintained to subsequent growth in warmer conditions which leads to the flowering stage. Thus, environmental conditions can tightly regulate the timing of developmental transitions. The stress memory formed by the changes in epigenetic marks are maintained longer than the memory formed due to the accumulation of metabolites and transcription factors (Crisp et al., 2016).

The occurrence of environmental stresses is not always persistent and sometimes it includes a period of recovery (Crisp et al., 2016). When plants have been primed to a specific stress, and they undergo a long period in absence of that specific stress; the attained-stress memory can be reset, and the plants are naïve to that specific stress again. The phenomena of losing stress memory has been recorded in research conducted by Ding et al. (2012) with *Arabidopsis* that has been primed to drought stress. After a 7-day period in the absence of stress, drought-stress-gene responses are reduced in comparison to the plants that repeatedly exposed to the stress. Wibowo et al. (2016) also showed that plants exposed to salt stress can maintain memory into the successive generations by controlling the epigenetic mechanisms; however, in the absence of salt stress, the salt stress memory is rapidly erased and reprogrammed to the basal level in which the plants become vulnerable to the salt stress again. The loss of stress memory is thought due to the reprogramming of epigenetic marks during reproduction to ensure the correct development phases takes place (Feng et al., 2010b). Another speculation is that the loss of epigenetic stress memory is perhaps due to random DNA damaged that is followed by the replacement of methylated cytosine to unmethylated cytosine during the DNA repair process (Blevins et al., 2014).

The evidence shown in this chapter indicate that stress memory is transient and it only lasts for only a few generations. The mechanisms by which stress memories are propagated stably into a successive generation without being reset currently remains unknown.

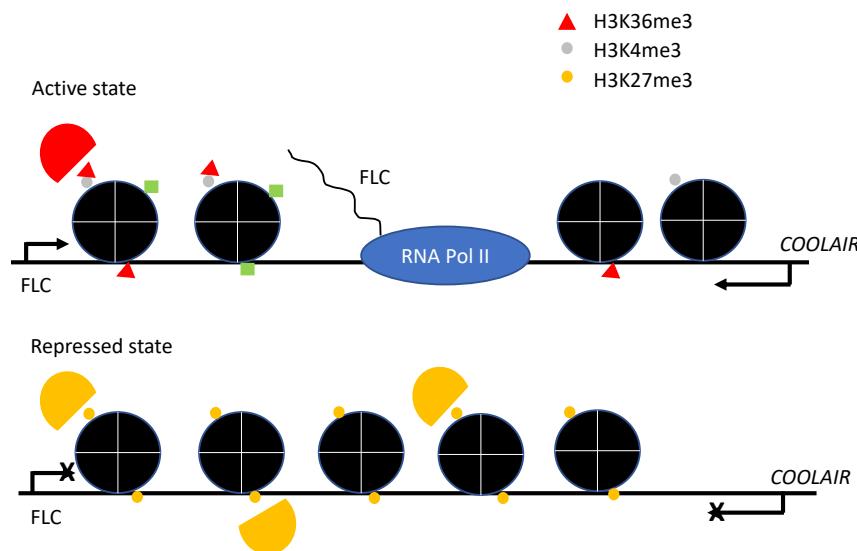


Figure 4.1 *FLC* expression and chromatin status during vernalisation. The high expression of *FLC* chromatin is characterized by H3K4me3, H3K36me3, histone acetylation, and active transcription by polymerase II. After winter, repression may be promoted by EARLY FLOWERING 6 (ELF6), which mediates a switch from H3K36me3-rich to H3K27me3-rich chromatin. At the same time, expression of *COOLAIR* is increased. For loci in the repressed state after cold exposure, H3K27me3. In this repressed state, both *FLC* and *COOLAIR* transcription are reduced (modified from: (Berry & Dean, 2015)).

4.1.3 Chapter Aims

This chapter aims to study transgenerational adaptability of regenerated-plants from leaf and root tissue that have undergone exposure to mild stress during somatic embryogenesis by using overexpression of RKD4 in *Arabidopsis*.

4.2 Results

4.2.1 Treatment with Salt Stress During Somatic Regeneration Confers Adaptation in Root-Regenerated Plants

The ability of plants to form transgenerational memories after exposure to mild abiotic or biotic stress has been well documented; the progenies of these plants even show a strong improvement in adaptability to stress (Boyko & Kovalchuk, 2010; Boyko et al., 2007; Molinier et al., 2006). Repeat exposure to the same type of stress is crucial for the establishment of stable transgenerational stress memory. However, environmental stress appears to be non-persistent (Crisp et al., 2016). Wibowo et al. (2016) have shown that when the descendants of salt stress-treated *Arabidopsis* are no longer exposed to salt stress, the salt stress memory is gradually lost. The loss of epigenetic marks is thought to be responsible for the process of forgetting the stress memory (Bruce et al., 2007). This process of reprogramming occurs during sexual reproduction, and it is essential for maintaining genome integrity (Kawashima & Berger, 2014). To assess whether the stress memory introduced during asexual reproduction can bypass the epigenetic reprogramming and become heritable to the successive generation, *Arabidopsis thaliana* overexpressing *RWP-RKD4* was used. When treated with dexamethasone (DEX), the overexpression of *RWP-RKD4* causes the formation of somatic embryos in young primordial leaf and root apical meristem. During the process of somatic embryogenesis initiation, mild salt stress (75mM NaCl) introduced, and the embryos were collected from root and leaf tissue after seven days of exposure. The embryos were regenerated into fully functional plants, and the plants were normally grown to successive generations without further exposure to the stress. To evaluate whether the root-regenerated plants (RO) and leaf-regenerated plants (LO) treated with 75mM NaCl during somatic regeneration possess any resistance to salinity stress, the second generation (F2) seeds were grown in media with 175mM NaCl (Fig. 4.2 A), 50 seeds per line with four technical repeats. The survival rate was counted by scoring the plants with green cotyledon after salt stress, and the experiment was repeated twice to reduce the data bias. The result indicated that four out of five of the RO lines significantly better adapted when exposed to high salinity with the highest survival rate found in the RO line 5 ($P \leq 0.001$), compared to the control root-

regenerated plants (Control RO, no stress applied during somatic embryogenesis). Intriguingly the exposure of salt stress during somatic embryogenesis did not contribute to the establishment of stress memory in the LO plants, as none of F2 LO plants had any significantly improved response to osmotic stress.

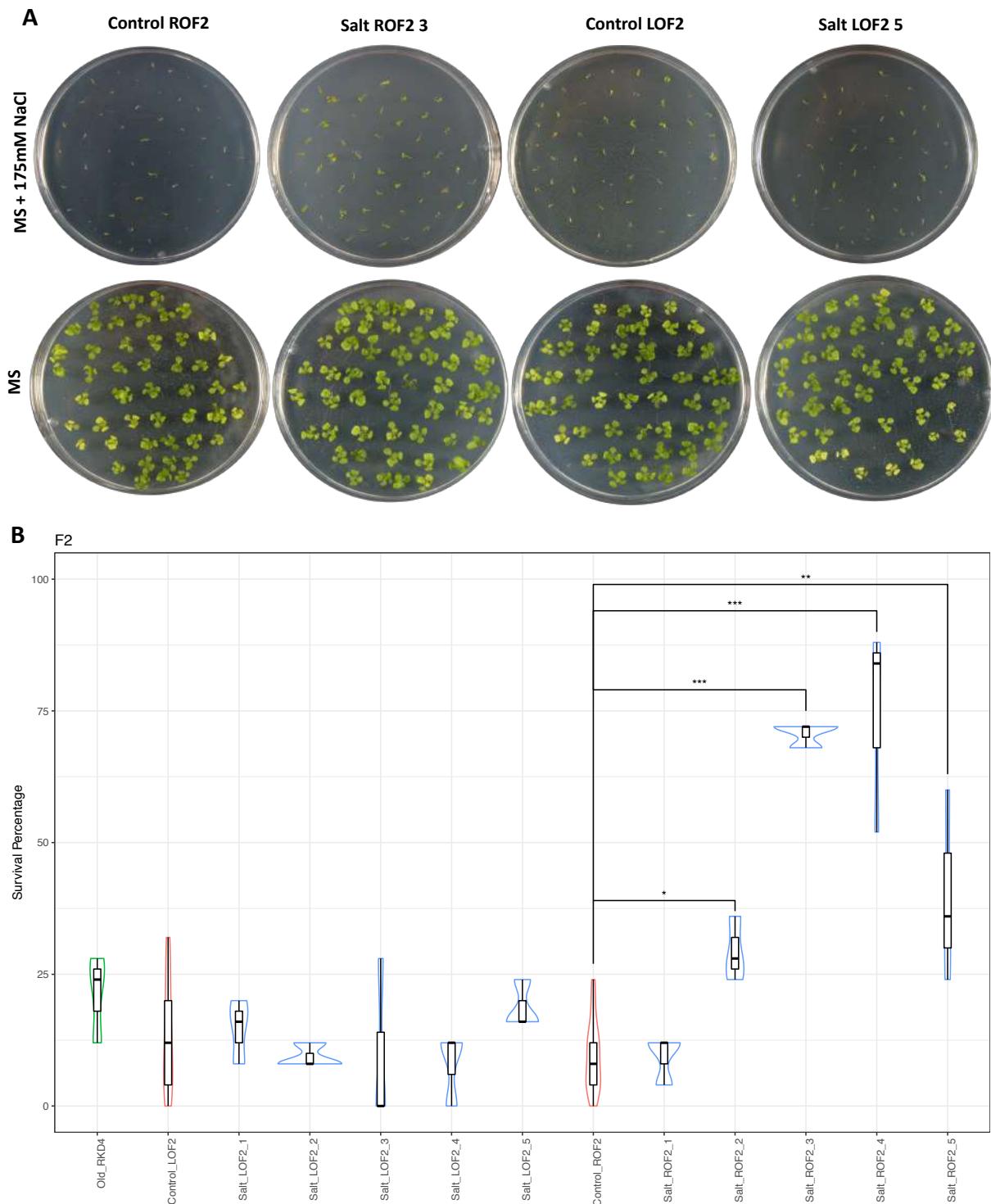


Figure 4.2 Transgenerational salt stress adaptation in regenerated plants in F2. Salt survival assay of the F2 progenies of control and salt-treated regenerated plants from leaf and root. (A) Seeds were germinated on MS and MS with 175mM NaCl; the images were captured two weeks after sowing. (B) The survival rate of F2 was analysed after two weeks on MS with 175mM NaCl by scoring plants with green cotyledon. Each line containing 50 seeds per plate with 4 technical repeats. Asterisk indicate a significant difference to the control (unpaired Student's *t*-test; *** p<0.001; ** p<0.01; *p<0.05; while old RKD4 (WT) (F0); RO (root origin plants); LO (leaf origin plants)), violin box indicates the data distribution

To test whether the tolerance to high salinity is a stably heritable trait in the RO plants, the survival rate was studied to a greater extent by growing the third generation (F3) seeds of RO and LO plants on media with 175mM NaCl (Fig 4.3 A). The result shows that the majority of the RO (line 1, 3, 4 and 5) and LO (line 1, 2, 3, and 5) plants had the same survival rate when compared to the control experiment, with one line from each RO (line 2) and LO (line 4) showing better survival. This may suggest that the salt stress memory is erased to non-stress levels in the F3 plants.

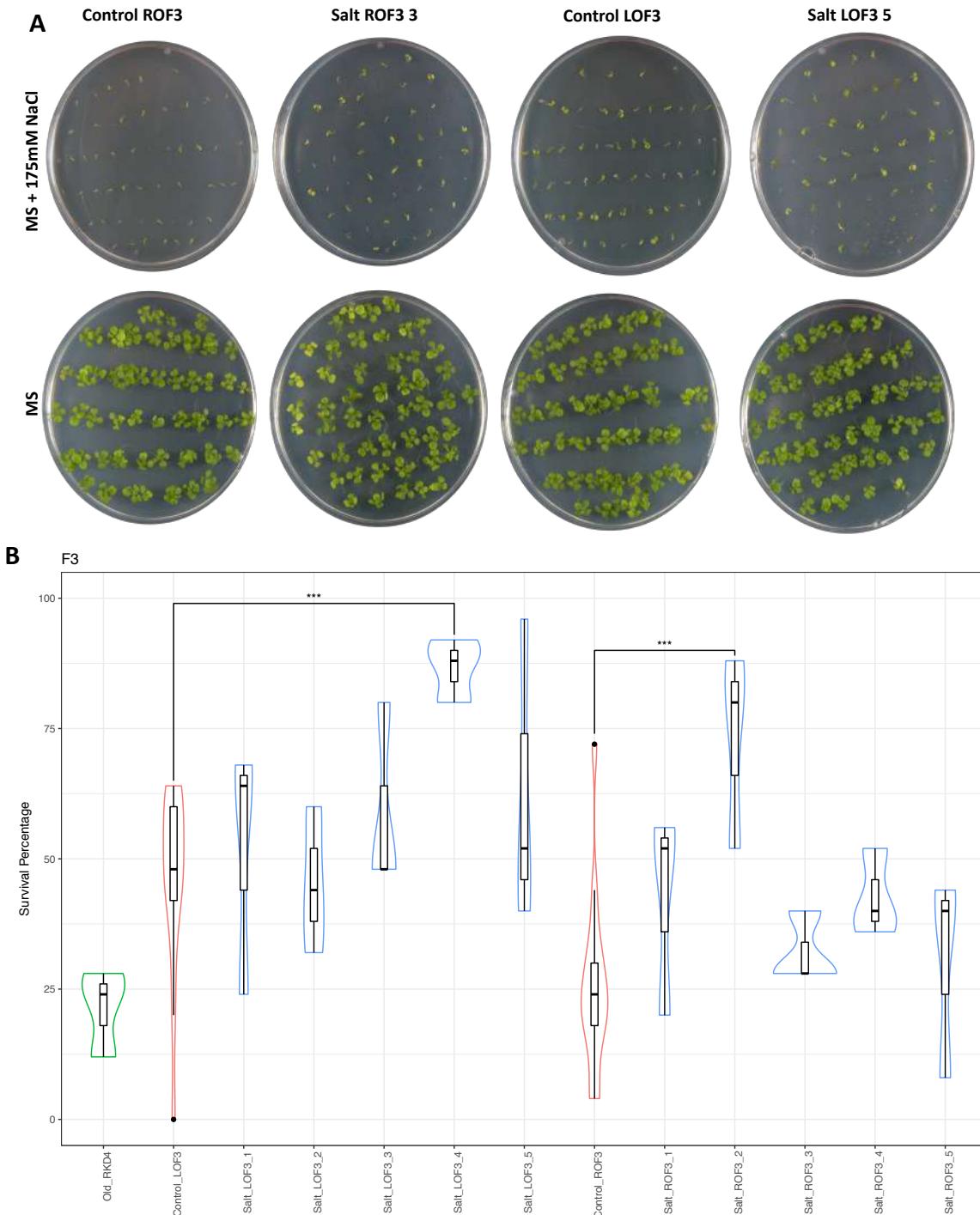


Figure 4.3 Transgenerational salt stress adaptation in regenerated plants in F3. Salt survival assay of the F3 progenies of control and salt-treated regenerated plants from leaf and root. (A) Seeds were germinated on MS and MS with 175mM NaCl; the images were captured two weeks after sowing. (B) The survival rate of F3 was analysed after two weeks on MS with 175mM NaCl by scoring plants with green cotyledon. Each line containing 50 seeds per plate with 4 technical repeats. Asterisk indicate a significant difference to the control (unpaired Student's *t*-test; *** p<0.001; ** p<0.01; *p<0.05; while old RKD4 (WT) (F0); RO (root origin plants); LO (leaf origin plants)), violin box indicates the data distribution

4.2.2 Regenerated Plants from Leaf Tissues Show Transgenerational Adaptability to Low-Temperature Stress

The developmental effect of the exposure to cold has been well studied, for instance, the vernalisation memory is formed after prolonged exposure to cold that involves silencing of the *FLC* gene through H3K27me3 (Lämke & Bäurle, 2017). Exposure to mild cold stress in plants can trigger a primed state that contributes to survival in further exposures to extreme cold, and the molecular responses to cold stress have been well elucidated (Chinnusamy et al., 2007b). However, the information of transgenerational effect subject to extreme temperature is only limited to the heat stress (Migicovsky et al., 2014), and very little is known regarding the transgenerational memory to cold stress.

To understand transgenerational cold stress memory in regenerated plants, *RWP-RKD4* was overexpressed to form somatic embryos in leaf and root tissues. At the same time, mild cold stress (4°C) was introduced for 48 hours with 24 hours of recovery at normal growing temperature, this cycle was repeated for seven days. The plants then developed from the somatic embryos from leaf and root tissue, and were grown to the next generation without further exposure to stress. To elucidate whether the regenerated plants exposed to cold stress during embryogenesis carry transgenerational stress tolerance, the F2 seeds were grown on MS media for two weeks and challenged the plants to low temperature by lowering the temperature by -1°C per hour until it reached -5°C. The experiment was conducted with 50 seeds per line and four technical repeats. To reduce the data bias, the experiment was repeated for seven times. The survival rate was counted after three days of recovery (Fig. 4.4 B) by scoring the number of green plants after three days of recovery. Interestingly, after the F2 plants were subjected to the extremely low temperature, two out of five lines of LO plants showed resistance to freezing stress with two lines (line 2 and 3) having significant improvement in survival in comparison to the control experiment with only 15% survival rate. LO line 3 had the strongest resistance, with survival rate around 30%. However, none of the RO plants indicated any resistance after being primed during somatic embryogenesis.

To further investigate whether the phenotype observed in the F2 is stably inherited to next generation, the F3 seeds were challenged with freeze stress. After the survival rate was analysed (Fig. 4.5 B), two out of five F3 LO plants had enhanced cold stress tolerance with the strongest improvement found in LO line 3; however none of RO line had any improvement. This indicated that the cold stress memory is transgenerationally inherited in some of LO lines but not in the RO lines.

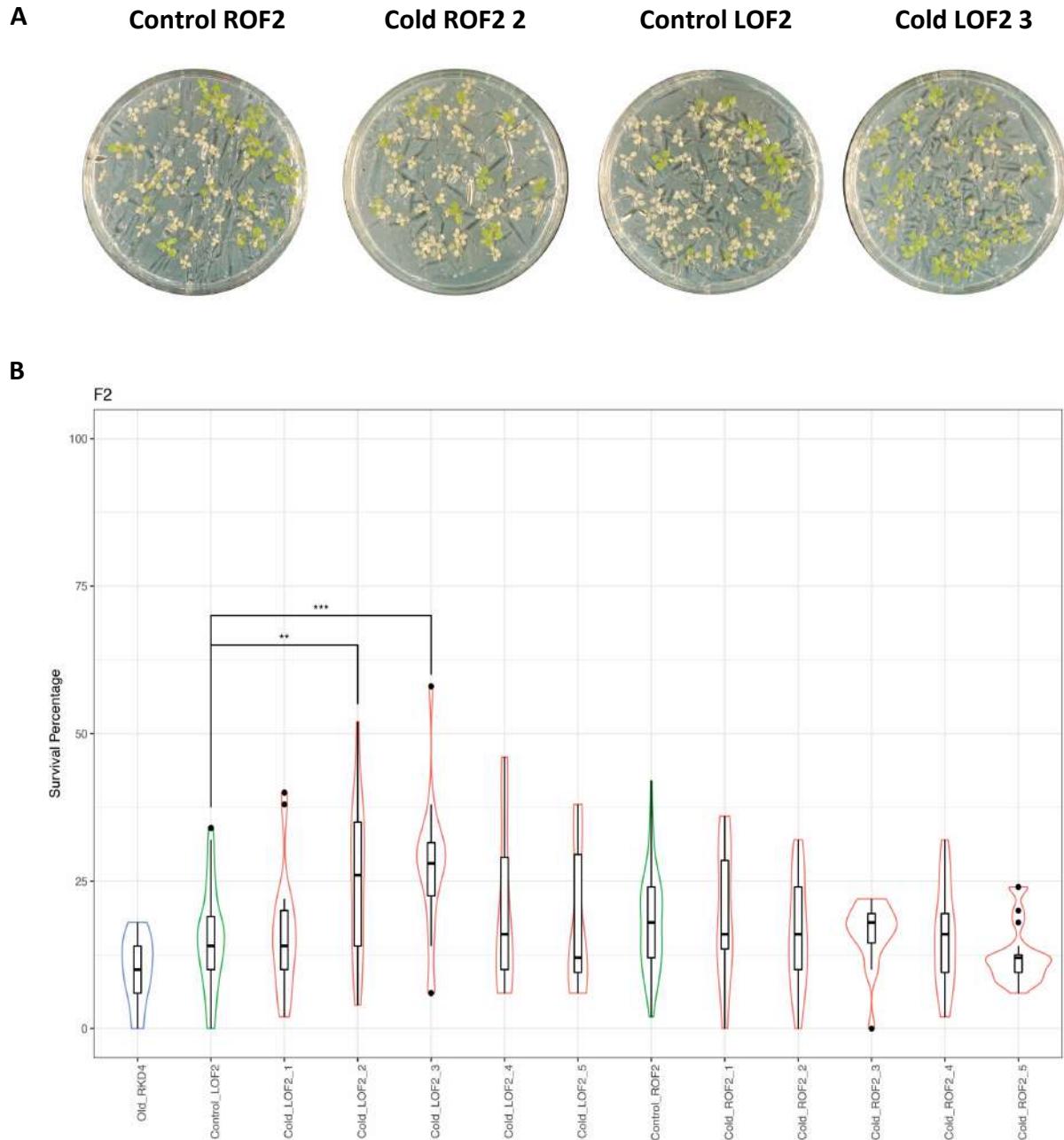


Figure 4.4 Transgenerational cold stress adaptation in regenerated plants in F2.
 Freezing tolerance assay of the F2 progenies of control and cold-treated regenerated from leaf and root. (A) The seeds were germinated on MS media. The pictures were taken after two days of recovery from freezing stress (-5°C). (B) The freezing survival rate of F2 was analysed after two days of recovery from freezing treatment (-5°C). For each line, 4 plates were analysed with 50 seeds per plate. Asterisk indicate a significant difference to the control (unpaired Student's *t*-test; *** p<0.001; ** p<0.01; *p<0.05; while old RKD4 (WT) (F0); RO (root origin plants); LO (leaf origin plants)). Violin box plot indicates the data distribution.

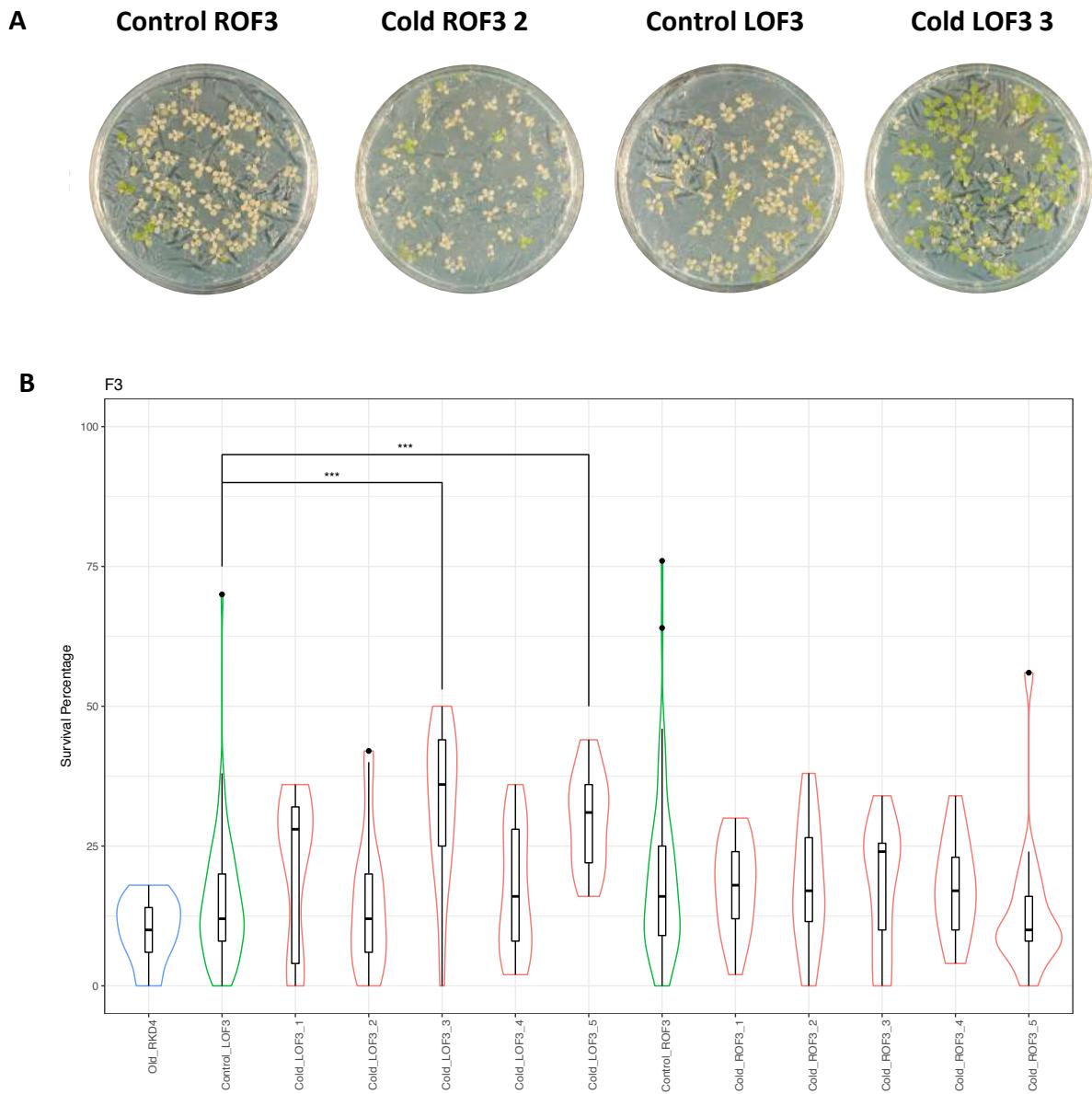


Figure 4.5 Transgenerational cold stress adaptation in regenerated plants in F3.
 Freezing tolerance assay of the F3 progenies of control and cold-treated regenerated from leaf and root. (A) The seeds were germinated on MS media. The pictures were taken after two days of recovery from freezing stress (-5°C). (B) The freezing survival rate of F3 was analysed after two days of recovery from freezing treatment (-5°C). For each line, 4 plates were analysed with 50 seeds per plate. Asterisk indicate a significant difference to the control (unpaired Student's *t*-test; *** p<0.001; ** p<0.01; *p<0.05; while old RKD4 (WT) (F0); RO (root origin plants); LO (leaf origin plants)). Violin box plot indicates the data distribution.

4.2.3 Regenerated Plants Primed with flg22 During Somatic Organogenesis Have Disease Resistance to *Pseudomonas syringae* pv. *tomato* DC3000

Both mammals and plants have an immune system to protect themselves against a wide range of infectious agents. Even though they have immune mechanisms against diseases, the immune responses are different in many ways between mammals and plants. Unlike mammals, plants do not possess adaptive immunity that depends on the activity of immune cells. Thus, plants completely rely on innate immunity in defense against pathogens (Quintin et al., 2014). The ability of plants to build an immune response memory has been well documented; the exposure to weakened microorganisms has allowed plants to develop a long period of protection against subsequent infections (Durrant & Dong, 2004; Quintin et al., 2014).

To elucidate the transgenerational immune response in regenerated plants, somatic embryos were treated with 1 μ M flg22 for seven days. The somatic embryos developed from leaf and root were collected and allowed to regenerate to fully functional plants.

To assess the immunity towards pathogenic bacteria, the F2 seedling were grown for 4-5 weeks on a short-day condition. Then two fully expanded leaves were challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), and the infection rate was measured with a fluorimeter to quantify the chlorophyll fluorescence measurement (fv/fm) (Fig 4.6). The experiment was repeated for seven times to reduce the data bias. Measuring fv/fm values allowed to monitor the progression of the *Pseudomonas* infection and chlorosis; the lower the fv/fm value, the more progressed the disease is. *Pto* DC3000 infection caused a reduction of fv/fm value over time, where the *fls2* mutant as the positive control showed the lowest fv/fm value which dropped below 0.6 in line 1,2,3 and 4 but not in line 5 (line 3, 4 and 5 are in Appendix 8.1). In line 1 and 2, all the control experiment (RKD4, Control RO, Control LO) had fv/fm values below 0.7 where the flg22 treated RO and LO had fv/fm values above 0.7. This implies line 1 ($P \leq 0.001$ (RO line 1) and ($P \leq 0.01$) LO line 1) and line 2 ($P \leq 0.05$) have better immune responses towards *Pseudomonas* infection. However, flg22 treated RO and LO in line 3, 4 and 5 did not show any improvement in the immune responses. The results suggest that the flg22 treatment during

somatic regeneration was able to induce the formation of an immune memory, and this memory persists until the second generation of regenerated plants. However, the disease resistance phenotype was only observed in two independent lines, line 1 and line 2, whereas the other lines did not show any altered resistance towards *Pto* DC3000. In addition, there is the difference in disease resistance between flg22 treated RO and LO plants in line 1 and line 2; whereas line 1 did not show any differences defense response between flg22 RO and flg22 LO, in line 2, flg22 RO has slightly better responses in comparison to flg22 LO. The variance in responses is perhaps due to different cellular responses to flg22, as the application of flg22 was topical. (direct application to the plants surface).

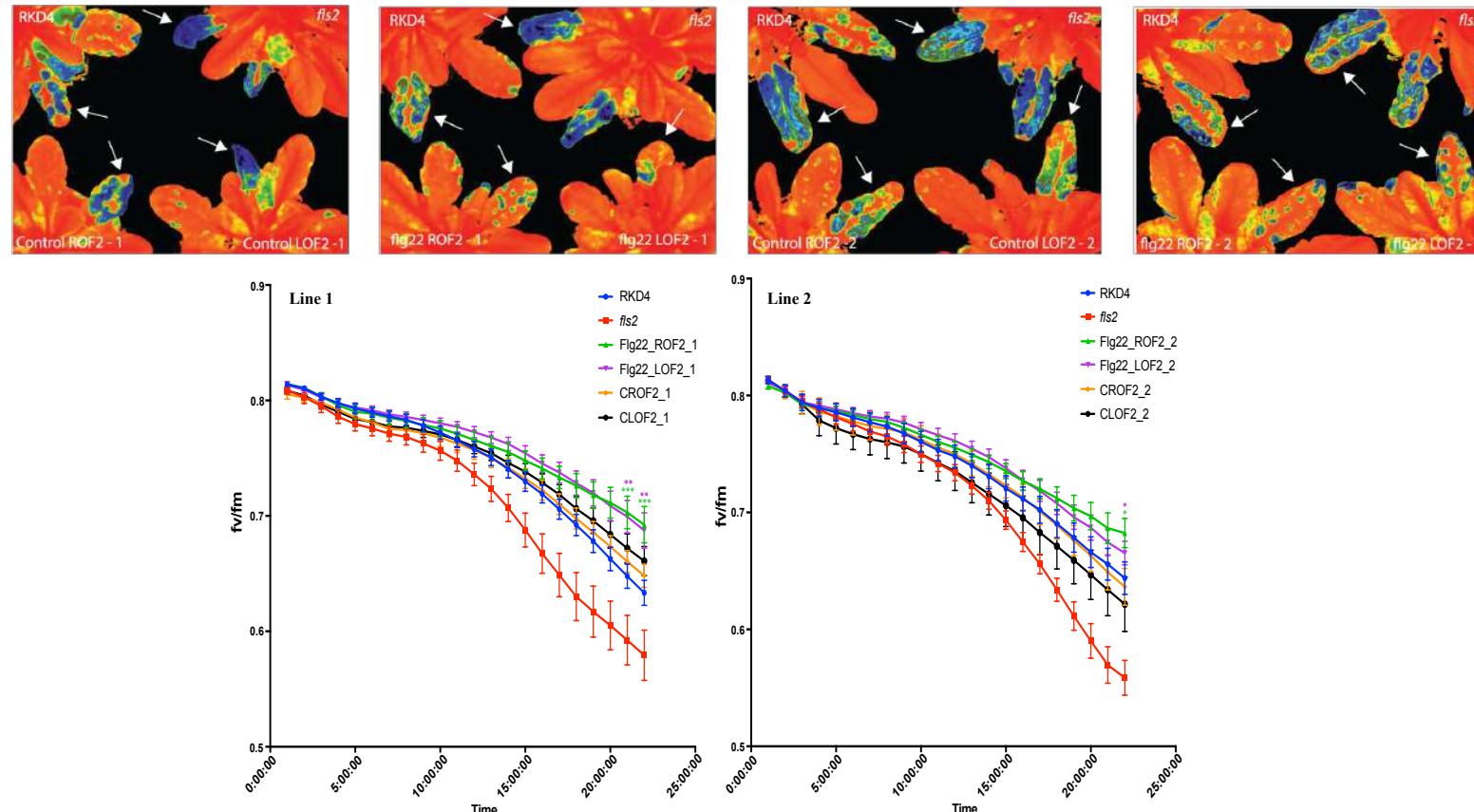


Figure 4.6 Adaptive immunity in regenerated plants. Fluorimager images for *Pst* DC3000 tolerance assay of the F2 progenies of control line 1 (A) and line 2 (C) with flg22-treated regenerated plants line 1 (B) and line 2 (D). Five weeks old of plants were infiltrated with *Pst*DC3000. The fv/fm value was calculated from the images generated by Fluorimager (Technologica). In this experiment, seven leaves were analysed from line 1 and line 2. RKD4 (WT); Root Origin (RO); Leaf Origin (LO); Control RO (CRO); Control LO (CLO). Two-way ANOVA; *** p<0.001; ** p<0.01; * p<0.05 (green asterisk: flg22 ROF2 vs control ROF2; purple asterisk: flg22 LOF2 vs control LOF2). Arrow indicates leaf infiltrated with *Pst* DC3000.

4.3 Discussion

This study has revealed that when four-leaf stage seedlings of *Arabidopsis* were pre-treated with a mild concentration of sodium chloride and cloned by somatic embryogenesis, some of the progeny showed long-term adaptation to high salinity stress. Groot et al. (2016) showed that salt treatment had persistent offspring phenotype in three consecutive generations. However, Wibowo et al. (2016) proved that the transgenerational salt responses were lost in the second generation when the salt stress was absent. In this study, when the salt stress was introduced during somatic regeneration, and the plants were regenerated from these somatic embryos; the transgenerational salt stress memory was maintained for up to the second generation when the progenies were grown in salt-free conditions (Fig. 4.2). Interestingly, this work revealed that the salt response phenotype is only transmitted in plants that come from roots cell somatic embryos (RO plants), where none of the plants regenerated from leaf cell somatic embryos (LO plants) were able to transmit the salt stress memory. The differences in the transmission of salt stress between RO and LO plants perhaps lie in where the salt stress occurs and which tissue perceives the stress. The exclusion of Na^+ is a crucial step in adapting to salt stress, and the SOS pathway is one of the mechanisms by which plants export Na^+ from the cells (Quintero et al., 2002). The exposure to high salt concentration is known to cause transcriptional changes of *SOS1*, *SOS2* and *SOS3* genes in a different root cell types, primarily in cortex and endodermis (Ji et al., 2013). In addition, a study in halophytes revealed that *CBL10* and *CIPK3* were upregulated in the epidermal bladder cells. These genes are involved in the pathway similar to the SOS pathway (Shabala et al., 2015). These findings indicate that the salt stress-sensing mechanisms are established in the root cells. The upregulation of SOS pathway in root cells may allow the RO plants to sense the high concentration of Na^+ and efflux them out from the cells. The lack of the SOS genes expression in leaf cells may explain the lack of salt stress adaptation in the LO plants. In addition, the results also indicated that the salt stress memory erased to the non-stress level in the F3. The erasure of stress memory is crucial in adaptive processes as stress is recurring in nature; and the ability to perceive new environmental cues is important for survival (Crisp et al., 2016). The transcriptional changes which occurred under stress response may not provide a fitness advantage to the plant under control conditions.

The ability of plants to sense and respond to low temperature has been well studied (Chinnusamy et al., 2007b). Migicovsky et al. (2014) showed that when plants were treated with cold stress, the first progeny of cold stressed plants had a reduced number of leaves, and this phenotype was passed to the second generation. However, the experiment did not test transgenerational adaptation to cold stress in the cold stress progeny. In this study, the transgenerational cold stress memory was observed in regenerated plants that undergone cold stress during somatic regeneration. When the second-generation plants were treated with extremely low temperature, they exhibited tolerance towards the stress and this phenotype was even observed in the third-generation (Fig. 4.5). While there is limited information regarding transgenerational adaptation to low temperature in plants, Wang et al. (2016) proved that high-temperature priming in wheat caused the primed progeny to developed tolerance to post-anthesis high-temperature stress. The heat stress also did not affect the grain yield of the primed progeny. In contrast to the salt-treated regenerated plants, the cold stress memory was transmitted only in some of regenerated plants from the leaf (LO plants), and the stress memory could be stably inherited to the third generation of LO plants (line 3). However, none of RO plants gained cold stress memory after being primed with mild low temperature during somatic regeneration. Research in *Santalum album* indicated that four *CBF* genes: *CBF1*, *CBF2*, *CBF3* and *CBF4* were upregulated significantly in leaf after 12 hours exposure to mild cold stress (4°C), but the *CBF* gene family was not expressed after the early exposure of cold stress in roots (Zhang et al., 2017). This indicates that early perception of cold stress is established in leaf but not in the root, and this early perception may be crucial in the development of cold stress adaptation and memory. Probably, the ability of the leaf to recognise and respond to cold stress has allowed the LO plants to establish transgenerational cold stress memory.

The ability of plants to respond to infection of pathogenic bacteria is well understood (Henry et al., 2013). Plants treated with flg22, activate rapid stomatal closure to prevent plants from being infested by pathogenic bacteria (Pastor et al., 2013). The same response has been observed when BABA was used to prime plants which even show

transgenerational immune memory in the progeny (Slaughter et al., 2011). In addition, Luna et al. (2012) reported that primed Arabidopsis with *PstDC3000* showed transgenerational systemic acquired resistance (SAR) sustained over one stress-free generation. In this experiment, the ability of plants to sustain SAR was observed after seven days of treatment with flg22 during somatic embryogenesis. The F2 progeny were challenged with *PstDC3000* after the parents were grown in a stress-free condition. The progenies from line 1 and line 2 showed significant resistance to *PstDC3000* (Fig. 4.5). The treatment of flg22 should give equal prime responses in both LO and RO lines, since studies have indicated that plants infested by root pathogens have the ability to develop an immune responses in the shoot to protect the upper ground organs from infection (Kaplan et al., 2008; Pieterse et al., 2002). The inverse response, promotion of defence responses in roots, has also been recorded when leaves are being infected with pathogenic microorganisms or attacked by foliar-feeding organisms (Bezemer & Vandam, 2005). However, in my experiment, transgenerational immune responses were only observed in both RO and LO line 2 while for line 1 it was only detectable in RO but not in LO plants. The differences in developing transgenerational immune responses between line 1 and line 2 are perhaps due to different cellular responses upon direct application of flg22. To overcome this issue in the future, more replicates may be necessary with repeated treatments of flg22.

4.4 Summary

In summary, the findings have identified possible tissue-specific stress adaptation in regenerated plants, and that the early perception of the stress signals is crucial in developing transgenerational stress memory in the corresponding responsive tissue. Some regenerated plants can generate transgenerational adaptive responses to salt, cold and a pathogen elicitor. However, transgenerational adaptations are dependent on the stress type and the specific tissue that perceived the stress signals. For instance, salt stress memory propagated by RO plants whereas LO plants showed improved perception of cold stress.

5. Transcriptome-wide Analysis of Regenerated Plants from Leaf and Root

5.1 Introduction

5.1.1 Transcriptional Changes in Response to Biotic and Abiotic Stress

Plants deal with adverse environmental conditions on a daily basis. In response to these changes, plants have evolved developmental plasticity in the way they adjust to the environmental stimuli. The process of adaptation involves physiological and molecular changes that confer in a superior phenotype to the negative conditions in nature.

High salt content in soil adversely affects water homeostasis and ion distribution (Zhu, 2001). As adaptive responses to osmotic stress, plants reduce the photosynthetic rate (Kawasaki et al., 2001) to decrease the growth rate and leaf expansion; it also affects stomatal closure to limit carbon dioxide uptake (Zhu, 2001). These are a physiological adaptations in response to salt stress. The main mechanism in salt tolerance is by the efflux of sodium ions from the cells into the environment; this will prevent water from diffusing out from the cells. When plants sense a high concentration of Na^+ in the soil, it affects the molecular landscape within the cells by changing the expression of various salt stress response genes. As described in chapter 4.1.1, high salt concentrations trigger the activation of the SOS pathway. The activation of *SOS3* by cytosolic Ca^{2+} triggers *SOS2* to initiate phosphorylation to activate *SOS1* which is a Na^+/H^+ antiporter (Xiong et al., 2002b). The active form of *SOS3-SOS2* also regulates the expression of *HKT1* which is a Na^+/K^+ co-transporter (Rubio et al., 1995; Xiong et al., 2002b). In *Arabidopsis thaliana*, the expression of *AtHKT1* causes NaCl sensitivity in *sos3* mutants, indicating that *SOS3* inhibits *HKT1* activity (Rus et al., 2004; Xiong et al., 2002b). However, *HKT1* is predominantly expressed in the phloem (Berthomieu et al., 2003) which translocases Na^+ from shoot to root where the Na^+ diffused out from the root phloem (Rus et al., 2004).

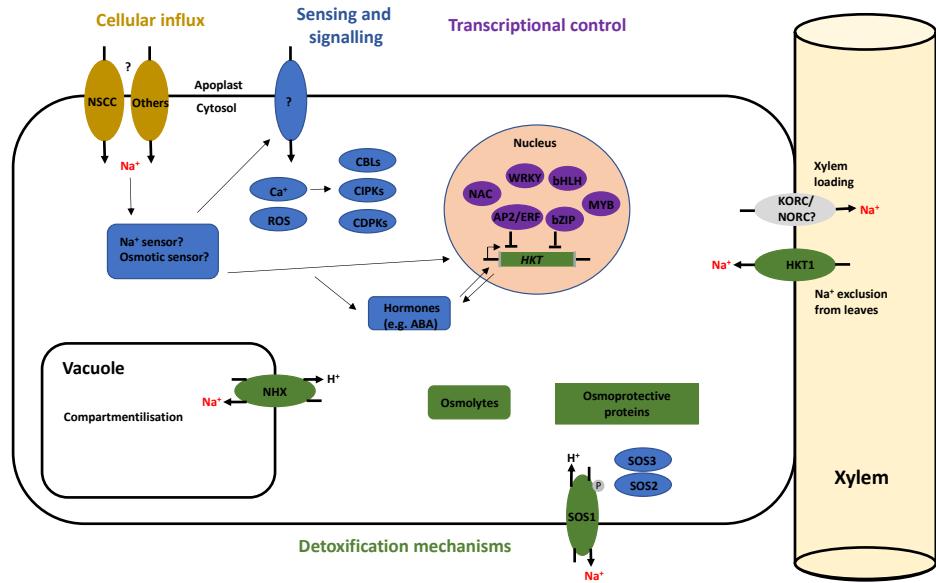


Figure 5.1 Cellular Na⁺ transport mechanisms and components of the salt stress response network in plant root cells. Na⁺ enters the cell via NSCCs and other membrane transporters (cellular Na⁺-influx mechanisms highlighted in brown). Inside the cell, Na⁺ is sensed by unidentified sensory mechanism. Then, Ca²⁺, ROS, and hormone signalling cascades are activated. CBLs, CIPKs, and CDPKs are part of the Ca²⁺-signalling pathway (sensing and signalling components highlighted in blue), which change the global transcriptional profile of the plant (transcription factor families in the nucleus depicted in purple; an AP2/ERF and a bZIP transcription factor that negatively regulate *HKT* gene expression are shown as an example). These early signalling pathways result in expression and activation of cellular detoxification mechanisms, including HKT, NHX, and the SOS Na⁺ transport mechanisms as well as osmotic protection strategies (cellular detoxification mechanisms highlighted in green). In addition, the Na⁺ in the plant is regulated in a tissue-specific manner by unloading of Na⁺ from the xylem. NSCCs, nonselective cation channels; ROS, reactive oxygen species; CDPKs, calcium-dependent protein kinases; CBLs, calcineurin B-like proteins; CIPKs, CBL-interacting protein kinases; AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR; bZIP, basic leucine zipper; NHX, Na⁺/H⁺ exchanger; SOS, SALT OVERLY SENSITIVE. (modified from: Deinlein et al., 2014).

Another molecular pathway conferring response to salt stress is the ABA signalling response. The accumulation of ABA in response to osmotic stress upregulates the gene *DREB2* (Tuteja, 2007). DREB2 is a DRE/CRT binding protein, and these proteins have a conserved AP2 DNA-binding domain (Shinozaki & Yamaguchi-Shinozaki, 2000). Salt-stress response genes such as *RD29A* and *RD29B* also contain an AP2 DNA-binding motif. Thus the expression of these genes is dependent on ABA signalling (Yamaguchi-Shinozaki & Shinozaki, 1994). In addition, ABA signalling can also activate the expression of a leucine zipper (bZIP) transcription factor that binds to ABA Responsive Elements (ABRE) and activates genes with ABRE-driven promoters (Uno et al., 2000). These genes, mostly involved in the salt stress response, include homeodomain transcription factor-encoding genes *ATHB7*, *AREB1* and *AREB2* (Liu et al., 2007; Uno et al., 2000).

Plant responses to freezing stress have the same molecular mechanisms to osmotic stress. Exposure to low temperatures affects membrane rigidity which stimulates the production of ABA (Shinozaki & Yamaguchi-Shinozaki, 2000). The accumulation of ABA increases the level of Ca^{2+} that impacts the cold signalling pathway (Chinnusamy et al., 2007a). The accumulation of Ca^{2+} induces the expression of *ICE1* (*INDUCER OF CBF EXPRESSION1*), and in Arabidopsis, the overexpression of *ICE1* results in an improved the freezing tolerance (Chinnusamy et al., 2003). ICE1 plays a role in controlling the expression of *CBF2* and *CBF3*, with CBF2 which specifically regulate the expression of genes with DRE/CRT motifs upstream of the promoter region. This motif is present in most cold-inducible genes such as *LEA* and *KIN* (Chinnusamy et al., 2007; Yamaguchi-Shinozaki & Shinozaki, 2006). Furthermore, CBF3 controls the expression of *COR* (*COLD RESPONSIVE*) genes. A well-studied member of the *COR* gene family, *COR15a*, has a significant role in freezing tolerance. When *COR15a* is expressed in Arabidopsis, it improves freezing tolerance by 1-2°C over the range of -4 to -8°C (Thomashow, 1999). It is predicted that COR15a reduces the tendency of membranes to form hexagonal II phase lipids in response to freezing (Thomashow, 1999). In addition, there are CBF independent pathways in the regulation of freezing tolerance in plants. It was found that the *eskimo1* (*esk1*) mutant of Arabidopsis showed enhanced freezing tolerance by accumulating high

levels of free proline. However, the expression of *ESK1* is not affected by cold stress (Chinnusamy et al., 2007). Moreover, *HOS9* and *HOS10* overexpression can improve cold stress tolerance by regulating *COR* genes (Chinnusamy et al., 2007; Zhu et al., 2005; Zhu et al., 2004).

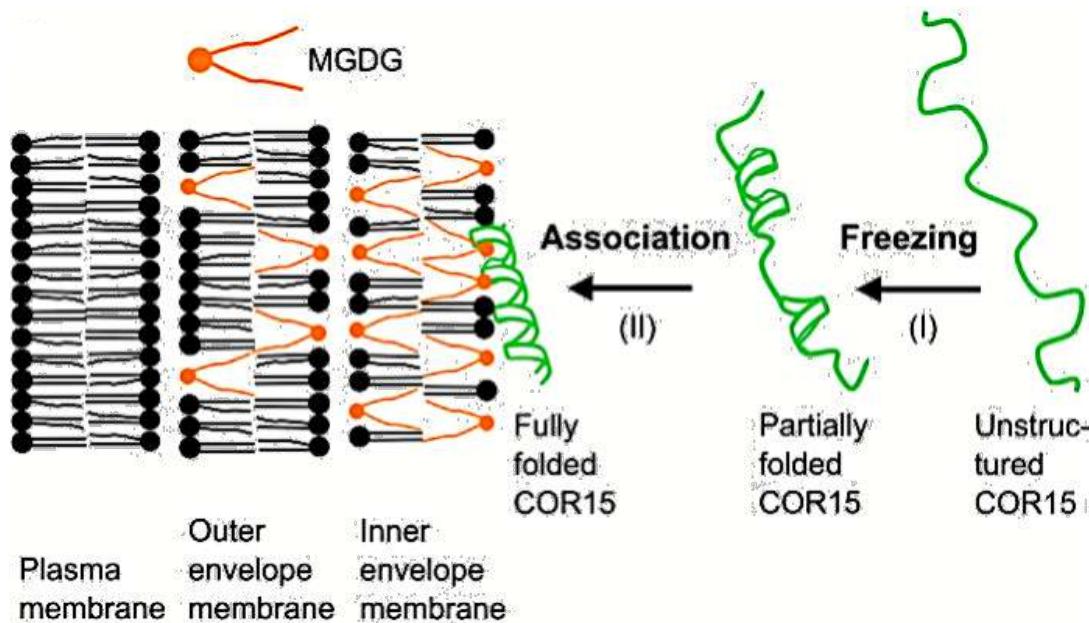


Figure 5.2 Model of COR15 protein function. Protein binding stabilises the inner envelope membrane against the formation of hexagonal II (HII) phase lipid domains involving adjacent membranes such as the chloroplast outer envelope and plasma membrane. This results in maintenance of plasma membrane integrity during freezing, which can be measured as reduced electrolyte leakage. Monogalactosyldiacylglycerol (MGDG) (obtained from: Thalhammer & Hincha, 2014).

Plant responses to biotic stress govern similar hormone responses to abiotic stress; these include jasmonic acid (JA), salicylic acid (SA) and ethylene signalling pathways. The plant defence mechanism activated under biotic stress is dependent on the invasive organism, whether they are herbivore, pathogenic bacteria or fungi. In response to herbivore attack and necrotising infection, high levels of JA are produced (Pieterse & van Loon, 1999). In *Arabidopsis*, the treatment with methyl jasmonate activates both thionin gene *Thi2.1* and defensin gene *PDF1.2*. These genes are also systemically activated after infection with a necrotising pathogen (Penninckx et al., 1996; Pieterse & van Loon, 1999). In the SA signalling pathway, the production of SA is induced after pathogen recognition.

The accumulation of SA activates the WRKY18 transcription factor, which will bind to two GACC/T motifs in the 5`UTR of *Nonexpresser of Pathogenesis-Related gene 1* (*NRP1*). *NRP1* then translocates into the nucleus and bind with TGA factors to form NPR1-TGA complexes. These complexes then bind to the TGA box in the promoter of *PR1* gene and regulate the expression of *PR1* (Mou, Fan, & Dong, 2003). Overexpression of *PR1* has improved plant resistance to virulent *P. syringae* (Eulgem, 2005). Although SA has a significant role in triggering plant defence responses, research done by Chinchilla et al. (2008), it is part of FLS2 defence mechanism. FLS2 is an LRR receptor-like kinase and perceives flagellin, the main protein of bacteria flagella (Gómez-Gómez & Boller, 2000). In Arabidopsis, the *fls2* mutant showed a reduction in defence responses to pathogens. This indicates the importance of *FLS2* expression during early defence responses. The recognition of bacterial flagellin (active epitope flg22) by FLS2 allows the formation of FLS2-BAK1 hetero-oligomer which transduces phosphorylation cascades to mitogen-activated protein kinases (MAPKs), mitogen-activated protein kinase kinases (MAPKKs) and calcium-dependent protein kinases (CDPKs) which the process activate the expression of flg22-responsive genes (Belkhadir et al., 2014; Robatzek & Wirthmueller, 2013). There is a crosslink between FLS2 defence responses and SA. There is evidence showing SA acts partially by enhancing accumulation of *FLS2* mRNA in early flg22 responses (Yi & Kwon, 2014). Furthermore, there is evidence for the involvement of ethylene in triggering defence responses in tomato by rapidly induced the expression of a *Pti4* transcription factor that can bind to GCC-box containing *PR* genes (Wang et al., 2002).

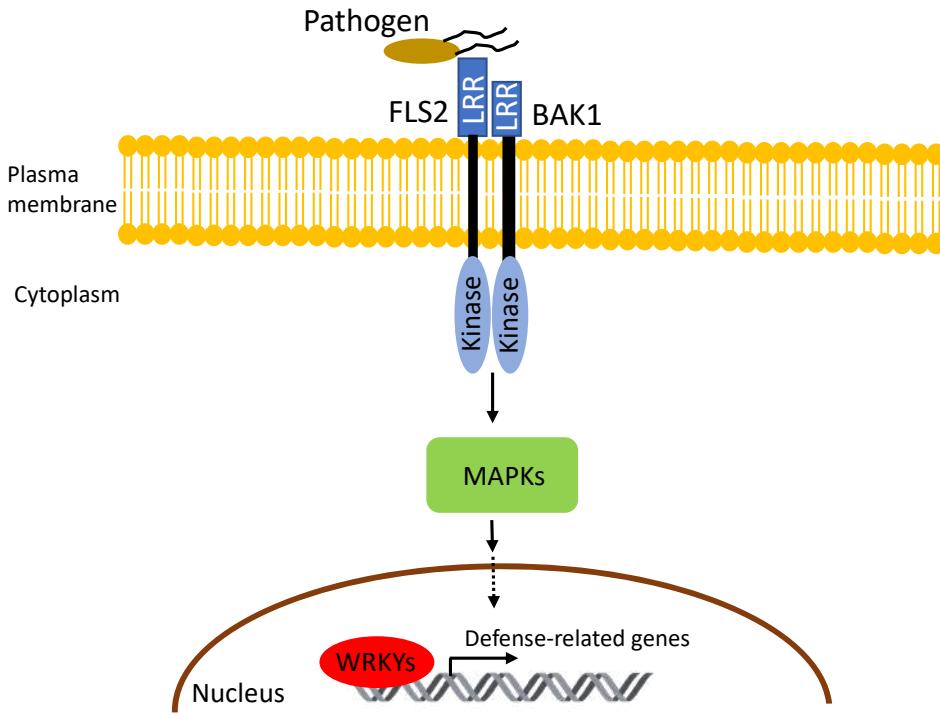


Figure 5.3 FLS2 signaling responses. FLS2 recognises distinct epitopes of bacterial flagellin. Transmembrane pattern recognition receptors (PRRs) have extracellular leucine rich repeat (LRR) receptor domain but different cytoplasmic domain. The binding of bacterial flagellin to FLS2 forms hetero-oligomer of FLS2-BAK1. FLS-BAK1 transduces phosphorylation to mitogen-activated protein kinases (MAPKs) which will activate the expression of flg22-responsive genes/defense-related genes.(modified from: (Haney, 2014)).

5.1.2 Molecular Mechanisms in Developing Transcriptional Memory

Transcriptional memory serves a pivotal function in adaptation towards rapid changes in environmental condition. The first evidence of transcriptional memory was observed in the *Saccharomyces cerevisiae GAL1* gene. When the budding yeast is first treated with galactose, it takes approximately one hour for the expression of *GAL1* to reach its peak, but when the expression of *GAL1* is repressed by growing the cells in the media with glucose, and when the budding yeast is then re-exposed to galactose, the transcription of *GAL1* resumes rapidly and only takes ten minutes to reach its maximum transcription (Kundu et al., 2007).

It was found that gene loops are important in maintaining transcriptional memory (Brickner, 2009). The formation of a gene loop between the promoter of *GAL1* and its 3` end allows faster RNA polymerase II recruitment after a period of gene repression (Wong et al., 2009). The loop formation is tethered to the nuclear pore complex, and it is bound to the nuclear pore by the interaction with Mlp1. During the initial formation, the loop does not directly interact with Mlp1. Instead, the binding occurs only after transcriptional repression (Wong et al., 2009). The binding to Mlp1 is crucial for maintaining the loop structure and transcriptional gene memory during a period of transcriptional repression (Wong et al., 2009). Furthermore, the chromatin state plays a role in the rapid reactivation of transcription after a period of repression. It was found that histone variant H2A.Z maintains long-term transcriptional memory by binding to the promoter of repressed genes such as *GAL1* and *INO1* and incorporating into the nucleosome; the genes that corporates to nuclear periphery significantly reactivated faster than the genes in the nucleoplasm (Brickner et al., 2007). In addition, SWI/SNF, a family of the ATP dependent chromatin remodelling complexes with HAS and BROMO domain (Tang et al., 2010), is important in controlling transcriptional memory by antagonising the function of ISWI-like chromatin remodelling enzymes (Kundu et al., 2007). Kundu et al. (2007) also found that the deletion in *SWI* did not affect the transcription of *GAL1* instead it abolished rapid re-induction of *GAL1* after repression.

Transcription memory upon stress has also been identified in plants. In Arabidopsis, the dehydration stress progenies in generation 2 (F2) and generation 3 (F3) have four distinct memory response pattern compared to the first stress (F1) generation (Avramova, 2015). Moreover, there are more than 2000 Arabidopsis genes showed memory responses to the dehydration stress with all of these genes have enrichment in gene ontology (GO) terms for increased synthesis of protective, damage-repairing and detoxifying functions, coordinating growth and photosynthesis under repetitive stress, re-adjusting interactions between dehydration and other stress-regulated pathways (Avramova, 2015). In maize, repeated dehydration stress displayed similar transcription memory responses to those of *A. thaliana* (Avramova, 2015). This indicates evolutionary conservation of stress memory in eudicot and monocot plants, and transcriptional stress memory is therefore a

biologically relevant mechanism that is conserved during evolution of land plants and regulates different responses to a single stress versus recurring stresses (Avramova, 2015).

Epigenetic events such as histone H3 lysine 4 methylation is important for transcriptional memory (D'Urso & Brickner, 2017). Loss of H3K4 methylation disrupts the binding of RNA Polymerase II (Pol II) to the promoter and transcriptional memory (D'Urso & Brickner, 2017). In *Arabidopsis*, during dehydration stress, the non-memory genes displayed dynamically changing H3K4me3 patterns correlating with the degree of transcription, while memory genes maintained increased H3K4me3 during recovery phase, when the gene expression was low (Avramova, 2015). Thus, in plants there is a correlation between transcriptional memory and epigenetic events, and it is clear that plants can develop temporal transcriptional memory upon exposure to stress, but the transgenerational transcriptional stress memory in the regenerated plants from root and leaf tissues are still unknown.

5.1.3 Chapter Aims

This chapter aims to study the transcriptional changes and transcriptional memory in regenerated plants from leaf and root tissue after exposure to mild salt, cold stress and pathogen elicitor (flg22) by performing RNA sequencing in the second generation (F2) of regenerated plants and assessing the differential gene expression between control and treated plants by using DeSeq2. The transcriptomic experiment is conducted to understand the molecular changes underpinning the phenotype found in the previous chapter.

5.2 Results

5.2.1 Differential Gene Expression in Regenerated Plants Exposed to Salt and Cold Stress

The exposure to abiotic stress such as cold and high salinity stress can change the underlying transcriptional state in different tissue types. Transcriptome profiling studies in *Arabidopsis thaliana* has found differential gene responses towards cold and salinity stress in roots and leaf tissues with only 30% of genes are shared by cold, salt and osmotic stress (Kreps et al., 2002). Moreover Wibowo et al. (2016) has shown heritable gene expression changes in response to salt stress, the tissue-specific heritable transcriptional memory remains unknown. To assess heritable transcriptome changes in regenerated-plants from leaf and root treated with mild cold and salinity stress during somatic regeneration, RNAseq was performed in three independent second generation (F2) lines (control RO-LO line 1, 2, and 3; salt RO-LO line 1, 2, and 3; cold RO-LO line 1, 2, and 3) with each sample containing leaf tissue from ten pooled-plants. The principal component (PC) analysis of gene expression in F2 salt and cold-treated regenerated plants (Fig. 5.4) showed clear groupings between non-stress regenerated plants (control) and stress-regenerated plants. To find the differentially expressed genes (DEGs), DeSeq2 (Love et al., 2014) was used with the $FDR < 0.01$ and $\log_{2}\text{fold}$ cut-off 1. The Figure 5.5 (A) indicates 225 genes respond differently to salt stress in RO plants, but only 32 DEGs genes are observed in LO plants. Meanwhile, cold stress affects the expression of 168 genes in LO plants and only 36 genes in RO plants (Fig. 5.5 B). The DEGs are further analysed to understand their expression value, Figure 5.5 (C) shows the actual expression value of DEGs in the F2 from salt-treated regenerated plants, while Figure 5.5 (D) shows the expression value of DEGs in the F2 from cold-treated regenerated plants. Hierarchical clustering analysis for both stress types indicates distinct clustering based on tissue origin and stress responses (Fig 5.5 C and D). The differentially expressed genes in F2 of root-origin (RO) salt-treated regenerated plants show 63 downregulation genes in comparison to the control experiments with 167 genes are upregulated while the cold-treated leaf-origin (LO) plants show 60 downregulated genes and 110 upregulated genes..

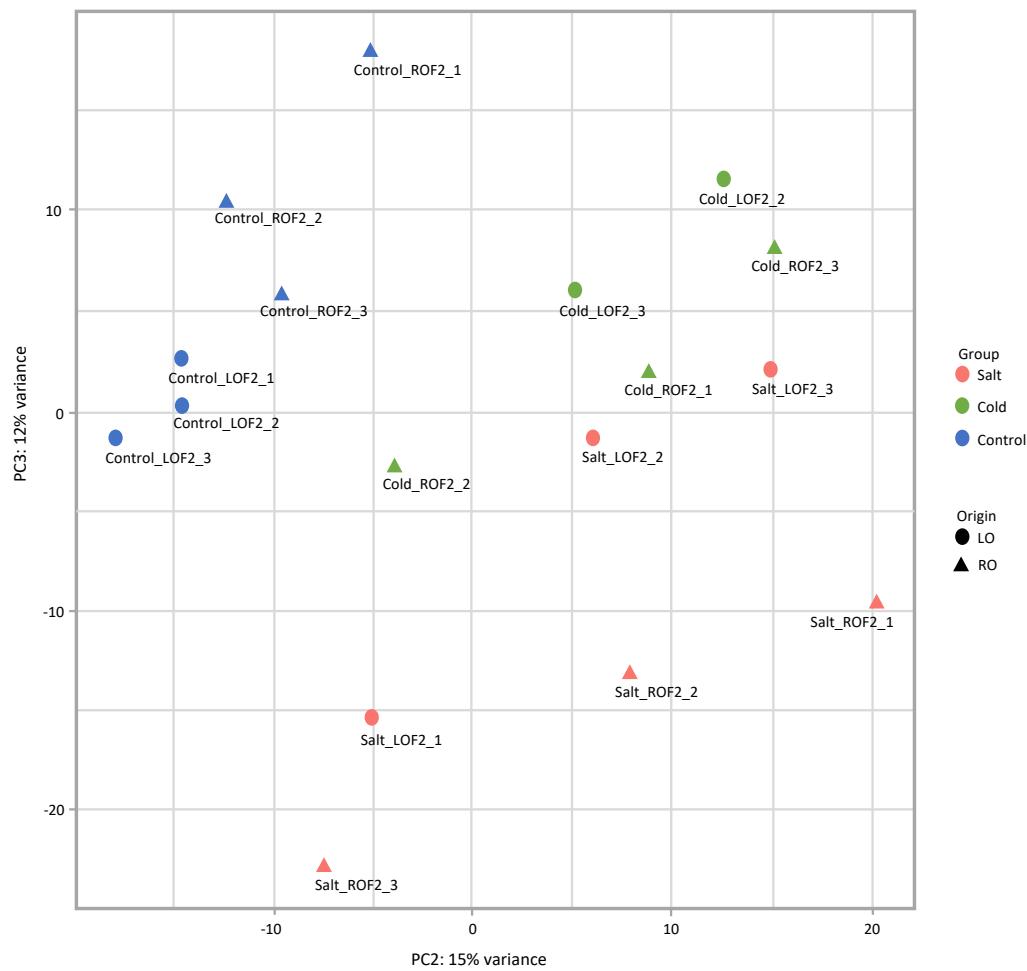


Figure 5.4 Principal component (PC) analysis of salt and cold-treated regenerated plants. The PC analysis between PC2 vs PC3 showed distinct clustering between control and stress (cold and salt) in the F2 regenerated plants based on their counts.

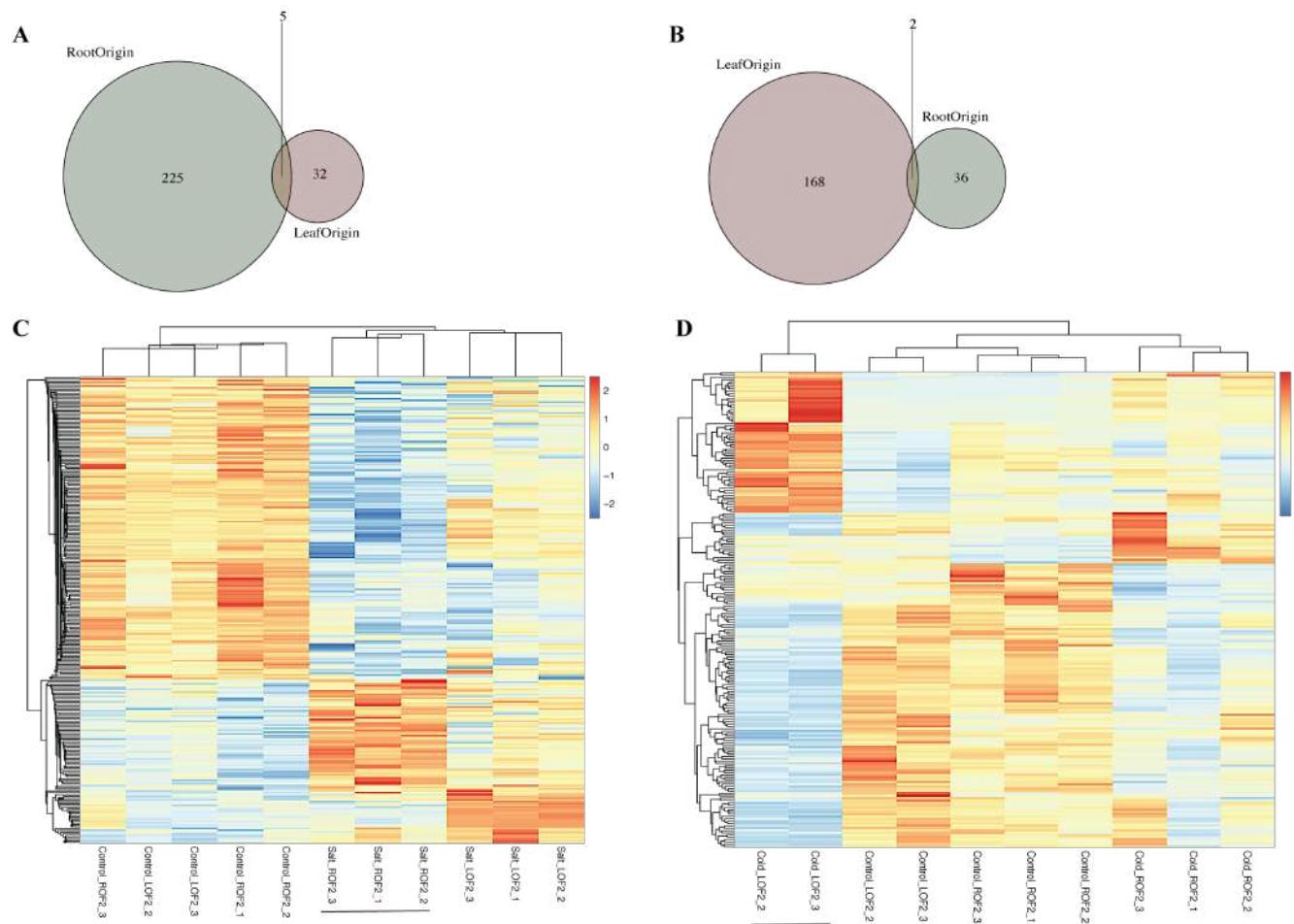


Figure 5.5 Analysis of salt and cold-induced differentially expressed genes (DEGs) in root and leaf regenerated plants. The Venn diagram showed the DEGs found in the F2 (A) salt-treated root (RO) and leaf (LO) plants, and (B) cold-treated RO and LO plants. (C), (D) Hierarchical clustering of pairwise correlation analyses based on DEGs log fold values in the second generation (F2) of salt-treated regenerated plants (A) and cold-treated regenerated plants (B). Scale bar indicates log₂fold.

By looking at the PC analysis in Fig. 5.4, there is variable between salt RO samples and cold LO samples. Thus a single comparison of DEGs between control RO-LO and stress RO-LO was performed. The analysis (Table 5.1) indicated that there are more downregulated genes found in salt ROF2 line 1, 2 and 3 compared when the analysis was performed by treated the sample as replicates. In addition, cold LOF2 line 1, 2 and 3 also showed more downregulated genes.

Table 5.1 Differentially expressed genes found in single comparison between control RO-LO with stress (salt RO and cold LO).

Sample Name	Upregulated Genes	Downregulated Genes
Salt ROF2 1	58	320
Salt ROF2 2	18	85
Salt ROF2 3	58	268
Cold LOF2 1	103	215
Cold LOF2 2	239	292
Cold LOF2 3	199	261

When the DEGs from salt ROF2 line 1, 2 and 3 (Fig. 5.6 A) intersected between each other, there are 26 DEGs common in all samples. Whereas in the cold LOF2 (Fig. 5.6 B), there are 134 genes shared between sample 1, 2 and 3. In addition, cold LOF2 2 and cold LOF2 3 shared 202 genes between them. These findings indicated that regenerated plants are able to maintain transcriptional changes in response to stress during somatic embryogenesis to the successive non-stress generation.

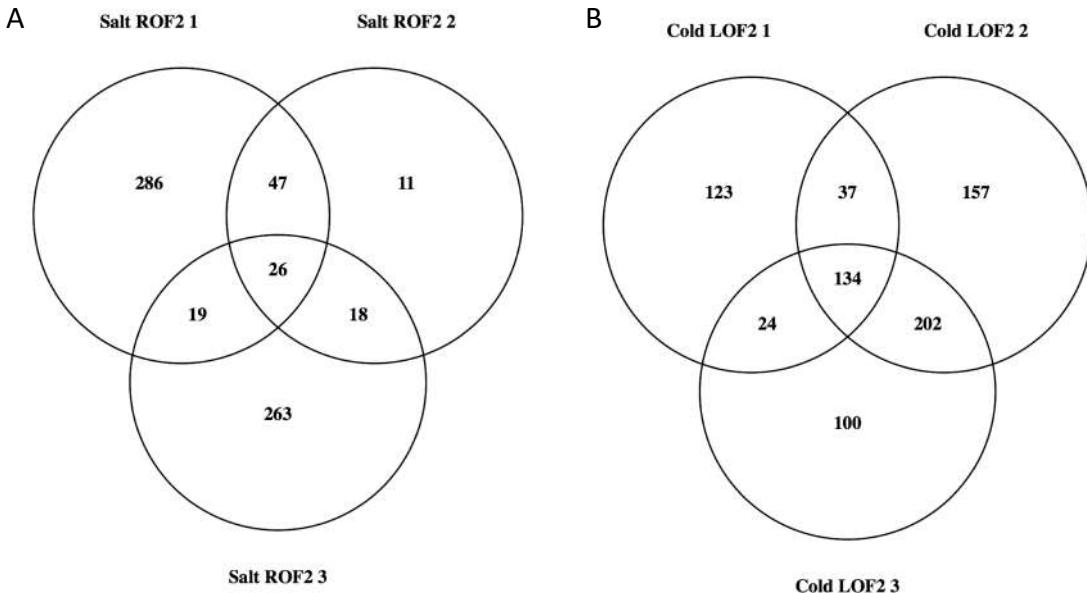


Figure 5.6 Intersection between differentially expressed genes (DEGs) in single sample of salt ROF2 and cold LOF2. DEGs in each sample of salt ROF2 (A) and cold LOF2 (B) was identified using DeSeq2 (Love et al., 2014). The upregulated and downregulated DEGs for each sample were combined and intersected by using Venny (Oliveros, 2015).

5.2.2 Flg22-Treated Regenerated Plants Do Not Show Significant Transcriptomic Changes

The induction of defence transcripts can be widely observed by treating plants with the bacterial elicitor flg22 (Denoux et al., 2008; Rosli et al., 2013). When *Arabidopsis* was treated with flg22 at the seedling stage, it triggered fast activation in defence signalling pathways (Denoux et al., 2008). Although plants can transgenerationally inherit the biotic stress responses (Alex et al., 2010), the transcriptomic memory induced by flg22 during the embryo stage remains unknown. The transcriptomic data was generated by analysing the RNAseq from F2 flg22-treated regenerated plants from root tissue (RO) and leaf tissue (LO) plants whereas the RNA was collected from pooled of 10 plants per line. The principal component (PC) was plotted between PC1-PC2 (Fig. 5.7). The PC1-PC2 analysis of flg22-treated RO and LO transcriptome did not show any clear clustering between control and flg22-treated plants. It indicated that there was no distinct difference between gene expression in the control and treatment. Thus further differentially

expressed genes analysis between control and flg22-treated regenerated plants was not possible to conduct.

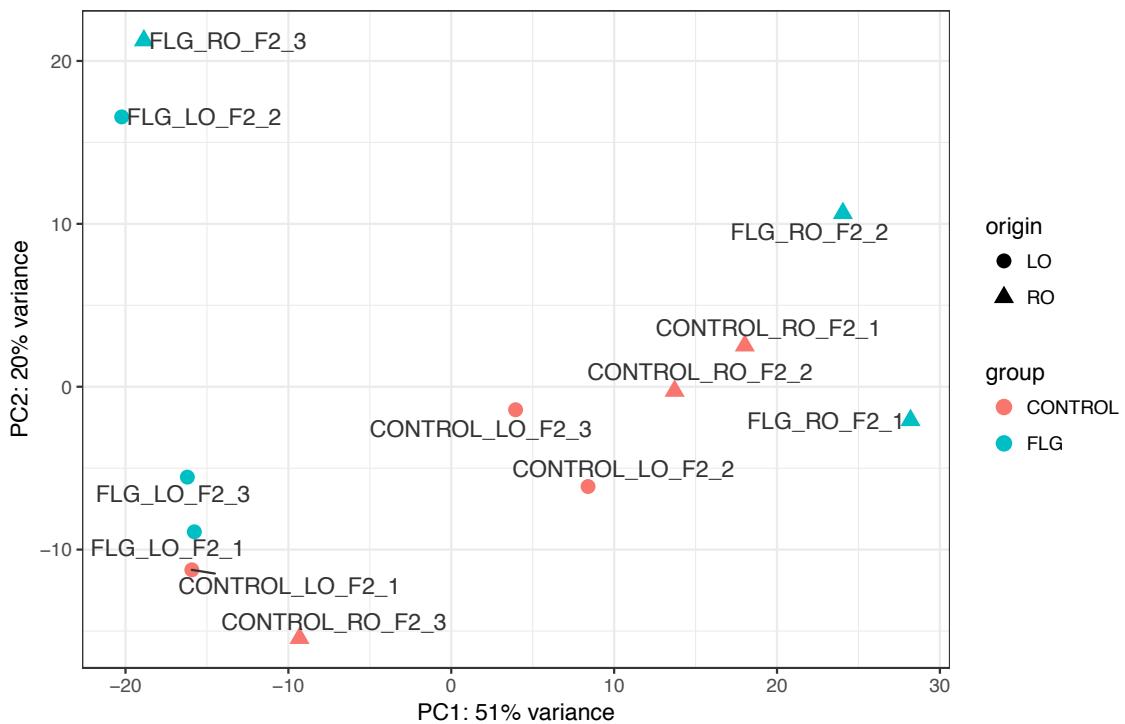


Figure 5.7 Principal component analysis of flg22-treated and control regenerated RO and LO plants. The first component with the highest variance (51%) is on the X-axis did not have clear separation between control RO-LO and flg22 RO-LO. The second component, with the second highest variance (20%), is on the Y-axis, depicting a maximum variation of the flg22 LOF2 2 and flg22 ROF2 3 samples from rest of samples. The samples and control did not form separate clusters on the PCA plot, indicating there is little transcriptional differences among different groups.

5.2.3 Gene Ontology Analysis of Differentially Expressed Genes in Regenerated Plants From Leaf and Root

The effects of environmental changes most of the time not only affect one specific gene, but rather it affects genes within a gene network (Vu et al., 2015). Elucidating the differentially expressed genes in response to the environmental cues is not sufficient enough to understand how the effect of transcriptional change is converted into a specific phenotype (Vu et al., 2015). To further understand how the differentially expressed genes affect the traits observed in both cold-treated leaf-regenerated plants and salt-treated root-regenerated plants, a gene ontology enrichment analysis and genes network analysis were performed.

Table 5.2 Salt stress-related genes found in the salt RO DEGs based on gene ontology analysis.

Name	Gene Annotation	Fold Changes
AT2G16500	ARGININE DECARBOXYLASE 1 (Do et al., 2014)	0.914479252
AT5G67300	MYB DOMAIN PROTEIN 44 (Shukla et al., 2015)	1.415877805
AT1G66400	CALMODULIN LIKE 23 (Munir et al., 2016)	1.57780022
AT5G37770	TOUCH 2 (Jung Jang et al., 1998)	1.80359214
AT1G27730	SALT TOLERANCE ZINC FINGER (Mittler et al., 2006)	1.807476761
AT5G24030	SLAC1 HOMOLOGUE 3 (Qiu et al., 2016)	2.015529834
AT3G43700	BTB-POZ AND MATH DOMAIN 6 (Weber & Hellmann, 2009)	-0.602406498
AT3G61890	HOMEobox 12 (Shin et al., 2004)	-1.758866374
AT2G47770	ATTSPo (Balsemão-Pires et al., 2011)	-1.874707602

To investigate if there was any gene term enrichment in differentially expressed genes both in cold and salt-treated regenerated plants, AgriGO (Tian et al., 2017) used with the DEGs found in cold LO and salt RO separately. The gene ontology (GO) analysis was using TAIR10 as reference (Fisher statistical test, yekuiteli multi_test adjustment method and significance level 0.05). The gene ontology analysis in salt-treated root-regenerated (RO) plants (Figure 5.8) shows enrichment in ion homeostatic, response to organonitrogen and phosphorelay signalling transduction. Furthermore, three significant enrichments are

found related to ethylene response and signalling transduction. Based on gene ontology enrichment analysis, seven genes are directly involved in salt stress response (Table 5.2), and the expression of six out of nine genes are mostly upregulated in comparison to the control experiment.

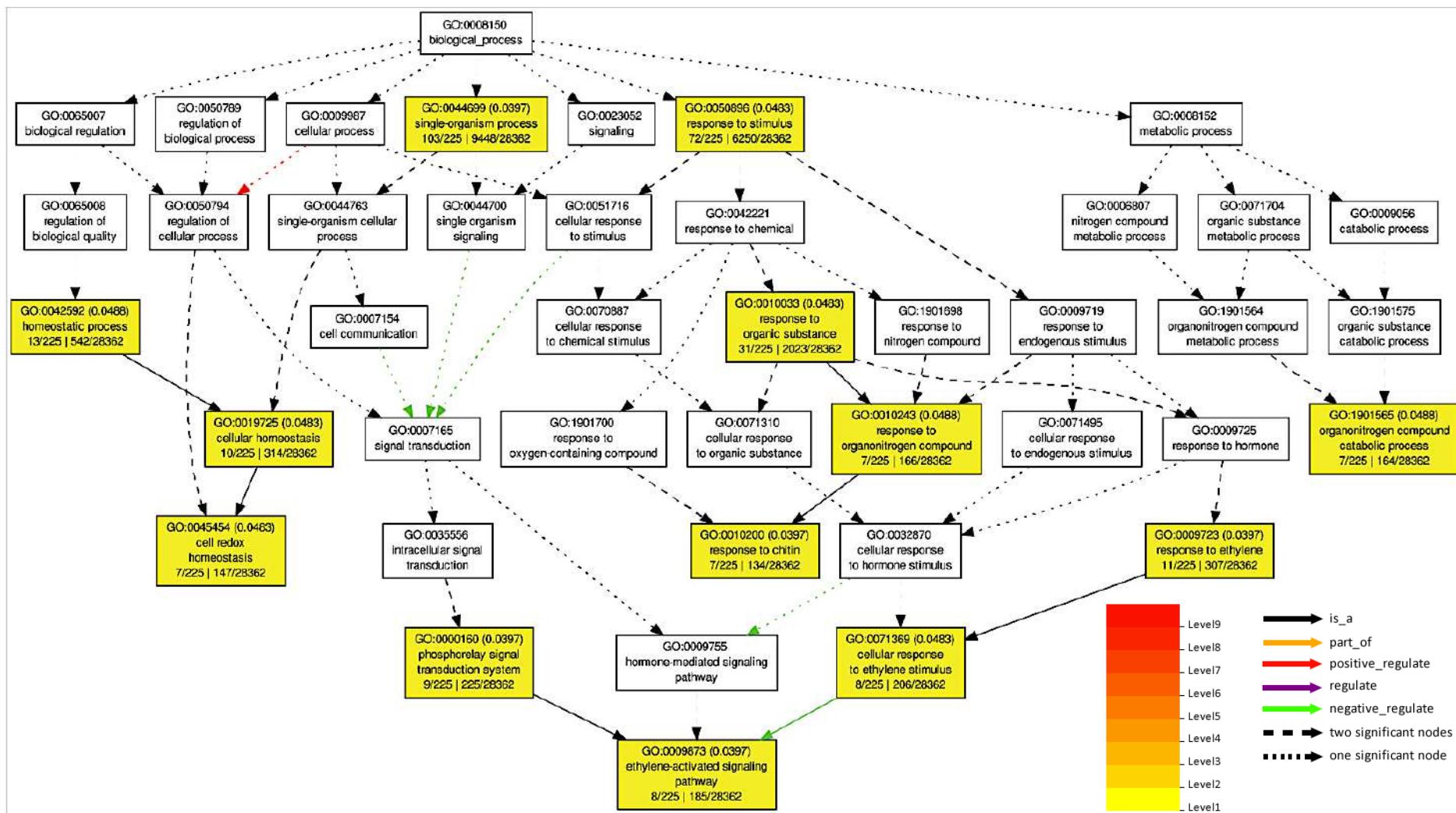


Figure 5.8 Gene Ontology (GO) analysis of DEGs found in F2 of salt-treated regenerated RO. The 230 DEGs found in the F2 salt-treated RO were loaded into AgriGO v2.0 (Tian et al., 2017) and the biological process was analysed. The heat-color legend indicates the significance level.

Table 5.3 Common DEGs found in all sample of salt ROF2 line 1, 2 and 3.

Gene Identifier	Gene Annotation
AT1G66160	CYS, MET, PRO, AND GLY PROTEIN 1 (CMPG1)
AT2G44080	ARGOS-LIKE (ARL)
AT5G20230	BLUE-COPPER-BINDING PROTEIN (BCB)
	BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2 (BCAT-2)
AT1G10070	CHLOROPHYLL A/B BINDING PROTEIN 3 (CAB3)
AT1G29910	CHLOROPHYLL A/B-BINDING PROTEIN 2 (CAB2)
AT1G29920	GENOMES UNCOUPLED 5 (GUN5)
AT5G13630	JUMONJI DOMAIN CONTAINING 5 (JMJD5)
AT3G20810	LATE EMBRYOGENESIS ABUNDANT 3 (LEA3)
AT1G02820	LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 6 (LHCB6)
AT1G15820	LOB DOMAIN-CONTAINING PROTEIN 38 (LBD38)
AT3G49940	MYB DOMAIN PROTEIN 77 (MYB77)
AT3G50060	NDR/HIN1-LIKE 13 (NHL13)
AT2G27080	NEET GROUP PROTEIN (NEET)
AT5G51720	NITRATE REDUCTASE 1 (NIA1)
AT1G77760	PHOSPHOENOLPYRUVATE (PEP)/PHOSPHATE TRANSLOCATOR 2 (PPT2)
AT3G01550	PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.1 (LHCB2.1)
AT2G05100	PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.2 (LHCB2.2)
AT2G05070	PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.3 (LHCB2.3)
AT3G27690	

The DEGs analysis also performed in individual samples of salt ROF2, and it was found that there are 26 common DEGs shared by all salt ROF2 samples (Table 5.3 (the unannotated genes were removed)). By using these common genes, the GO analysis was performed using agriGO. The GO analysis (Fig. 5.9) indicated that there was enrichment of photosynthesis genes in all salt ROF2 samples.

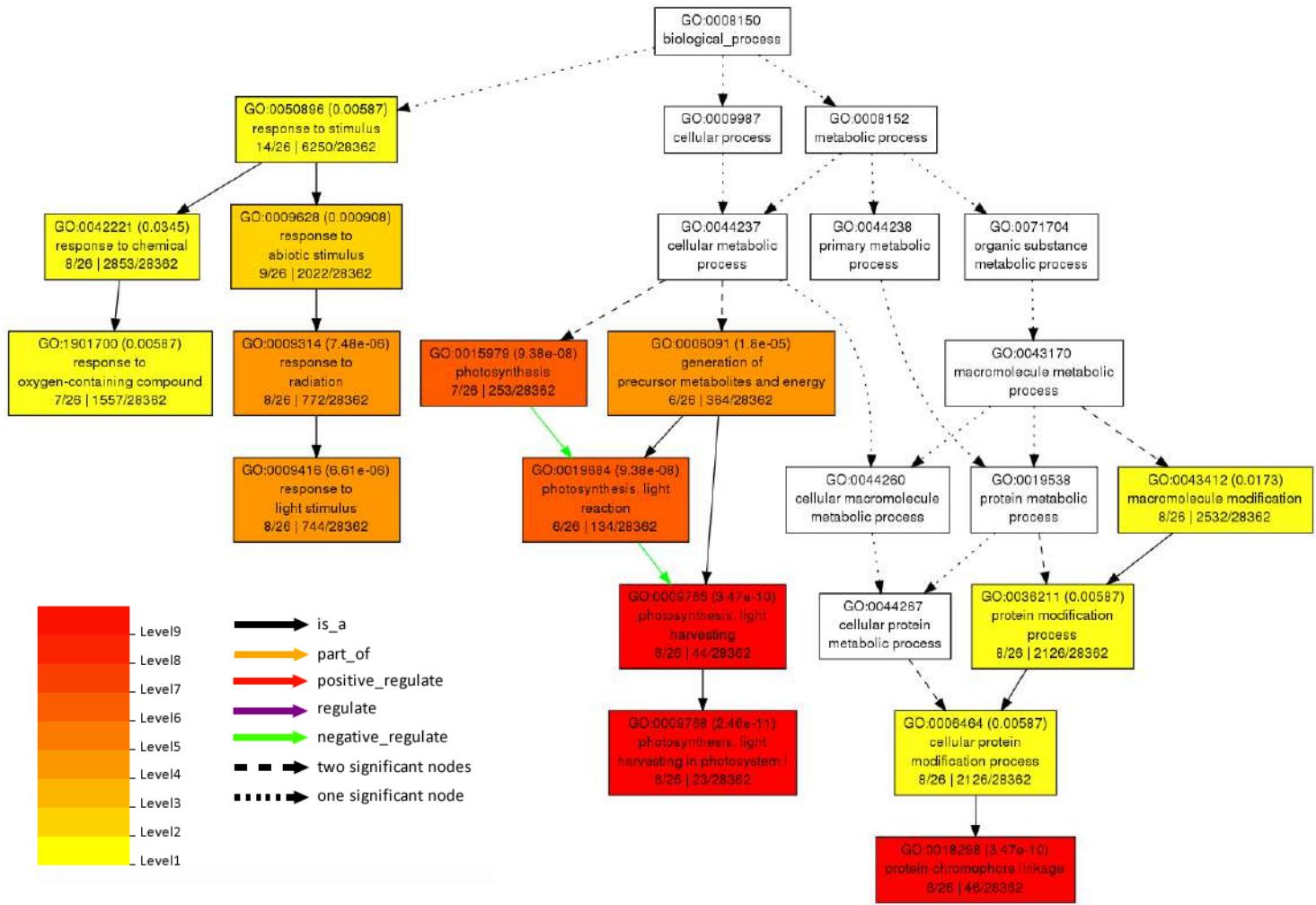


Figure 5.9 Gene Ontology (GO) analysis of common DEGs found in F2 of salt-treated regenerated RO line 1, 2 and 3. The 26 common DEGs found in the F2 salt-treated RO line 1, 2 and 3 were loaded into AgriGO v2.0 (Tian et al., 2017) and the biological process was analysed. The heat-color legend indicates the significance level.

We looked at the gene network analysis of the 230 DEGs found in the salt RO (Figure 5.10) using GeneMania (Warde-Farley et al., 2010). The genes database was created in the GeneMania by choosing the database that containing protein-protein interaction. This analysis revealed that 40 DEGs had known protein-protein interaction in a network. The gene network of salt RO revealed the presence of salt stress-related genes, such as *SLAC 1 HOMOLOGUE 2 (SLAH2)*, *SALT TOLERANT ZINC FINGER (STZ)*, *ETHYLENE RESPONSE 2 (ETR2)*, *NDRI/HINI-LIKE3 (NHL3)*. Most of the network responses were generated by the *NHL3* built up defence response network. The other significant network observed in the Salt RO was the *AUXIN RESISTANT 3 (AXR3)*, which indicates that salt stress may involve the auxin response.

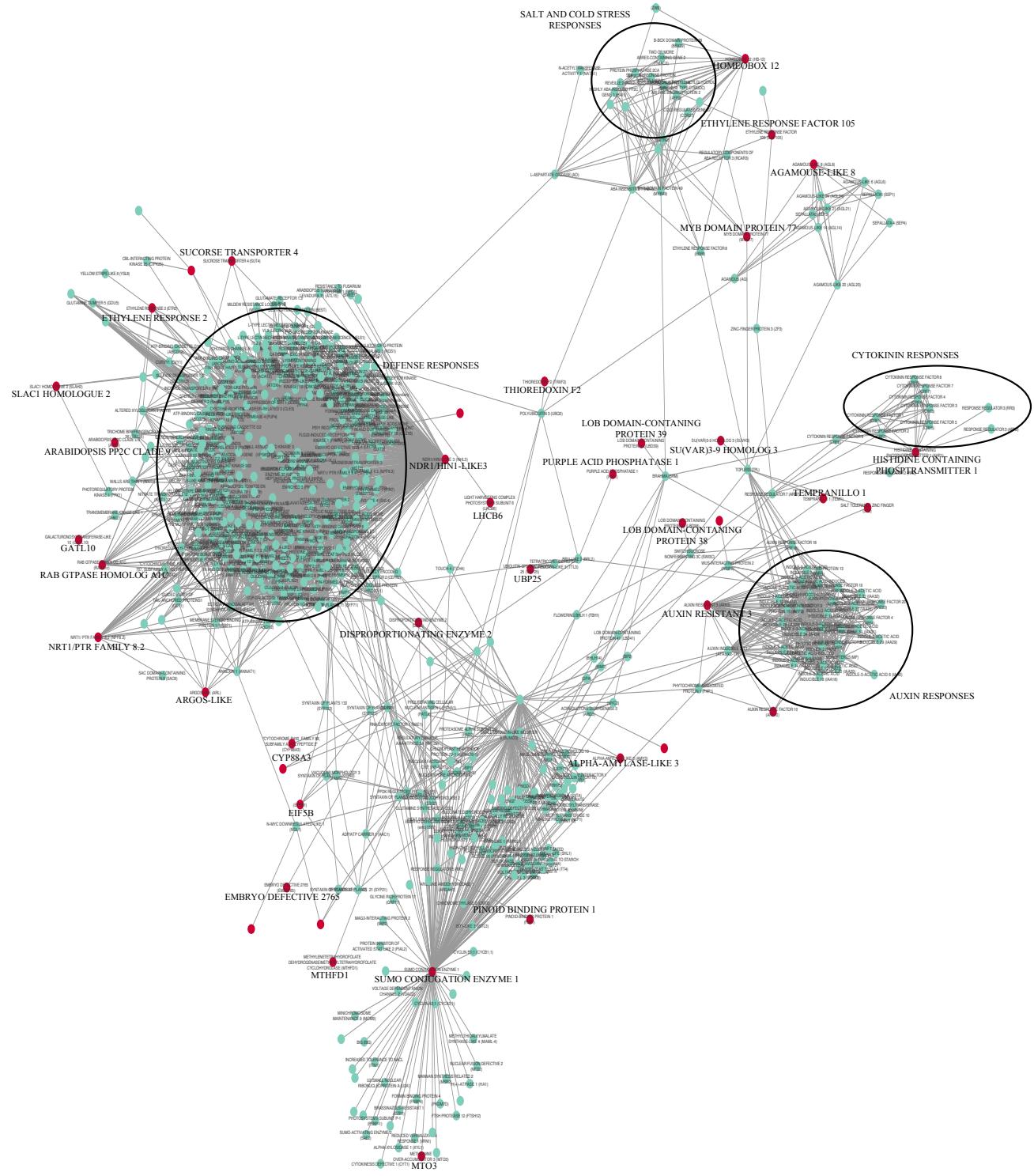


Figure 5.10 Gene network analysis of DEGs found in F2 salt-treated RO plants. The gene network within 230 DEGs in F2 salt-treated RO plants was visualised and analysed using GeneMania (Warde-Farley et al., 2010) in the Cytoscape. The network analysis indicated the protein-protein interaction from DEGs (red circles) intersected with the physical interaction database in GeneMania. Blue circles are gene that found directly interacted with DEGs.

The 170 differentially expressed genes from the cold-treated leaf-origin (LO) plants were loaded into AgriGO which detected a significant enrichment in both cell wall biogenesis and carbohydrate metabolic process (Figure 5.11). Based on the gene ontology enrichment, the genes that are directly involved in regulating cold stress responses did not presence in the analysis. However, some salt response genes were also found upregulated in the cold-treated LO plants. In addition, genes belonging to the water deficit response, ABA responses, carbohydrate and cell wall biosynthesis were also found to be upregulated. The 170 DEGs were also intersected with the database in AtGenExpress (Kilian et al., 2007), and 10 DEGs are associated with cold stress (Table 5.4).

Table 5.4 DEGs in F2 cold-treated LO plants that directly affected by cold stress according to AtGenExpress (Kilian et al., 2007).

Name	Gene Annotation	Fold Change
AT2G20680	MAN5-2	0.979644202
AT3G16670	POLLEN OLE 1	2.344466676
AT3G05730	DEFENSIN-LIKE FAMILY PROTEIN	2.528451521
AT4G23630	VIRB2-INTERACTING PROTEIN 1	-0.683006937
AT1G78070	WD40 REPEAT-LIKE SUPERFAMILY	-1.449179254
AT1G51090	ATHMAD1	-1.535683945
AT1G27200	GLYCOSYLTRANSFERASE FAMILY PROTEIN	-1.610015094
AT3G61890	HOMEobox 12	-1.615732825
AT1G56600	GALACTINOL SYNTHASE 2	-1.788792562
AT2G47050	PECTIN METHYLESTERASE INHIBITOR SUPERFAMILY	-1.794253212

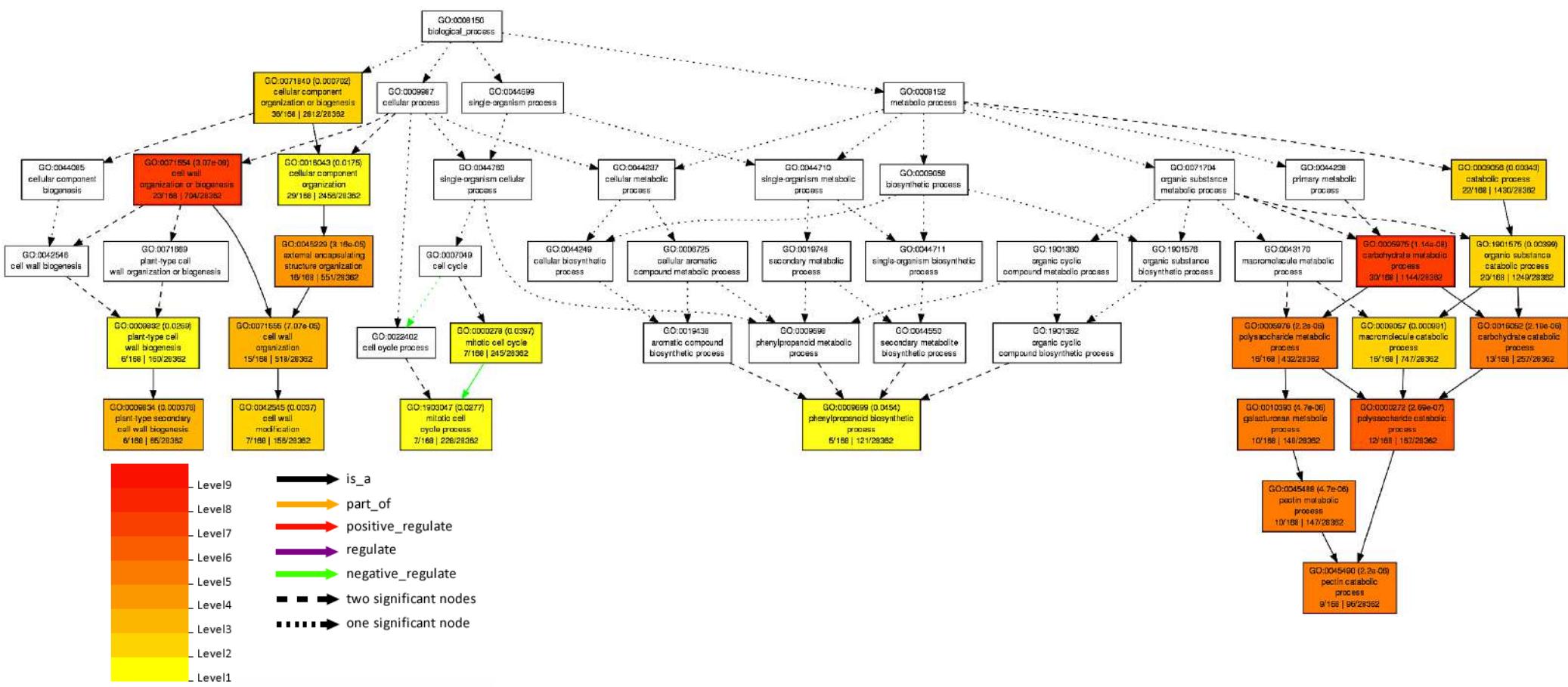


Figure 5.11 Gene Ontology (GO) analysis of DEGs found in F2 cold-treated regenerated LO. The 170 DEGs in the cold-treated LO were loaded into AgriGO v2.0 (Tian et al., 2017) and the biological process was analysed. The heat-color legend indicates the significance level.

To further understand how these differentially expressed genes affect the phenotype of the cold-treated LO plants, gene network analysis was carried out. The gene network analysis has clustered the differentially expressed genes into cellulose biosynthesis, cell wall biogenesis, lignin biogenesis, phenylpropanoid biogenesis, response to reactive oxygen and metabolic process. The network analysis of cold LO only found one DEG overlapping with the protein-protein interaction database in the GeneMania (Figure 5.12). The network for cold-stress responses in the LO only found *BEL1-LIKE HOMEO DOMAIN 3* (*BLH3*) which regulates the transition from vegetative to the reproductive state. Based on RNAseq data, the expression of *BLH3* was downregulated in the cold LO (log2fold -1.62).

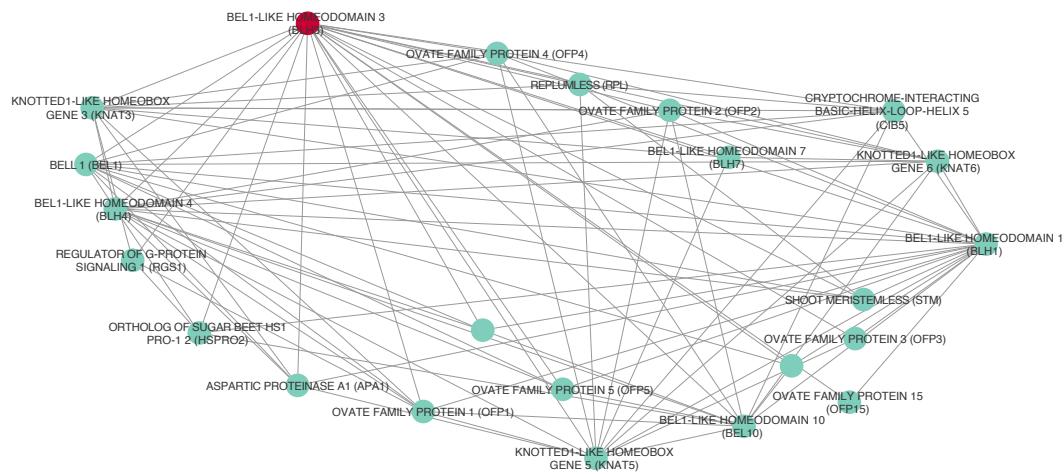


Figure 5.12 Gene network analysis of DEGs found in F2 cold-treated LO plants. The gene network within 170 DEGs in F2 cold-treated LO plants were visualized and analysed using GeneMania (Warde-Farley et al., 2010) in the Cytoscape. The network analysis indicated the protein-protein interaction from DEGs (red circles) intersected with the physical interaction database in GeneMania. Blue circles are gene that found directly interacted with DEGs.

The transcriptomic analysis was also performed in individual sample of cold LOF2 in line 1, 2 and 3. It was found that there were 134 common DEGs shared between cold LOF2 line 1, 2 and 3. These common DEGs were further used to generate GO analysis by using AgriGO. The GO analysis (Fig. 5.13) indicated the enrichment of genes involved in cell wall biosynthesis and carbohydrate metabolic processes. This finding was similar with the result when the lines were treated as biological replicate (Fig. 5.11)

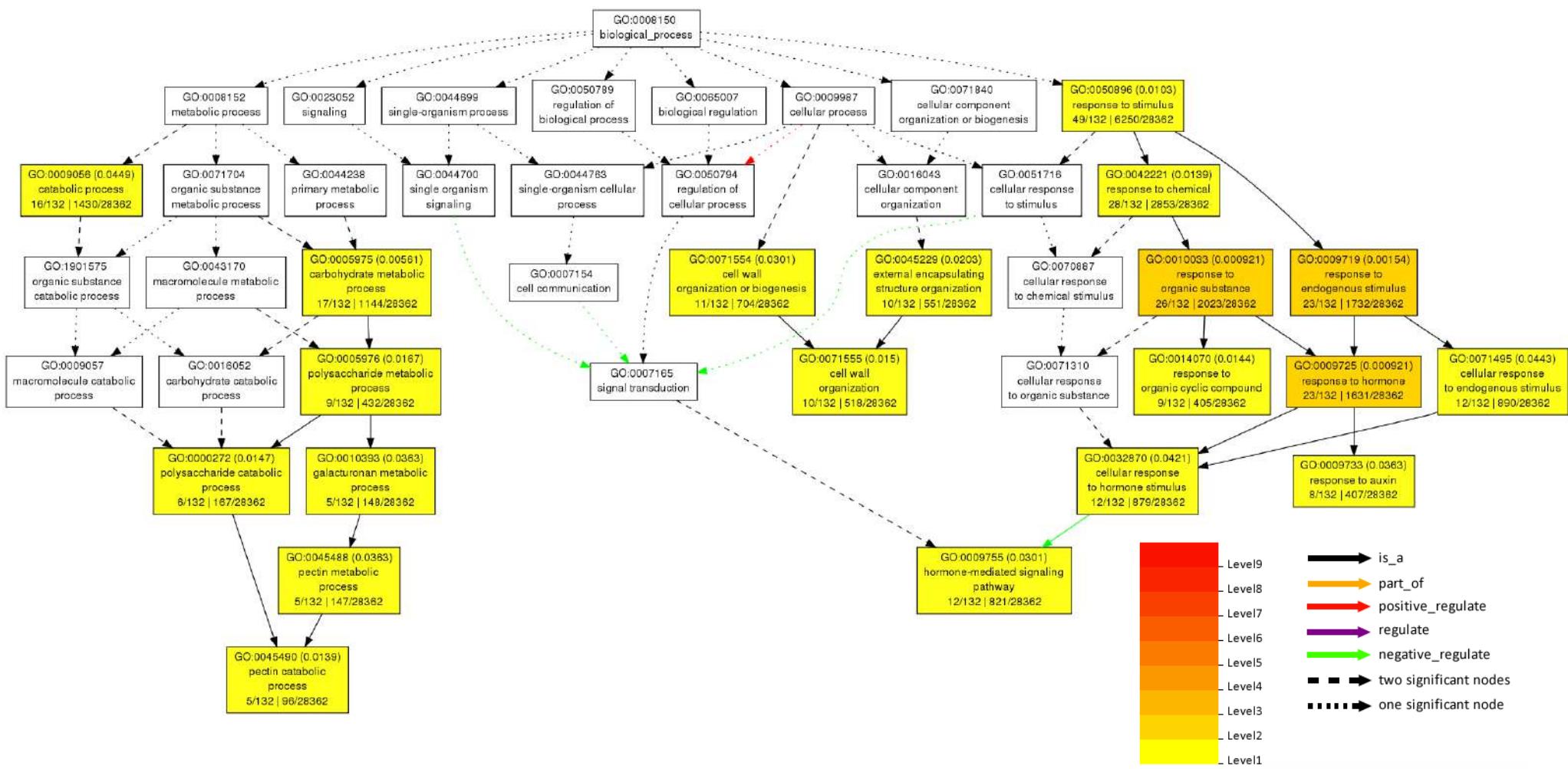


Figure 5.13 Gene Ontology (GO) analysis of common DEGs found in F2 of cold-treated regenerated LO line 1, 2 and 3. The 134 common DEGs found in the F2 cold-treated LO line 1, 2 and 3 were loaded into AgriGO v2.0 (Tian et al., 2017) and the biological process was analysed. The heat-color legend indicates the significance level.

5.3 Discussion

A study conducted by Kreps et al. (2002) has shown differential transcriptome changes in different tissues in response to salt, osmotic and cold stress in *Arabidopsis*. They found that the exposure to acute salinity stress gave differing responses to both root and leaf transcripts, where there were more changes in the root in comparison to the leaf. This finding is consistent with the current findings where the exposure of salinity stress during somatic embryogenesis has caused different gene responses in both root-regenerated (RO) plants and leaf-regenerated plants, with more in roots. It appears that RO plants can perceive the salt stress signals and affects the transcripts of salt-stress responses genes, where LO plants have less transcriptomic changes due to salt stress. Gene ontology enrichment analysis of salt-treated RO plants has found enrichment in ethylene responses and signalling pathways. Several published work have indicated the involvement of ethylene in regulating salt stress responses where upregulation of ethylene response genes reduces salt sensitivity in plants (Cao et al., 2007; Cao et al., 2008; He et al., 2005). The ethylene response genes are under direct control of MAPK signalling genes. Xu et al. (2008) also found that MAPK cascades, especially MKK9, play a crucial role in regulating the expression of the genes that are responsible for ethylene biosynthesis and ethylene responses. In addition, there are two DEGs (Table 5.2) belonging to the nitric oxide metabolic process that are highly upregulated by salt stress in the salt RO. These genes are CML23 and TCH2 which are involved in regulating reactive oxygen metabolic processes and ion homeostasis. The accumulation of nitric oxide has been shown to induced salt resistance by influencing ion homeostasis in *Arabidopsis* callus (Wang et al., 2009). Both CML23 and TCH2 are calmodulin-like proteins that are crucial in the Ca^{2+} sensor which is involved in modulating plant stress tolerance (Munir et al., 2016). The regulation of Ca^{2+} ion homeostasis is crucial to prevent ionic toxicity at the cellular level during salinity stress. It is proposed that CML23 and TCH2 affect the accumulation of NO by modulating non-enzymatic NO production (Galon et al., 2010). In addition, there are many pieces of evidence indicating that TCH2 plays an important role during high osmotic stress and ion stress by sensing the presence of Ca^{2+} ion (Delk et al., 2005; Jang et al., 1998).

A large proportion of the salt RO gene network was generated by *NHL3*, which is a member of *NDR1/HIN1-like* genes. *NHL3* links to the increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (Shapiro et al., 2001). It indicates that salt RO may also have improve tolerance to *Pst* DC3000. This finding is also similar to the finding by Kamei et al. (2005) where they found upregulation of *NLH3* in the *sos2* mutant treated with 250 mM NaCl. Ma et al. (2006) also found the presence of defence genes during salt stress responses. In addition, auxin responses were also detected in the salt RO gene network. It is known that salt stress significantly reduces root meristem size by downregulating the expression of PINFORMED (PIN) genes, thus reducing auxin levels (Liu et al., 2015). Salt stress also promoted stabilisation of AUXIN RESISTANT3 (AXR3)/INDOLE-3-ACETIC ACID17 (IAA17), which represses auxin signalling (Liu et al., 2015). However, SOS system genes that are crucial in salt tolerance are not found in the DEGs; perhaps this is due to the SOS pathway only functioning at the level of protein modification and not at the transcript level (Ma et al., 2006). Instead, there are nine genes (Table 5.2) that directly regulate salt stress responses. In addition, when the transcriptomic was analysed on single sample of salt ROF2, it is found that genes related to photosynthesis enriched in all salt ROF2 samples. Chaves et al., (2009) found that salt stress induces down regulation of large number of genes related to photosystem 2 (PSII) and photosystem 1 (PSI). Salt stress increases the accumulation of NaCl in the chloroplast of higher plants, and it inhibits PSII activity (Sudhir & Murthy, 2004). The decreasing of PSII activity during salt stress is proposed due to the dissociation of 23 kDa polypeptide extrinsically bound to PSII (Sudhir & Murthy, 2004).

Cold-treated regenerated plants show a different response in transcripts between leaf-origin (LO) and root-origin (RO) plants. There are 168 differentially expressed genes found in LO plants compared to only 36 in RO plants. Even though Kreps et al. (2002) found that there is no transcriptional different between leaf and root in responses to cold stress at 4°C exposure, their finding is not consistent with the current result. The differences in these findings were perhaps due to the stress introduced during early somatic embryogenesis where the cell fate was set back to naïve pluripotent state. It is known that the expression of the CBF gene family is upregulated in the leaf during early

exposure to cold stress, while the expression of CBFs in the root is delayed (Zhang et al., 2017). Most probably the ability to perceive the cold-stress signal during naïve state is important to establish cold-stress transcript responses. After the late phase of somatic embryogenesis when the cell fate was defined, the information received during naïve state is being fixed. This may cause the differences in response between LO and RO plants. The gene ontology enrichment analysis of cold-treated LO plants indicated enrichment in genes that are involved in cell wall biosynthesis and metabolic processes. The transcriptomic analysis of single sample from cold LOF2 also indicated genes enrichment related to cell wall biogenesis and metabolic processes in all samples. When the DEGs from the F2 cold-treated regenerated LO plants were loaded to the AtGenExpress (Kilian et al., 2007), ten genes were directly associated with cold stress responses (Table 5.4). Interestingly, the *GolS2* was found to be downregulated in the cold-treated LO plants. Galactinol synthase is an enzyme that plays a role as an osmoprotectant, and the overexpression of this gene confers cold resistance in *Arabidopsis* and rice (Nishizawa et al., 2008; Shimosaka & Ozawa, 2015). The result of the current experiment did not fit with the existing findings, the down-regulation of *GolS2* should make the cold-treated LO plants more chilling sensitive, however cold-treated LO plants were cold resistant (Figure 4.4). It is possible that other molecular responses compensate for the down-regulation of *GolS2*. Furthermore, Fowler & Thomashow (2002) found that long-term exposure to cold stress upregulated transcription factor homeobox-leucine zipper protein (ATHB12). However, I found that this transcription factor was downregulated in the F2 cold-treated LO plants. The expression of *ATHB12* in data generated from Fowler & Thomashow (2002) showed the fluctuation of *ATHB12* at a longer period of cold exposure. It may indicate that *ATHB12* may not be crucial for transgenerational adaptation to cold stress.

The GO enrichment analysis of upregulated genes in cold-treated LO plants indicated enrichment in carbohydrate metabolic process, secondary metabolites and cell wall biogenesis (data not shown). According to proteomic studies on cold exposure in plants, the proteins that belong to primary metabolism, cellulose biosynthesis and membrane proteins are upregulated in cold stress (Ghosh & Xu, 2014). The gene network analysis showed the involvement of *BEL1-LIKE HOMEODOMAIN 3 (BLH3)* in the cold LO gene

network. This gene is involved in regulating the timing of the transition from vegetative to reproductive phase, and it interacts with Ovate Family Proteins (OFPs) to regulate this transition phase (Zhang et al., 2016). Furthermore, the *BEL1-LIKE HOMEO DOMAIN* gene, *BLH6* showed interaction with *KNOTTED ARABIDOPSIS THALIANA 7 (KNAT7)* which contributes to the negative regulation of secondary cell wall biosynthesis in Arabidopsis (Liu et al., 2014). The cell wall plays a pivotal role in regulating cold acclimation in plants. By regulating cell wall thickness it can affect the cell volume to develop organs that are responsible for cold-stress tolerance, in addition, the thickening of the cell wall prevents the development of extracellular freezing during cold stress (Rajashekhar & Lafta2, 1996). Lignin biosynthesis (*LACCASE 17 (LAC17)*, *PINORESINOL REDUCTASE 1 (PRRI)*, *LACCASE 11 (LAC11)*, *CHITINASE-LIKE PROTEIN 2 (CTL2)*) appear to be enhanced in the cold-treated LO plants. It has been shown that lignin composition in the cell wall can be affected by the exposure to cold stress, and enhanced lignification is crucial during cold acclimation (Domon et al., 2013; Tenhaken, 2015).

The transcriptomic analysis of flg22-treated regenerated plants both from the root (RO) and leaf (LO) failed to display any significant differences in gene expression due to low counts differences between control-regenerated and flg22-regenerated plants. It may be due to the early response genes to the flg22 is transient, and the expression of these genes lost after 24-hours of induction (Denoux et al., 2008). Since the induction of somatic embryos takes seven days, perhaps flg22 responsive genes reset their expression rapidly and are not inherited. Thus, another approach to induce mild biotic stress for example using methyl jasmonate should be consider as another option.

The transcriptional changes initiated by salt and cold stress during somatic embryogenesis have been transmitted to the offspring even in the absence of stress. The gene ontology analysis and genes network analysis has shown on enrichment for genes involved in salt and cold stress. These findings indicate the ability of plants to acquire a transcriptional stress memory that is stably heritable to the next non-stress generation. The molecular mechanisms involved in the formation of transcriptional memory are thought to involve

epigenetic changes such as DNA methylation and histone methylation (Francis & Kingston, 2001). However, further investigation is needed to elucidate the involvement of epigenetic mechanisms in regulating these transcriptional changes.

When the transcriptomic analysis of single samples carried out for salt ROF2 and cold LOF2, the DEGs found in salt ROF2 was different compared to when the samples were treated as replicates. Although the DEGs found in cold LOF2 did not differ in two method of analysis (separated each sample or combined each sample as replicates). However, the transcriptomic outcome from this single sample comparison cannot be fully considered. Because without replication it will be difficult to estimate the genes that are differentially expressed in individual lines, and the analysis will only calculate fold change based on normalise read counts (Schurch et al., 2014).

5.4 Summary

The phenotypic changes observed after mild exposure to stress during somatic regeneration have been associated with the transcriptional changes of stress response genes. Tissue responsiveness transmitted the transcriptional memory of stress response genes during somatic embryogenesis, in which the transcriptional changes affected by salt stress have been inherited by root-origin (RO) plants, while the cold signals were propagated in leaf-origin (LO) plants. Since transcriptional memory has been inherited to the second generation of stress-free RO and LO plants, the ability of this transcriptional memory to be passed to the successive generations may involve epigenetic mechanisms such as DNA methylation and histone marks. However, further investigation is needed to elucidate the involvement of DNA methylation in these transcriptional changes.

6. DNA Methylation Changes are Stably Inherited in Regenerated Plants

6.1 Introduction

6.1.1 DNA Methylation is Affected by Environmental Stimuli

DNA methylation is a process of addition of methyl group to the C-5 of the cytosine ring of DNA (Jin et al., 2011). The function of the DNA methylation is to control gene expression and contribute to gene stability. To adapt to stress, plants need a versatile control of gene expression. The first evidence of methylation regulating stress responses in plants was discovered by Steward et al. (2002). They found that when maize is exposed to cold stress, demethylation in root tissue was observed. They identified a gene expressing a putative protein and part of a retrotransposon-like sequence, *ZmMII*, was demethylated. Interestingly, the expression of *ZmMII* was induced by cold (Steward et al., 2002). Thus, this indicates the importance of regulation of *ZmMII* during cold stress adaptation in maize. In tobacco plants, the expression of *Nicotiana tabacum GLYCEROPHOSPODIESTERASE-LIKE PROTEIN NtGDPL* was expressed during aluminum stress, and it also found that the genomic loci of *NtGDPL* was demethylated at CCGG sites within one hour of aluminum stress treatment (Choi & Sano, 2007a).

The effect of salt stress on the methylation state of the genome has been well studied. The exposure of *Triticum aestivum* to salt stress caused global DNA hypermethylation in CCGG sites of both two different cultivars used in the study (Zhong et al., 2002). The finding was similar to the methylation state found in a halophyte *Mesembryanthemum crystallinum* where under salt stress condition the level of CNG-methylation was doubled, while the hypermethylation was also found in satellite DNA and caused photosynthesis changes from C₃ to the crassulacean acid metabolism (CAM) by the formation of a specialised chromatin structure regulating the expression of a large number of genes in *M. crystallinum* related to photosynthesis and CAM pathways (Dyachenko et al., 2006). While in *Arabidopsis thaliana* the salt stress-induced methylation changes largely in gene body compared to the promoter (Bilichak et al., 2012). However, Wibowo et al. (2016) found that the methylation changes due to salt stress in *Arabidopsis thaliana* were mainly at TEs and intergenic regions. Although Bilichak et al. (2012) and Wibowo et al. (2016) applied the same salt stress treatment, the differences in their finding maybe due to the

different method they used to analyse the methylation data, where Bilichak et al. (2012) used Methyl-DNA Immunoprecipitation method while Wibowo et al. (2016) use whole-genome bisulphite sequencing coupled with statistically robust Hidden Markov Model algorithm. Their findings indicate that salt stress induces global hypermethylation changes in both genic and intergenic regions of the genome.

While salt stress induces global DNA hypermethylation, the response and adaptation to cold stress have induced global DNA demethylation. To understand the effect of cold stress to the methylation state of the genome, Shan et al. (2013) analysed methylation-sensitive amplified polymorphisms on maize; they found that cold accounted for 32.6% to 34.8% of DNA methylation polymorphisms where demethylation mainly contributed to the overall DNA methylation changes. The DNA demethylation in response to cold stress in maize was found to affect *Ac/Ds* transposon regions (Steward et al., 2000) and four genes (*MALATE DEHYDROGENAS 1 (MDH1)*, *POTASSIUM CHANNEL KAT1-LIKE PROTEIN (KAT1)*, *SERINE HYDROXYMETHYLTRANSFERASE 4 (SHM4)*, *4-COUMARATE-COA LIGASE 2 (4CL2)*) in *Brassica rapa* (Liu et al., 2017). In addition, in *Populus simonii*, cold stress contributed to the 1376 stress-specific differentially methylation regions (SDMRs) (Banerjee et al., 2017; Song et al., 2016). Among the SDMRs, 162 SDMRs contain non-coding RNAs such as microRNA and long-non-coding RNAs (Song et al., 2016). In nature, a prolonged exposure to cold during winter also regulated the expression of some genes, with one of these genes being Flowering Locus C (*FLC*). The exposure to prolonged Winter caused the repression of *FLC* by the accumulation of histone marks H3K27me3 on the promoter region of *FLC*, the accumulation of this histone marks silenced the *FLC* during winter, and the *FLC* re-expressed in summer by changing the histone marks from H3K27me3 to H3K4me2 (Zhu et al., 2015).

Plants show dynamic DNA methylation changes as a response in plant defense mechanisms. The ability of plants to inherit defense responses have been well studied (Sahu et al., 2013). The role of DNA methylation in plants exposed to pathogen attack was investigated by Dowen et al. (2012) where the loss function of DNA methylation in

met1-3 and *ddc* made the *Arabidopsis* be more resistant to *Pst* DC3000. It is found that the pathogen infection caused reduced DNA methylation in the gene body in *Pst* DC3000 treated *Arabidopsis* compared to the untreated *Arabidopsis*. The DNA demethylation appeared to restrict multiplication and vascular propagation of *Pseudomonas syringae* in leaves (Yu et al., 2013). In addition, the DmCs were enriched only in the gene-rich region in *Arabidopsis* (less than 5 Kb to the closest transcription start site) with the hypo-DmC was found more in the CG methylation while the level of hypo- and hyper-DmC in the non-symmetric methylation CHG and CHH were the same (Dowen et al., 2012). During antibacterial defense, not only the region of the gene body was demethylated, but it also affects TEs reactivation (Yu et al., 2013). The activation of TEs causes the down-regulation of key transcriptional gene silencing factors. Furthermore, the expression of immune responsive genes also affects the level of methylation at the specific methyl cytosines. This phenomenon was reported by Wang et al. (2013) the loss of function of *elp2* (a gene that forms an interaction with *NPR1*, a key transcription coactivator of plant immunity) decreased the average methylation levels of methylcytosines and alters methylation levels of specific methyl cytosines.

6.1.2 Transgenerational Inheritance of Stress-Induced DNA Methylation Changes

DNA methylation has served as a method for plants adaptation to environmental stresses (Bruce et al., 2007). Unlike mammals, plants can inherit a considerable proportion of DNA methylation marks from parents to offspring (Verhoeven et al., 2010). An extensive transgenerational epigenetic study in *Arabidopsis* indicated that 99.998% of methylated regions in the genome were stably inherited across successive generations which it indicated that spontaneous epialleles were uncommon (Hofmeister et al., 2017). The heritable changes of DNA methylation associated with stress have been recorded in plants. Nitrogen deficiency in rice altered locus-specific methylation in leaf-tissue in the stressed plants (S0), and these changes were stably inherited to the non-stress self-fed progeny first, second and third generation (Kou et al., 2011). Meanwhile, the same result was observed by Boyko et al. (2010) when *Arabidopsis thaliana* was exposed to salt stress; the global DNA methylation changes were transmitted to the non-stressed progenies.

These DNA methylation changes are found in promoter regions, gene-coding regions, transgenes and transposable elements (TEs) (Wibowo et al., 2016).

The mechanisms by which the DNA methylation changes in responses to the environmental cues inherited to the successive non-stress generation are thought to involve small RNAs in RNA directed DNA methylation mechanisms (Henderson & Jacobsen, 2007), and several studies have documented the RdDM as a key in epigenetic in transgenerational inheritance of stress responses (Alex et al., 2010; Ito et al., 2011). RdDM is dependent on the presence of small RNAs especially the 21-22 nt small interference RNAs (siRNAs) (Matzke & Mosher, 2014). The pathway begins when the single-stranded RNA (ssRNA) is produced by Pol IV and copied by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) to produce double-stranded RNA (dsRNA) (Fig. 6.1). The dsRNA is further processed by DICER-LIKE 3 (DCL3) into 24 nt siRNAs, methyl groups are added into these siRNAs and incorporated into ARGONAUTE 4 (AGO4). The AGO-siRNAs complexes bind to Pol V and activate *de novo* DNA methylation in all sequence context (CG, CHG and CHH) through the activity of DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Matzke & Mosher, 2014). The other pathway in RdDM involves RNA DEPENDENT RNA POLYMERASE 6 (RDR6). The Pol II transcribed a new synthesise RNA from a transposon, this RNA then processed by RDR6 to produce double-stranded RNA which will be cut by DCL2 and DCL4 into 21-22 nt siRNAs. These siRNAs are loaded into AGO1 to initiate post-transcription gene silencing (PTGS) or into AGO2 to form complexes with NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD) and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to start the process of *de novo* methylation (Matzke & Mosher, 2014). The involvement of RdDM pathways during heat stress tolerance in Arabidopsis was extensively investigated by Popova et al. (2013). Arabidopsis deficient in NRPD2 (the second largest subunit of Pol IV and Pol V) and Rpd3-type histone deacetylase HDA6 were hypersensitive to heat stress, but the single knockouts of either the *nrdp1* (Pol IV) or *nrpe1* (Pol V) were not. This indicated that the heat tolerance in Arabidopsis is partially dependent on the RdDM pathways (Popova et al., 2013). In addition, heat stress also activates the *Onsen* retrotransposition which is

under the direct control of RdDM pathways and developmental control (transgenerational transposition) (Ito et al., 2011; Mirouze & Paszkowski, 2011). The RdDM pathways have also been shown to play a pivotal role in *P. syringae* resistance (Agorio & Vera, 2007). The loss function of AGO4 in the *ago4-2* mutant in *Arabidopsis* exhibited improved disease tolerance to the virulent bacterium *P. syringae* pv *tomato* DC3000 and also to avirulent *P.s.t.* DC3000 carrying the *avrRpm1* gene, but the loss of other RdDM components that operating upstream of AGO4 such as RDR2 and DCL3, and downstream such as DRD1, CMT3, DRM1 and DRM2 did not compromise resistance to *P.s.t.* DC3000 (Agorio & Vera, 2007). The findings indicated that AGO4 works independently of other components of the RdDM pathway in mediating resistance to *P.s.t.* DC3000 (Agorio & Vera, 2007).

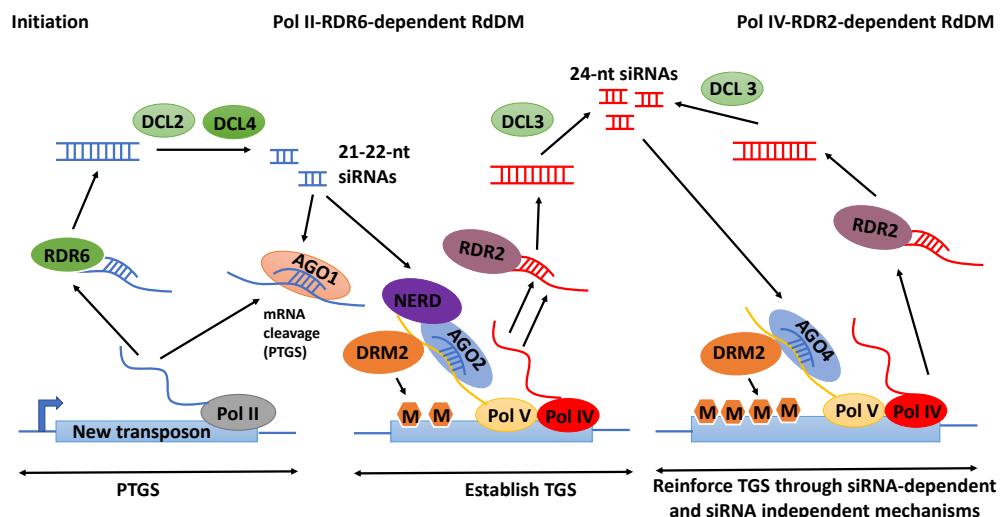


Figure 6.1 Non-canonical RdDM pathway. The newly synthesised RNAs by RNA polymerase II (Pol II) are copied to produced double stranded RNAs (dsRNAs) by RNA DEPENDENT RNA POLYMERASE 6 (RDR6). These dsRNAs are further processed by DICER-LIKE 2 (DCL2) and DCL4 to produce 21-22-nt siRNAs. These siRNAs are loaded into ARGONAUTE 1 (AGO1) and guide cleavage of transposons transcript in a typical PTGS pathway. Some of the 21-22 nt siRNAs can introduce low level DNA methylation by the helping of DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), Pol V, AGO 2 and NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD). The sporadic methylated DNA recruits Pol IV that transcribes single-stranded RNAs (ssRNAs). The ssRNAs are copied by RDR2 and cleavage by DCL3 to produce 24-nt-siRNAs. These siRNAs recruits DRM2 to densely methylate the DNA. PTGS (Post Transcriptional Gene Silencing); TGS (Transcriptional Gene Silencing) (modified from: Matzke & Mosher, 2014).

Epigenetic reprogramming is crucial during developmental stages and plays role in imprinting, controlling totipotency, pluripotency and TEs activity (Feng et al., 2010). Thus, epigenetic reprogramming is important to ensure correct development during embryogenesis and gametogenesis. Interestingly, Verhoeven et al. (2010) found that when genetically identical apomictic dandelions exposed to various environmental stresses, the epigenetics alteration caused by stress was propagated to the apomictic offspring in a common unstressed environment. Furthermore, Wibowo et al. (2016) showed that the epigenetic changes due to salt stress were reset to the original non-stress epigenetic state when the plants were sexually propagated in the absence of stress. It indicates that the epigenetic changes triggered due the environmental stimuli are reprogrammed during sexual propagation in a non-stress situation. During the plant developmental stages, active DNA reprogramming occurs during male gametogenesis (Gutierrez-Marcos & Dickinson, 2012), and during embryogenesis, the CG and CHH methylation is reprogrammed and reinitiated after embryogenesis (Kawashima & Berger, 2014).

6.1.3 Chapter Aims

This chapter aims to assess the transgenerational inheritance of DNA methylation from the regenerated plants exposed to different stresses during the process of somatic regeneration by using whole-genome bisulphite sequencing technique, and the differential methylated regions are analysed with Hidden Markov Model algorithm. The experiment is conducted to explain whether the changes in DNA methylation contributes to the transcriptomic changes found in salt ROF2 line 1, 2 and 3, and cold LOF2 line 1, 2 and 3 in the previous chapter.

6.2 Results

6.2.1 DNA CG Hypermethylation is A Common Feature of Stress-Exposed Regenerated Plants from Leave Tissue

Wibowo et al. (2016) found that methylation induced by salt stress is loss subsequently in the progenies not exposed to the salt stress. The finding contradicted previous studies that showed that methylation marks caused by stress are inherited to the next unstressed generation. However, the ability of plants to inherit the epigenetic marks upon stress exposure during somatic embryogenesis has never been studied.

In the previous chapter, this work showed that the progenies of RO (root) and LO (leaf) regenerated plants shows the ability to inherit the salt and cold transcriptomic memory to the next generation when the stress was exposed during somatic regeneration. To understand whether the transcriptomic changes is related to the changes in DNA methylation, whole-genome bisulfite sequencing analysis of control (non-stress) RO and LO, cold (4°C) RO and LO line 1, 2 and 3, and salt (75mM NaCl) RO and LO line 1, 2 and 3 in the second generation (F2) was performed. For each sample, three independent lines were sequenced where for each line leaves of 10 individual plants were pooled. By using a modified Hidden Markov Model (HMM) (Hagmann et al., 2015) with beta binomial distribution the methylated regions (MRs) were called in different methylation context (CG, CHG and CHH). The P values were generated by testing the scores against an empirical distribution of scores obtained by random permutation of all cytosines throughout the genome. After FDR calculation, consecutive stretches in high state with an FDR <0.05 are defined as methylated regions (MRs). Then selected MRs being in different or highly methylated states between strains and statistically tested them for differential methylation (including FDR calculation). Regions that showed statistically significant methylation differences between at least two sets of samples were identified as DMRs.

When *Arabidopsis* is exposed to salt stress, the results indicated that the stress induced hypermethylation in the genome (Bilichak et al., 2012) while the cold stress caused the

equal distribution of hypermethylation and hypomethylation within the genome (Liu et al., 2017). In the current experiment, when salt stress was induced during the somatic regeneration, in the F2 (Fig. 6.2), the differentially methylated regions (DMRs) in LO plants were CG methylation, while there was a small proportion of the DMRs was CG demethylated. A few DMRs were hypermethylated in CHG context while CHH methylation was mainly unchanged. In RO plants, the DMRs were almost equal between hypermethylation and hypomethylation region in CH, CHG and CHH methylation. In the cold treated RO and LO plants (Fig. 6.3), DMRs had similar CG methylation pattern to salt treated plants. However, there was more CHG hypermethylation DMRs in the cold LO.

The next analysis conducted was the identification of differentially methylated regions (DMRs). To identify the DMRs between samples, the three independent lines were considered as replicates. The DMRs then compared to the stress RO (either salt or cold) to the control RO and stressed LO (either salt or cold) to the control LO. After the analysis, 118 DMRs in salt RO, while salt LO contains 398 DMRs was found. Where cold RO has 185 DMRs, and cold LO has 560 DMRs. In the second generation of cold and salt regenerated RO and LO, the DMRs was accumulated mostly within the genic and intergenic regions (Fig. 6.4).

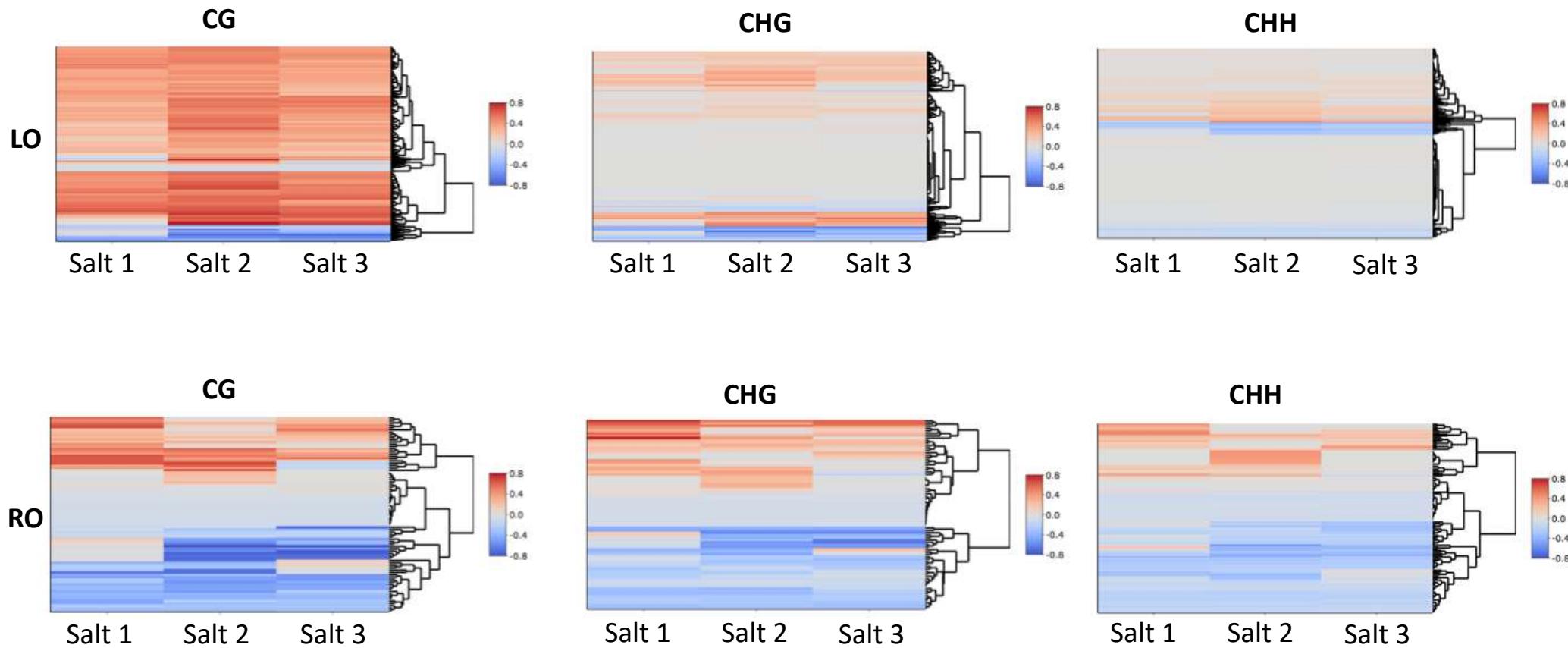


Figure 6.2 Relative methylation changes in DMRs in CG, CHG and CHH context in salt ROF2 and LOF2. One-directional clustering of DMRs in CG, CHG and CHH context in the second generation of salt RO (root) and LO (leaf) regenerated plants compared to the control regenerated RO and LO. The second generation of salt regenerated RO and LO. Red indicates hypermethylation; blue indicates hypomethylation; grey indicates unchanged. The scale bar indicates the methylation rate.

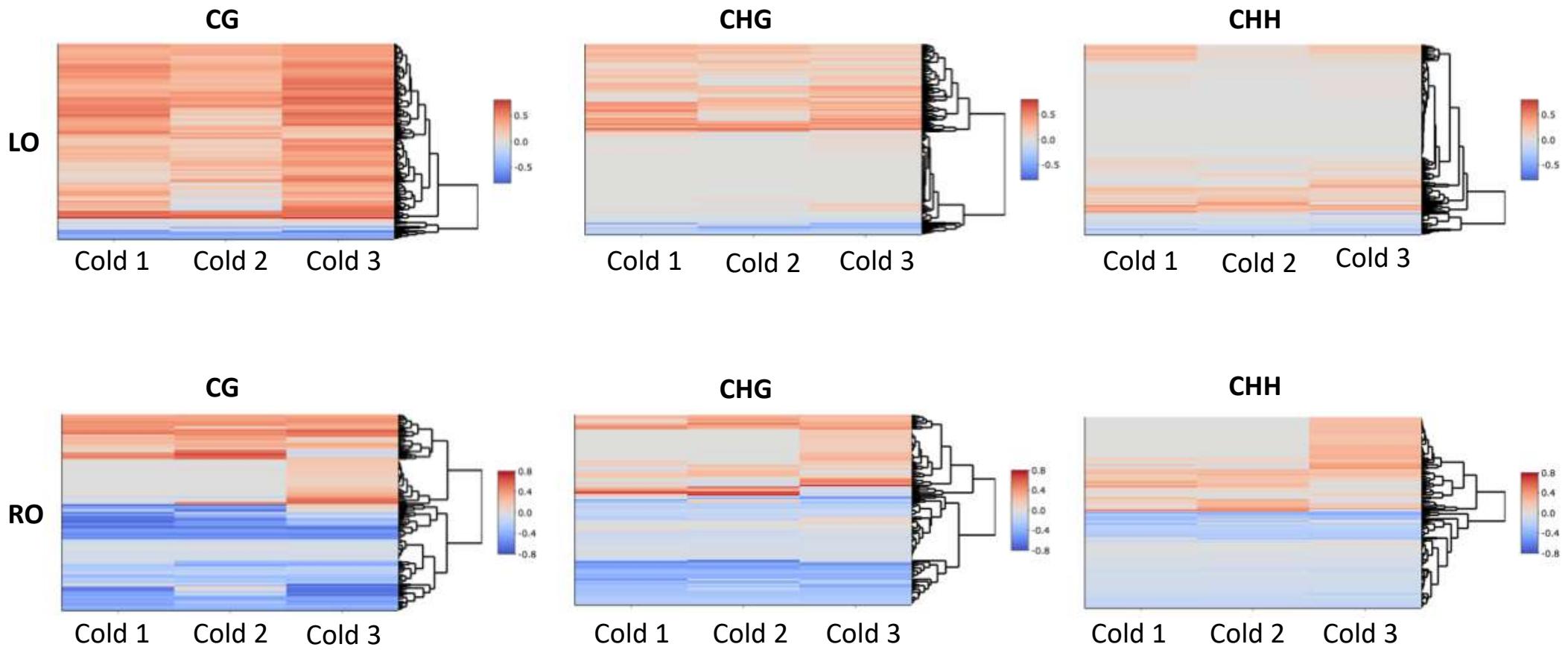


Figure 6.3 Relative methylation changes in DMRs in CG, CHG and CHH context in cold ROF2 and LOF2. One-directional clustering of DMRs in CG, CHG and CHH context in the second generation of cold RO (root) and LO (leaf) regenerated plants compared to the control regenerated RO and LO. The second generation of cold regenerated RO and LO. Red indicates hypermethylation; blue indicates hypomethylation; grey indicates unchanged. The scale bar indicates the methylation rate.

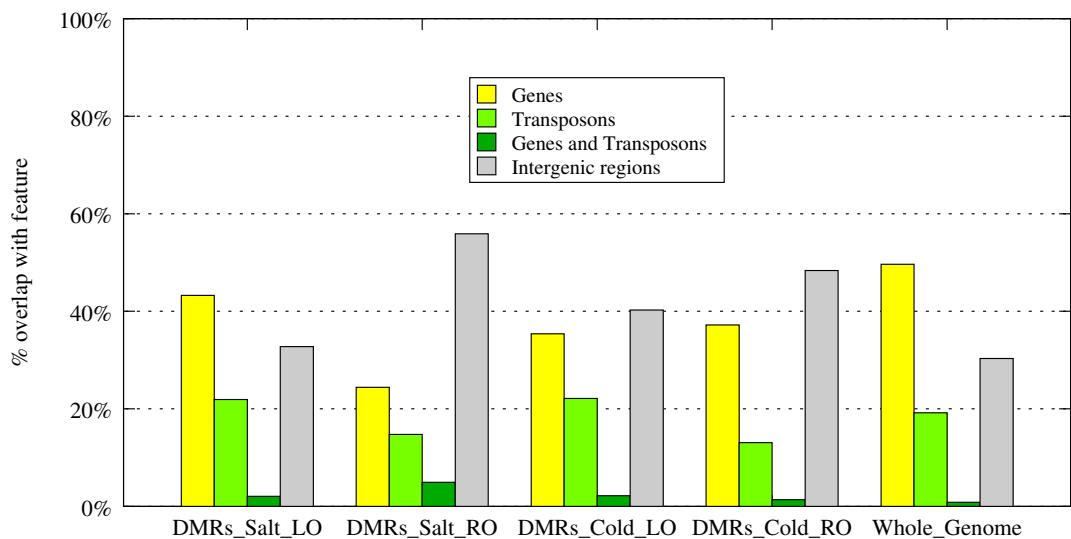


Figure 6.4 DMR distribution annotated to the different genomic regions. All DMRs found in the second generation of salt regenerated RO (root) and LO (leaf) plants, and cold regenerated RO and LO were annotated to the intergenic (grey), genes and transposon (dark green), transposons (light green), and genes (yellow) regions of the genome.

6.2.2 Differentially Methylated Genes are Over-Represented in the Leaf Regenerated Plants

DNA methylation is believed to play a role in repressing gene expression, probably by inhibiting the promoter which activates transcription factors that they bind to, but the exact role of DNA methylation in gene expression is unknown (Phillips, 2008). While Wibowo et al. (2016) showed that the methylation of the transposable element affected the expression of nearby genes. To understand the connection between the transcriptome and phenotypic changes generated by the exposure of salt and cold stress during somatic regeneration, a correlation between DMRs and genes within the *Arabidopsis* genome was established. The DMRs found in the second generation of cold and salt regenerated RO

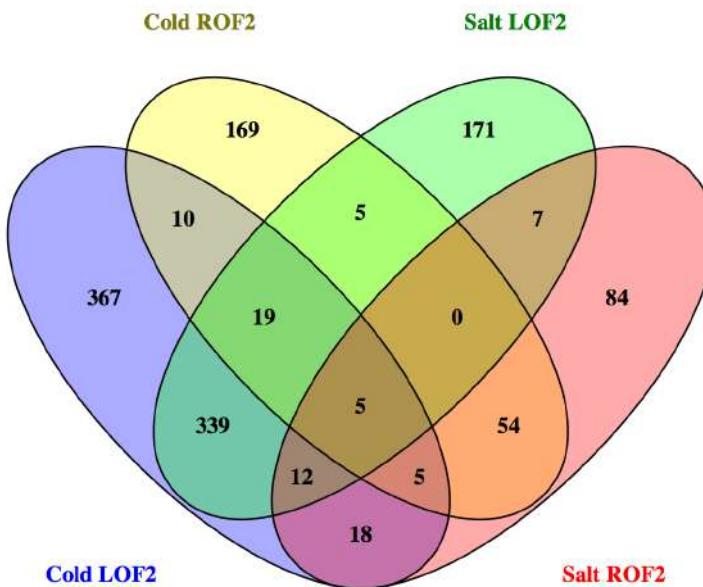


Figure 6.5 Putative Differentially Methylated Genes (DMGs) found in the second generation of stress-regenerated plants. DMGs were the genes found 2-kb upstream and downstream of the DMRs. The DMGs found in each sample were loaded into Venny, and Venn diagram was generated to show the intersection of DMGs between samples. (root) and LO (leaf) regenerated plants were intersected to the *Arabidopsis* genome (TAIR10) to find the intersection between DMRs with the genes found 2-kb upstream and downstream of the DMRs. There is no strict rule on choosing the distance between DMRs

Table 6.1 Putative DMGs found in salt ROF2, salt LOF2, cold ROF2 and cold LOF2.

Sample Name	Number of DMGs
Salt ROF2	185
Salt LOF2	558
Cold ROF2	267
Cold LOF2	775

and genes, but the 2 kb window was used based on a study conducted by Wibowo et al. (2016) where the 2 kb window was applied to identify genes that regulated by DMRs. The genes are found within 2-kb upstream and downstream of the DMRs are termed as putatively differentially methylated genes (DMGs). The data showed (Table 6.1) 775 DMGs in the cold regenerated LO plants and 267 DMGs in cold regenerated RO plants, where 558 DMGs were found in the salt regenerated LO and 185 DMGs in salt regenerated RO. Interestingly, 54 DMGs were shared between salt regenerated LO with cold regenerated LO plants, while 64 DMGs were shared between salt regenerated RO with cold regenerated RO (Fig. 6.5). To assess the biological function of the DMGs found between salt and cold regenerated RO and LO plants, gene ontology (GO) analysis was conducted by using AgriGO v2.0 (Tian et al., 2017). However, there was no significant enrichment of GO terms in all DMGs found.

There were also five DMGs that are common in all cold and salt regenerated RO and LO plants (Table 6.2). The three genes are AMINO ACID PERMEASE 1 that plays pivotal role in amino acid transport into the embryo for storage protein accumulation and seed yield (Sanders et al., 2009), EXORIBONUCLEASE 2 involved in degrading the 3' product of miRNA-mediated mRNA cleavage and that acts as endogenous post-transcriptional gene silencing suppressor (Zakrzewska-Placzek et al., 2010), and PHOTOSYNTHETIC NDH SUBCOMPLEX B 5 involved in transport of electron from photoproduced stromal reductants and ferredoxin to the intersystem plastoquinone pool (Ifuku et al., 2011). The transcript of At5g42530 increased in response to 2,4,6-trinitrotoluene (TNT) in *Arabidopsis* (Mentewab et al., 2005). In addition, based on

Klepivoka Atlas (Klepikova et al., 2016), At5g42530 highly expresses in the mature leaf of Arabidopsis, whereas At5g43755 highly expresses in stigma.

Table 6.2 Putative DMGs found in salt-cold regenerated ROF2 and LOF2.

Gene ID	Gene Description	Gene Symbol
At1g58360	Encodes AAP1, a neutral amino acid transporter expressed in seeds. Functions in amino acid uptake into embryos. The transporter also functions in the acquisition of glutamate and neutral amino acids by the root.	AMINO ACID PERMEASE 1 (AAP1)
At5g42530	hypothetical protein	
At5g42540	Encodes a protein with similarity to yeast 5'-3' exonucleases and can functionally complement the yeast mutations. In Arabidopsis, XRN2 acts as a suppressor of posttranscriptional gene silencing.	EXORIBONUCLEASE 2 (XRN2)
At5g43750	NAD(P)H dehydrogenase 18	PHOTOSYNTHETIC NDH SUBCOMPLEX B 5 (PnsB5)
At5g43755	non-LTR retroelement reverse transcriptase-like protein	

The DMGs were also analysed in the single samples of salt LOF2, salt ROF2, cold LOF2 and ROF2 (Table 6.3). The results of single samples analysis indicated that DMGs were double in the LO compared to the RO for both salt and cold regenerated plants. This findings were similar when the samples treated as replicates (Table 6.1). In addition, Venn diagram was used to find the intersection of between DMGs in all sample (Fig. 6.6). The result showed that there were 146 common DMGs in cold LOF2, 42 common DMGs in cold ROF2, 146 common DMGs in salt LOF2 and 52 common DMGs in salt ROF2. It indicates that DMGs are mostly unique to the each sample.

Table 6.3 Single samples analysis of putative DMGs.

Sample Name	Number of DMGs
Salt ROF2 1	498
Salt ROF2 2	647
Salt ROF2 3	588
Salt LOF2 1	527
Salt LOF2 2	742
Salt LOF2 3	807
Cold ROF2 1	451
Cold ROF2 2	409
Cold ROF2 3	719
Cold LOF2 1	826
Cold LOF2 2	583
Cold LOF2 3	914

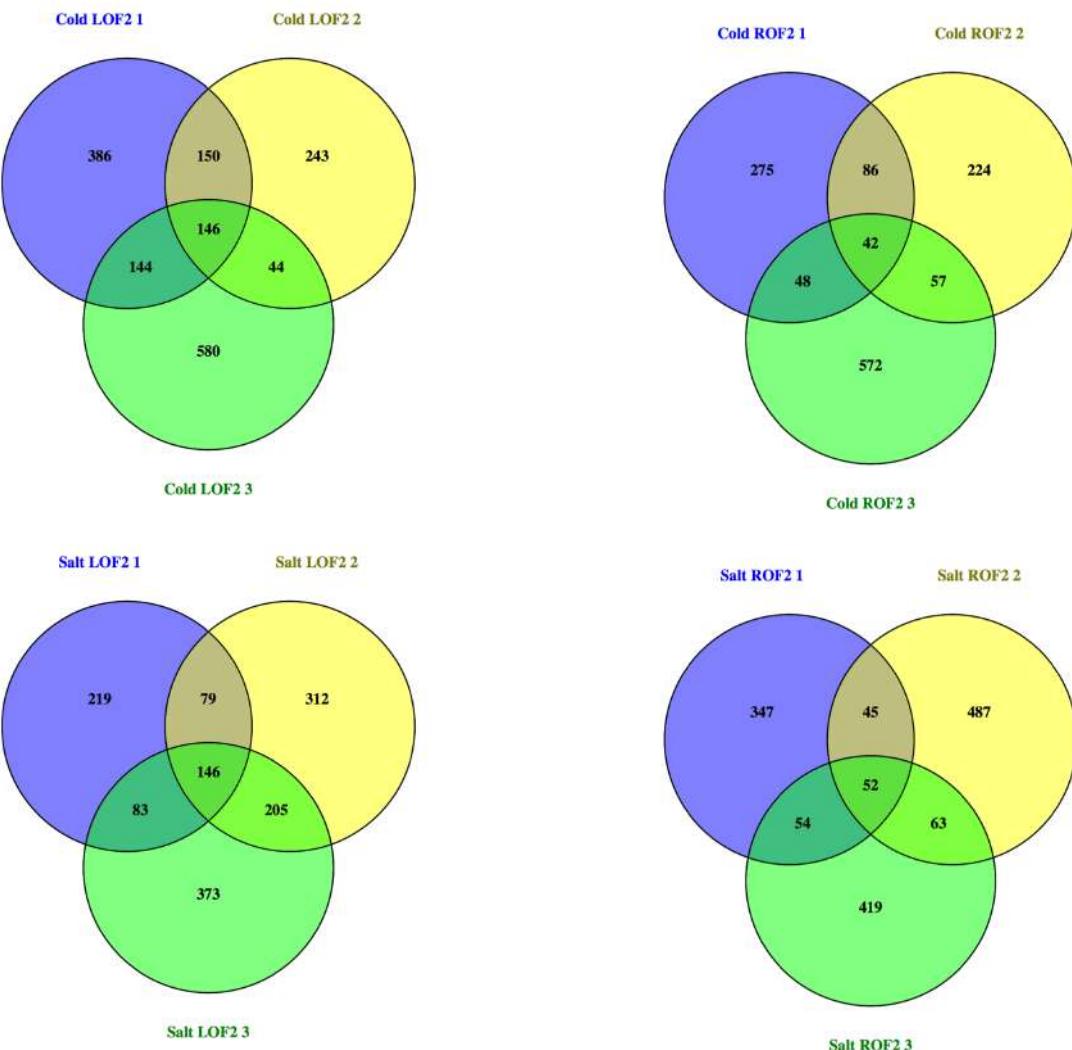


Figure 6.6 Putative Differentially Methylated Genes in the single sample of second generation of stress-regenerated plants. Single sample analysis of DMGs were performed in all sample of regenerated plants by finding genes located 2 kb upstream and downstream of the DMRs. Venny was used to generate Venn diagram to find the intersection of DMGs between samples.

6.2.3 Tissue of Origin Underpins Transcriptional and Epigenetic Changes After Regeneration

To understand the effect of methylation on the DMGs, on expression analysis of the DMGs was conducted by utilising the transcriptome information of the second generation salt-cold regenerated RO (root) and Leaf (LO) plants (Chapter 5). The differential expression of DMGs was compared between the control regenerated RO and LO with the stress (cold and salt) regenerated RO and LO. The genome is hypermethylated in both salt LO and cold LO (Fig. 6.2 and Fig 6.3), the expression of DMGs found in both salt LO and cold LO were equally divided between upregulated and downregulated DMGs (Fig 6.7 A and C). The same observation was found in the salt RO and cold RO (Fig. 6.7 B and D) where the expression of DMGs was equally distributed between upregulated and downregulated genes. This support the view that methylation works in two directions either promoting expression and/or repressing the expression of the genes. Intriguingly, the DMGs were also grouped based on their tissue origin not by stress treatment.

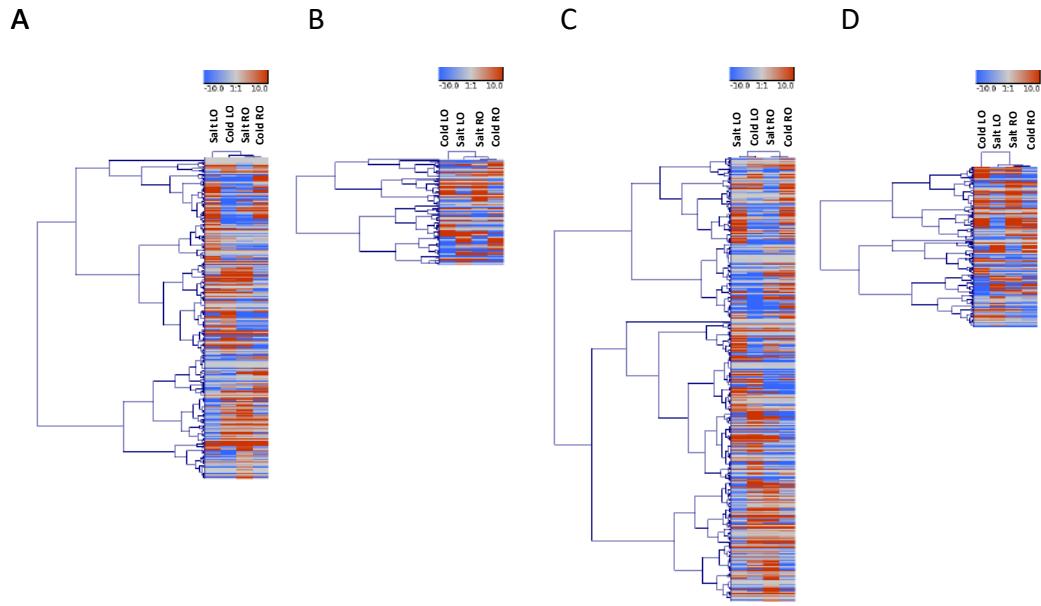


Figure 6.7 The expression of differentially methylated genes in the second generation of stress-regenerated plants. The relative expression of DMGs was done by comparing the expression of DMGs in the stress and the expression in the control experiment by using the counts generated from Chapter 5. Heatmaps were plotted to visualise the expression of DMGs found in (A) Salt LOF2, (B) Salt ROF2, (C) Cold ROF2, (D) Cold LOF2. The expression of DMGs in each sample were checked again the other sample (e.g. the expression of DMGs found in salt ROF2 is also checked in salt LOF2, cold ROF2 and cold LOF2). Red indicates upregulated compared to the control experiment and blue indicates downregulated compared to the control.

To assess if the DEGs may be affected by methylation, we looked for overlaps between DEGs and DMGs. In this analysis the DEGs were generated by using less stringent FDR (0.05) in DeSeq2 (Chapter 5 DEGs used FDR 0.01). Then the DMGs were intersected with DEGs by using Venny (Oliveros, 2015.). The intersection showed that there was limited connection between DMRs and DMGs, where salt ROF2 DEGs and salt ROF2 DMGs had four genes (Table 6.4) intersected whereas salt ROF2 DEGs and salt LOF2

DMGSS shared ten genes in common (Fig. 6.8 A). Meanwhile, cold LO DMGs and cold LO DEGs shared 15 genes (Table 6.5) in common, and only three genes were in common between cold RO DEGs and cold RO DMGs (Fig. 6.8 B). This indicated that there was a connection between methylome and transcriptome in the cold-treated regenerated plants, but there was no clear connection between methylation in the salt-treated regenerated plants.

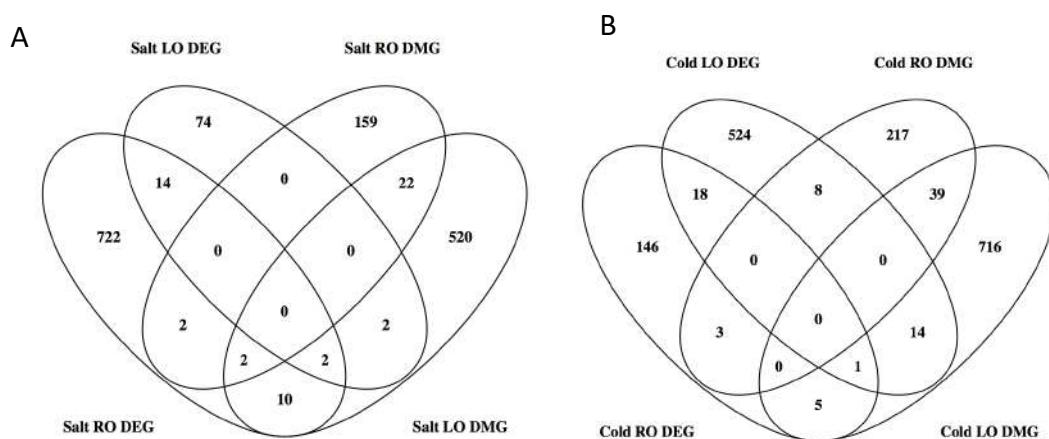


Figure 6.8 Intersection between differentially expressed genes and differentially methylated genes. The intersection of DEGs (FDR 0.05) and DMGs between (A) salt ROF2 and LOF2; and (B) cold ROF2 and LOF2 were generated by using Venny (Oliveros, 2015).

Table 6.4 Genes found intersected between salt ROF2 DMGs and salt ROF2 DEGs.

Gene Model Name	Gene Symbol
At5g42530	
At5g05300	INFLORESCENCE DEFICIENT IN ABSCISSION LIKE 6 (IDL6)
At3g45640	MITOGEN ACTIVATED PROTEIN KINASE 3 (MPK3)
At5g43750	PHOTOSYNTHETIC NDH SUBCOMPLEX B5 (PnsB5)

Table 6.5 Genes found intersected between cold LOF2 DMGs and cold LOF2 DEGs.

Gene Model Name	Gene Function
At3g14040	Pectin lyase-like superfamily protein
At3g43270	Plant invertase inhibitor superfamily
At5g62580	ARM repeat superfamily protein
At3g62530	ARM repeat superfamily protein
At3g48410	Alpha/beta-Hydrolases superfamily
At2g41640	Glycosyltransferase family 61 protein
At5g40020	Pathogenesis-related thaumatin superfamily protein
At5g35370	S-locus lectin protein kinase family protein
At1g69790	Protein kinase superfamily protein
At1g11785	Transmembrane protein
At1g58370	XYLANASE 1 (XYN1)
At1g64190	6-PHOSPHOGLUCONATE DEHYDROGENASE 1 (PGD1)
At1g80310	MOLYBDATE TRANSPORTER 2 (MOT2)
At3g16180	NITRATE TRANSPORTER 1.12 (NRT1.12)
At3g43210	TETRASPORE (TES)

In addition, some genes implicated in stress responses had associated DMRs. In this instance, it was observed that there was a DMR flanking upstream of the *TCF1* gene (Fig. 6.9) in the cold LO plant in which this gene is responsible for freezing tolerance in Arabidopsis. This DMR may serve as a distant regulatory element that controls the expression of *TCF1*. Moreover, the expression of *MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3)* gene was assessed in the control ROF2, salt ROF2 and WT (Fig. 6.10). The *MPK3* was gene within DMG and DEG list (each sample as replication analysis). In the wild type, there is methylation found 1 kb upstream of *MPK3* based on single-base resolution epigenome maps (Lister et al., 2008). The qPCR analysis of *MPK3* indicated that the expression of this gene was two-fold higher in the salt ROF2 line 1 and 2 compared to the WT. The expression of *MPK3* was three-fold higher in the control ROF2 compared to WT.

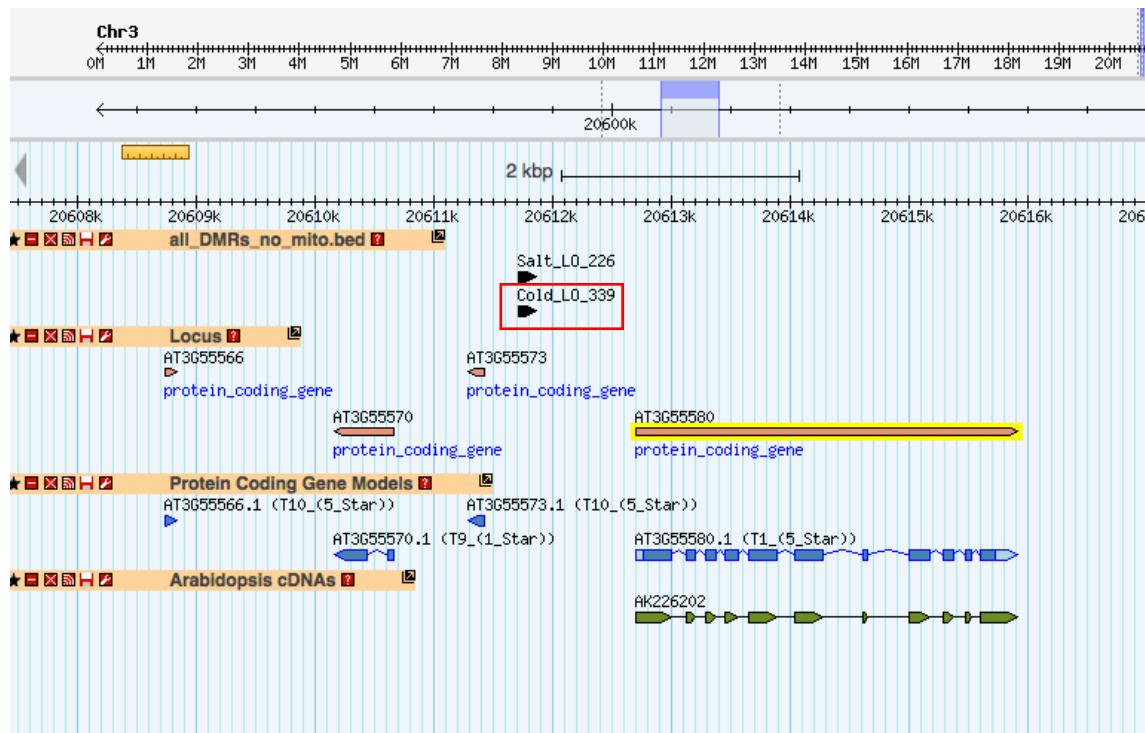


Figure 6.9 Genome browser view of the genomic region flanking *TCF1*. The DMR (black arrow in red-box) is found upstream of *TCF1* (AT3G55580) (blue) in the cold LO plant. The DMR has a potential to affect the expression of *TCF1* as a long distance regulator.

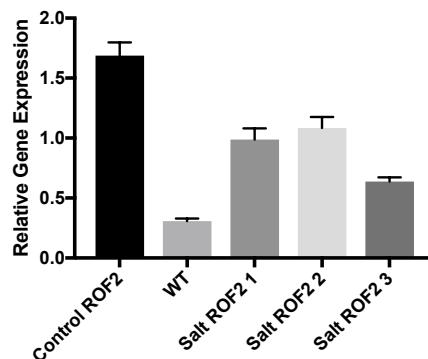


Figure 6.10 qPCR analysis of *MPK3* expression in each sample of salt ROF2. *MPK3* (At3g45640) was on the list in DMGs and DEGs in salt ROF2. The qPCR was done to quantify the absolute expression of *MPK3* in control ROF2, salt ROF2 line 1, salt ROF2 line 2, salt ROF2 line 3 and WT (old RKD4). The expression was normalised using *PP2A* (At1g13320) and *GAPDH* (At1g13440) and analysed using $\Delta\Delta CT$ method (Schmittgen & Livak, 2008).

6.3 Discussion

To understand the effect of stress exposure during somatic embryogenesis on transgenerational epigenome memory, *Arabidopsis* regenerants were exposed to salt and cold stress. Plants regenerated from somatic embryos were grown to the successive generation without further exposure to the respective stress. Wibowo et al. (2018) found that the methylation changes in all sequence context during somatic regeneration in RO and LO are stably inherited from first generation (F1) to second generation (F2), thus in this experiment, the methylation analysis was focused on the second generation (F2) of regenerated plants. To enable the application of robust statistical analysis and to exclude spontaneous individual epimutations which could arise in the course of few generations (Becker et al., 2011), for each treatment group, leaf samples from ten plants were collected and pooled.

The methylome data in this study showed that the methylation changes triggered by exposure to stress during somatic embryogenesis were able to persist two generations later. Epigenetic marks induced by the exposure to stress can be mitotically maintained to the daughter cells allowing plants to develop adaptability to the corresponding stress. These epigenetic changes also trigger a “primed” state to the plants that make them readily to respond to the stress, thus improving the survival rate of plants when exposed to the stress (Bruce et al., 2007). The inheritance of these stress-induced epigenetic changes to the progenies has been observed in which the parental epigenetic states passed to the progenies allowing the progenies to have the same adaptability response to stress. Some studies conducted in plants reported that DNA methylation changes induced by stress responses in plants could be propagated to the non-stress conditions for several generations. The evidence includes the inheritance of paternal DNA methylation induced by heavy metal in rice to the non-stress progenies, and this confers heavy metal adaptation of the progenies (Ou et al., 2012). In addition, over ~45% of differentially methylated cytosine positions occurred during salt stress were inherited to the non-stress progenies (Bilichak et al., 2012). On the other hand, Wibowo et al. (2016) conducted transgenerational salt stress experiments, and found that salt-stress-induced DNA methylation changes are gradually lost in non-stress generations. This indicates that the

stress-induced DNA methylation changes were reset after meiosis and/or embryogenesis. The resetting perhaps occurs during reproduction stage and embryogenesis (Kawashima & Berger, 2014) where most of the methylation marks are thought to be reprogrammed.

However, a study in bread wheat revealed that when drought and salt stress were introduced during the reproductive stage and maintained until physiological maturity when these seeds were sown, the progenies showed improved tolerance to drought and salt (Tabassum et al., 2017). This showed that molecular changes induced during reproduction stage were more likely to be propagated stably to the successive generation, and were able to escape the epigenetic reprogramming during gametes fusion. This study supported my findings that the stress-induced DNA methylation changes during somatic embryogenesis were able to be retained in the second generation of non-stress regenerated plants.

In this study, the methylation data for regenerated plants exposed to salt stress was rather unexpected due to large number of DMRs found in the salt LOF2 than in salt ROF2. However, this finding is supported with a study where salt stress increased DNA methylation in the shoot of almost all rice ecotype tested, while in the root, the level of DNA methylation is reduced (Karan et al., 2012). In addition, by looking at the DEGs (Chapter 5) where it showed that there were more transcriptomic changes in cold LOF2 than cold ROF2. The methylation data of cold LOF2 showed the same enrichment as the transcriptome where DMRs were predominantly found in cold LOF2 than cold ROF2. This indicates that the DNA methylation changes are largely due to tissue specificity.

The results obtained also showed CG hypermethylation in both salt-treated regenerated LO plants and cold-treated regenerated LO plants in comparison to the control experiments, and equal methylation state between hyper- and hypomethylation in salt-cold-treated regenerated plants. The global methylation between roots and shoots showed no difference in CG, CHG and CHH methylation between these tissues, but CG methylation remains the most abundant methylation marks found in roots and shoots, followed with CHG and CHH methylation (Widman et al., 2014). In *Medicago truncatula*

roots exposed to salinity stress showed a high level of CHH methylation (Yaish et al., 2018). Although the CHH methylation marks induced by salt stress erased in subsequent non-stress progenies (Wibowo et al., 2016). However, in this study, salt-treated regenerated RO plants maintained the CHH methylation, although CHH methylation was less prominent than CG and CHG methylation salt ROF2. Whereas the demethylation of RO plants in all sequence context in response to cold was supported with the findings by Steward et al. (2002) in which maize showed genome-wide demethylation occurred in the root tissues in response to cold stress.

The DMRs analysis in this study indicated that DMRs were mostly found in the genic and intergenic region of the genome when the stress was induced during somatic regeneration. This finding was in contrary with the study conducted by Wibowo et al. (2016) where they found that stress-induced DMRs are mostly enriched within TEs in the genome. The enrichment of DMRs in the genic and intergenic region in this study may be due to the abundance of CG methylation found. This view is supported by study conducted by Widman et al. (2014) where they found that DNA methylation located within the genes was mainly CG methylation, where CHG and CHH methylation was mainly found at a very low level within the genes but at a very high level in intergenic and repeat-rich regions of the genome. In addition, when *Arabidopsis* was exposed to biotic stress CG methylation was enriched in the intergenic region while CHH methylation was mainly found in the transposons (Dowen et al., 2012). Only small changes in CHH methylation was observed in this experiment, which may explain the reason of lack enrichment of DMRs on repeat-rich regions, known to be target of the RdDM (Matzke & Mosher, 2014).

The putative DMGs analysis was performed in this experiment whereas it defined as genes that located 2-kb upstream and downstream of the DMRs. The data showed that there were more DMGs found in the LO compared to the RO in both salt-treated regenerated plants and cold-treated regenerated plants. In addition, the results indicated that there are 54 DMGs shared between salt ROF2 and cold ROF2, and 339 DMGs shared between salt LOF2 and cold LOF2 (Fig. 6.4). It may be due to cold and salt are categorised as osmotic and dehydration stress to plants, both of them induce transient Ca^{2+} influx to the cytoplasm

(Xiong et al., 2002b). Thus it may indicate that salt and cold stress may have potential to induce similar methylation responses within the genome on similar tissue type.

In addition, the single sample analysis of putative DMGs indicated that there was only small proportion of common DMGs found in all sample. It indicated that the DMGs are unique to specific samples. The variation maybe due to the plants were regenerated from different cell type of root and leaf. Kawakatsu et al. (2016) reported that different root cells have different methylation pattern. Thus, the finding also indicated that cell specific methylation can be partially inherited to the next progeny, in which this view is supported by Han et al. (2018) where over 90% DNA methylation gain and 75% DNA methylation losses are inherited from generation 0 to the generation 1 in the regenerated maize from tissue culture. However, the methylation analysis was done without replication in this analysis, which it may present false positive result. Thus to limit the bias, more biological replications are needed for each sample in this experiment.

The results showed that there five DMGs are shared in all sample of salt-treated regenerated plants and cold-treated regenerated plants (Table 6.2). These DMGs were linked to stress responses. *AMINO ACID PERMEASE 1 (AAPI)* is an amino acid transporter that localised to the embryos, and it plays a significant role in mediating uptake of amino acids by the embryo that crucial for storage protein synthesis and seed yield (Sanders et al., 2009). This protein also involved in increasing of proline uptake during salt stress in Arabidopsis and promoted salt stress tolerance (Wang et al., 2017). NADPH dehydrogenase is an important reductive coenzyme that is required by the antioxidative system (Corpas & Barroso, 2014). This enzyme is also a crucial element in supporting the mechanism of response to nitro-oxidative stress situation in plants (Corpas & Barroso, 2014). This enzyme also an important antioxidant system that protects the cells against salt-induced oxidative stress (Valderrama et al., 2006). Finally, exoribonucleases, especially Arabidopsis 5'-3' exoribonuclease (AtXRN4) involved during heat stress by mediating mRNA degradation of HSFA2 that linked to suppression of heat acclimation (Nguyen et al., 2015). These may indicate that all of these genes might be involved in regulation of stress responses in high salinity and low temperature. This hypothesis can

be further tested by using the mutant of these genes, and the salinity and freezing can be performed in these mutants.

The expression data of the DMGs (Fig. 6.5) suggest that there were differences between methylation and expression, which indicated that methylation marks did not always suppress the gene expression. A study elsewhere showed that the location of DNA methylation is important to determine the effect of methylation on a gene, the impact of DNA methylation on expression is greater when it is in the transcription unit, but methylation outside of the transcription unit may potentiate the effect of the methylation within the transcription unit (Irvine et al., 2002). Therefore, to explain this finding further, it is crucial to understand where the methylation was found in the DMGs.

In addition, the intersection results between DMGs and DEGs indicated that there was a small connection between methylation and transcriptome expression for the cold-treated regenerated plants and salt-treated regenerated plants. A small intersection is found between salt RO DEGs and salt RO DMGs perhaps due to a few changes found between salt ROF2 and control ROF2 in term of their methylation differences (Appendix 8.8). Surprisingly, salt LOF2 clustered together with cold LOF2, it suggests that they share common differentially methylated marks. Thus perhaps cross phenotypic testing can be done to assess whether salt LO has improved tolerance to cold stress, and cold LO has increased adaptation to high salinity.

In addition, to further understand the connection between transcriptome and methylation, the expression of *MITOGEN-ACTIVATED PROTEIN KINASES 3 (MPK3)* were tested in salt ROF2 line 1, 2 and 3. This gene is found both in the DEG and DMG list of salt ROF2. In the wild type *Arabidopsis*, there is methylation found 1 kb upstream of *MPK3* gene (Lister et al., 2008). The DMR found 2 kb upstream of *MPK3* which it indicates there is methylation differences between salt ROF2 and control ROF2. Based on qPCR result, it indicates that the methylation upstream of *MPK3* does affect the expression of *MPK3*. This finding is supported with Wibowo et al. (2016) where that methylation marks located

upstream or downstream of a gene could act as a distant regulatory system of that particular gene.

The finding of *TOLERANT TO CHILLING AND FREEZING (TCF1)* gene in the Cold LO DMGs may also offer a link between methylation, transcription and phenotype. *TCF1* is an RCC1 family protein that interacts with histone H3 and H4 and associated with chromatin containing a target gene, *BLUE-COPPER-BINDING PROTEIN (BCB)* that regulates lignin biosynthesis (Ji et al., 2015). The study showed that suppression of *TCF1* is linked to a reduction in lignin biosynthesis, allowing cell permeability and protecting the cells from freezing damage (Ji et al., 2015). Wibowo et al. (2016) showed that DMRs that located upstream or downstream of genes had a direct effect on the expression of that gene. The DMRs for *TCF1* were found upstream of the gene, thus to understand further the link between methylation, transcription and phenotype, an experiment to remove this DMR by using CRISPR-Cas9 or introducing this DMR into the genome at the exact position found in the cold LO plant by using an inverted repeat hairpin (Wibowo et al., 2016). Similar experiment can also be conducted for *MPK3* in salt ROF2. *MPK3* was indicated to be associate with priming in Arabidopsis where the *MPK3* was found more strongly in primed (with abiotic and biotic stress) plants compared to the non-primed plants (Beckers et al., 2009). Therefore, this experiment will allow us to understand whether the DMR found upstream of *TCF1* and *MPK3* has regulatory effect in controlling the expression of this gene, and to show if the changes in *TCF1* and *MPK3* expression corresponds to the increase or decrease freezing tolerance in the cold LOF2 and salt tolerance in salt ROF2.

6.4 Summary

Stress-induced methylation changes is still observable in the second generation of salt-treated regenerated plants and cold treated-regenerated plants. The methylation changes in regenerated plants from leaf (LO) are CG hypermethylation in both salt and cold stress, whereas root regenerated plants (RO) have equal CG and CHG hypo- and hypermethylation. The DMRs are predominantly found in the LO plants compared to the RO plants, with most of the DMRs found in the genic and intergenic region of the genome.

DMGs analysis found five DMGs shared in all samples, and these four of these DMGs are related to stress. In addition, the differences in the expression of DMGs indicate that methylation is rather dynamic in regulating gene expression, suggesting that DNA methylation can either promote expression of a gene or repress the expression.

7. General Discussion

7.1 Stress-Induced during Somatic Embryogenesis Transmitted to the Non-Stress Offspring

As non-motile organisms plants have evolved several strategies to adapt to adverse environmental changes. These strategies include epigenetic modification such as DNA methylation (Kumar et al., 2017), and this epigenetic modification serves as the basis of stress memory in plants (Bruce et al., 2007). A previous approach introduced drought stress during the reproductive stage of wheat, and the stress was maintained until maturity; the plants were able to inherit this drought memory into the next generations (Tabassum et al., 2017). However, the study did not test for multigenerational drought adaptation. Thus the transgenerational stress memory induced during the reproductive stage and/or embryogenesis remains unknown. Several studies have observed that stress exposure can induce transgenerational changes in DNA methylation to the successive generation (Boyko et al., 2010; Wang et al., 2016). However, a recent study by Wibowo et al. (2016) revealed that exposure to salt stress in one generation was not enough to induce transgenerational adaptation to the offspring, where the acquired salt stress tolerance was rapidly lost in the subsequent generations. This suggested inability of plants to produce stable modifications after stress exposure. It was thought that perhaps the epigenetic reprogramming during gametogenesis and embryogenesis has a significant role in resetting this acquired stress because it is important for the plant to reset any epigenetic marks acquired during developmental stages to ensure that their progeny will have the correct developmental information (Kawashima & Berger, 2014).

To address this issue, a new approach was developed by using the overexpression of *RKD4* in *Arabidopsis* to evaluate the impact of stress induced during somatic regeneration (Wibowo et al., 2018). This study used a genome-wide transcriptomic analysis to elucidate the differential expressed genes on the second generation of salt and cold-treated regenerated plants. Based on the transcriptomic analysis, the phenotypic assay and methylome analysis were performed to further understand the transgenerational salt and cold stress resistance in the regenerated plants.

Our finding show that when either salt and cold stress memory is induced during somatic regeneration, and that this memory can be inherited to the next unstressed generations. However, the inheritance of the stress adaptation depends on the tissue from where the somatic embryos come from. Salt stress tolerance is inherited primarily in root-regenerated plants (RO), where cold stress tolerance is transmitted best on leaf-regenerated plants (LO) and flg22 induces diseases resistance in both RO and LO. Wibowo et al. (2016) showed that salt stress responses lost immediately after one generation without stress. However, the data shows that stress tolerance can be partially inherited until the third generation (F3), at least for cold stress. Assessing the cold tolerance in the F4 and F5 generations will help us to understand the stability of cold-stress memory.

Improvement in the response to stress is usually followed by growth and development trade-off. For instance, manipulation of a plant hormone pathway can improve disease resistance in plants, but it also has a negative effect on plant growth (Denancé et al., 2013). Meanwhile, developing salt tolerance traits by changing the expression of *DREB1*, *ADR1* and *AtNHX1* causes imbalances in plants development and physiology (Denby & Gehring, 2005). However, even though stress-induced regenerated plants show improved tolerance to salt and cold, the negative effects from this adaptation to the growth and development of these plants was not observed. Although the yield of these plants was not counted, the visual examination revealed that these plants have similar growth and development in comparison to the control lines. This finding was similar to the study conducted by Wibowo et al. (2018) where there is no morphological differences between RO, LO and wild type. The lack of growth and development trade-off maybe due to when stress was induced during the regeneration, the regenerated plants are primed to the stress. Thus the regenerated plants are more ready to adapt when exposed to the stress. This hypothesis is supported by Karasov et al. (2017) where transgenerational priming becomes one of mechanisms for plants to limit the growth trade-off. During primed-state stalled RNA polymerase II and H3K4me3 accumulate as at the primed-genes before actual transcription takes place, allowing plants to express stress-related genes faster (Liu & Avramova, 2016).

7.2 Transgenerational DNA Methylation Inheritance in Regenerated Plants Induced by Stress During Somatic Regeneration

The ability of environmental cues to cause changes on the epigenetic state of the genome is well studied. A wide-range of environmental stresses have been linked to the changes in chromatin and associated epigenetic marks (Eichten et al., 2014). Furthermore, drought, flooding, nutrient deficiency, temperature shock, pathogen infection, high salinity, heavy metal exposure, ultraviolet radiation, and herbivory can induce DNA methylation changes (Boyko et al., 2010; Crisp et al., 2016). These stress-induced DNA methylation changes can be inherited to the subsequent generations (Boyko et al., 2010; Suter & Widmer, 2013; Wang et al., 2016). However, Wibowo et al. (2016) proved that salt stress-induced DNA methylation changes are dynamic and that the stress-induced DNA methylation is reversible when plants do not experience the same type of stress. In plants, epigenetic marks are actively removed during sexual reproduction (Kawashima & Berger, 2014), but this epigenetic reprogramming in plants seems to be incomplete (Paszkowski & Grossniklaus, 2011). Current data indicates that when stress is induced during somatic embryogenesis, the changes in DNA methylation (CG and CHG) are inherited to the second generation. The CG and CHG methylation are passed on to the newly synthesis DNA from the paternal DNA strands during DNA replication, while CHH methylation is *de novo* synthesised by the RdDM pathway (Bond & Baulcombe, 2014). In addition, the RdDM pathway also can *de novo* synthesise the methylation at CG and CHG (Matzke & Mosher, 2014). The putative mechanisms of transmission of transgenerational stress memory involves RdDM pathway (Bilichak & Kovalchuk, 2016). Thus it is possible that RdDM pathway plays important role in the maintenance of CG and CHG methylation in the current study. However, to test this hypothesis, RdDM pathway mutants should me be used with similar experimental setup, and the level of CG and CHG methylation should be assessed.

The impact of stress-induced DNA methylation is mainly enriched on repetitive elements and TEs throughout the genome (Secco et al., 2015; Tan, 2010). Wibowo et al. (2016) also observed the salt stress-induced accumulation of DNA methylation in TEs. However, current study indicated when the stress is induced during somatic embryogenesis, the

DNA methylation is mainly found in the intergenic and genic regions of the genome. The abundance of CG and CHG methylation is responsible for the enrichment of DNA methylation in the intergenic and genic region. CHH methylation is known to affect TEs and repetitive elements, but not genic regions (Bond & Baulcombe, 2014) which this supports current finding where little CHH methylation may contribute to the less DMRs found in the TEs. In addition, a study conducted to understand cell-specific DNA methylation in roots found that there was widespread cell type-specific DNA methylation in six different root cells (Kawakatsu et al., 2016). However, the global methylation of CG and non-CG methylation between root and shoot did not differ significantly. Instead one in every 173 cytosines were differentially methylated between root and shoot (Widman et al., 2014).

Current study outcome indicates that most DMRs found in the LO plants for both salt-treated regenerated plants and cold-treated regenerated plants are enriched in genic and intergenic regions. The exact explanation of this finding is still unknown due to the lack of other studies investigating stress-induced DNA methylation in different tissue types. Perhaps, tissue-specific methylation has a greater contribution than stress-specific methylation. Another possibility stress-mediated differences in DNA methylation between RO and LO plants are due to Ca^{2+} influx in the cytoplasm activating secondary messengers (Xiong et al., 2002a). These secondary signals initiate the activation of MAPK signalling cascades that triggers the activation of stress response genes (Zhu, 2016). A study has linked the effect of secondary messengers, such as reactive oxygen species to DNA methylation. Reactive oxygen species generated by paraquat efficiently induced DNA demethylation at coding regions, in which the effect was similar to the demethylation patterns induced by salt and cold treatments (Choi & Sano, 2007b). Similar findings were also observed during wounding in maize, in which wounding induced oxidative stress by the production of reactive oxygen species (Savatin et al., 2014). The wounding responses also decreased the global DNA methylation level by 20-30% after an hour of the wounding (Lewandowska-Gnatowska et al., 2014). Thus, since both salt and cold stress triggers the accumulation reactive oxygen species, there is possibility that it may induce similar epigenetic changes.

7.3 Stress-Induced Transcriptional Memory in Regenerated Plants Exposed to the Stress During Somatic Embryogenesis

Our experiments have shown that stress-induced during somatic embryogenesis can improve stress adaptation in regenerated plants, and that this adaptation remains in the second generation. This improved adaptation is usually associated with changes in the transcriptome in absence of stress resembling “priming effects”. Cold stress leads to more transcriptomic changes in the leaf-regenerated plants, while salt stress has altered the transcriptome in the root-regenerated plants. Induction of cold stress during somatic embryogenesis caused the upregulation of cold stress-related genes in the LO plants, where salt stress exposure during somatic regeneration upregulates salt stress response genes in the RO plants. This indicates that stress, induced during somatic embryogenesis, develops primed-regenerated plants in a tissue-specific manner. Although salt and cold stress have different responses in gene expression between root and leaf (Kreps et al., 2002), the mechanisms of how stress-induction during somatic embryogenesis can lead in the transgenerational transcriptome changes in plants are still unknown.

RNA decay is an essential mechanism for resetting the stress memory by clearing the stress-responsive transcripts (Crisp et al., 2016). In plants, mRNA is further stabilised by the addition of 5' cap, 7-methyl guanine residue connected to the mRNA via 5'-5' triphosphate bond, and the 3' poly(A) tail (Crisp et al., 2016). RNA decay occurs by removal of either 5' cap, 7-methyl guanine residue or 3' poly(A) tail, or degradation by endonuclease activity that predominantly happens in the cytoplasm and proceeds by either 5'-3' or 3'-5' exoribonuclease decay (Belostotsky & Sieburth, 2009; Christie et al., 2011; Crisp et al., 2016). Stress exposure can also affect the process of RNA decay. In *Arabidopsis*, dehydration stress activates MPK6 to phosphorylate serine 237 of *Arabidopsis DCP1* and phospho-DCP1, related to DCP5, promotes mRNA decapping (Jun et al., 2012). This process is important for dehydration stress adaptation as the *dcp5-1* was hypersensitive to dehydration stress (Xu & Chua, 2012). Thus to establish transcriptional memory upon stress induction, plants need to inhibit RNA decay or stabilise specific transcripts (Crisp et al., 2016). One hypothesis is that regenerated plants can inhibit the

process of RNA decay and stabilise stress-specific transcripts produced during somatic embryogenesis. This is supported by our finding that *EXORIBONUCLEASE 2* is differentially methylated in stress-regenerated plants suggesting that there is a link between DNA methylation and the control of transcriptional memory by preventing RNA decay of stress-related transcripts. However, further experiments to check the induction time of stress-related genes between the control-regenerated plants and stress-regenerated plants needs to be carried to test this hypothesis.

Environmentally induced gene expression changes can also be passed through to the next generation by the inheritance of chromatin states. Tao et al. (2017) studied the epigenetic inheritance of *FLC*, and found that the seed-specific transcription factor LEAFY COTYLEDON1 (*LEC1*) was expressed at the pro-embryo stage similar to the *FLC*, and the expression of *FLC* was dependent on *LEC1* because expression of *FLC* was suppressed in the *lec1* at the pro-embryo stage. This indicates that *LEC1* activates and promotes *FLC* expression as early as the pro-embryo stage. The activation of *FLC* by *LEC1* was also followed by changes in chromatin to the active state by the accumulation of H3K36me3 at the *FLC* locus. The expression of *FLC* mediated by *LEC1* was maintained from the pro-embryo until the seedling stage followed by the accumulation of H3K36me3. Thus, probably when stress is induced during somatic embryogenesis, it is possible it activates certain transcription factors (TFs) that are expressed during somatic embryogenesis (Fig. 7.1). The activation of these TFs may induce the expression of stress-response genes that can be passed on from pro-embryo to post-embryonic stage. However, to confirm this further hypothesis the same experiment should be conducted by using mutants that affect cold sensing (*ice1*), salt sensing (*sos1/2/3*) and flg22 sensing (*fls2*).

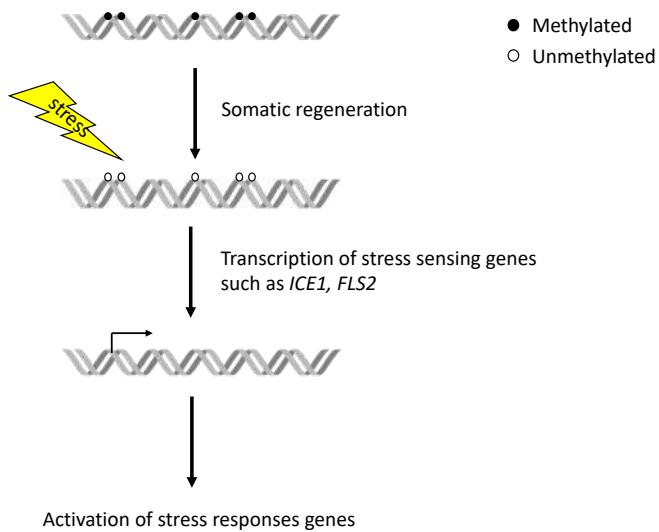


Figure 7.1 Hypothesis mechanism of transgenerational stress-tolerance inheritance in the regenerated plants. Before undergone somatic regeneration, some part of the genome are methylated. This may affect the expression of stress sensing genes such as *ICE1* or *FLS2*. During somatic regeneration, some part of the genome becomes unmethylated which it may activate the transcription of stress sensing genes. If the stress is introduced during the somatic regeneration, it will further increase the transcription of stress sensing genes where it will affect the downstream signaling cascades and activation of stress responses genes. The responses may be fix when the tissue fate has been defined after the regeneration completed.

7.4 Stress-Induced DNA Methylation May Affect the Expression of Stress Responses Genes

My data indicate that DNA methylation changes in response to stress, introduced during somatic embryogenesis, can persist to the second generation of non-stress regenerated plants. These DNA methylation changes found may affect genes located within 2-kb upstream and downstream of DNA methylation marks. This DNA methylation is not found to affect TEs predominantly, but mostly affects genic and intergenic regions. The expression of DMGs also differs for each methylation-affected gene. This indicates that methylation is a dynamic process that does not always have a negative effect in gene expression. Zhang et al. (2006) conducted genome-wide DNA methylation analysis in *Arabidopsis* to understand the relationship between DNA methylation and gene expression. They compared the sites of DNA methylation with microarray expression data from 79 different tissue conditions and found that when DNA methylation was located

within the gene body, the expression of these genes was significantly higher than unmethylated genes. However methylation at the promoter of the genes resulted in lower expression. This finding inferred that gene body methylation had higher expression and promoter-methylated genes are expressed in tissue-specific manner. The function of genic methylation was further assessed and it was found that tissue-specific genic methylation might reduce or improve transcription elongation efficiency (Maunakea et al., 2010). DNA methylation at the intergenic region is related to controlling the expression of non-coding RNA (Suzuki & Bird, 2008).

These methylation changes were also found to affect the expression of stress-related genes. In cold LO plants, DNA methylation marks were detected upstream of *TOLERANT OF CHILLING AND FREEZING 1* (*TCF1*). Wibowo et al. (2016) found salt stress DMR downstream *CARBON/NITROGEN INSENSITIVE 1* (*CNI1*). When T-DNAs were inserted in the transcript body of *CNI1* and the downstream DMR, these caused misregulation of *CNI1* in response to stress. Furthermore, the deletion of *CNI1* DMR reduced downregulation of sense *CNI1* transcript. This indicates that methylation marks can serve as a long-distance regulatory elements. To further understand whether the DMR upstream of *TCF1* has a direct effect in the *TCF1* transcript, CRISPR/Cas9 deletion of DMR could be used, and the transcript level of *TCF1* could be assessed after the deletion of the DMR. *TCF1* is thought to regulate freezing tolerance by reducing lignin content in the cell wall. Reduction of lignin content in *Arabidopsis* also enhances the elasticity of the cell wall to accommodate the growth of ice crystals with less damage to the cellular level (Ji et al., 2015).

7.5 Concluding Remarks

In summary, this work indicated that stress-induced during somatic embryogenesis is to induce transgenerational adaptation in regenerated plants. This enhanced stress tolerance phenotype is inherited to non-stressed progenies over multiple generations. The phenotype changes occur in a tissue-specific manner, where salt-stress induced adaptive phenotypes are found in regenerated plants which come from root tissue (RO plants), whereas cold-stress improved freezing tolerance in the regenerated plants which come from leaf tissue (LO plants). These phenotypic changes occur are associated changes in the transcriptome of regenerated plants. A transcriptome-wide analysis of second-generation stress-treated regenerated plants shows that stress induction during somatic regeneration is enough to cause transcriptomic changes in the regenerated offspring. The transcriptomic memory is still maintained in the second generation of non-stress regenerated plants. This indicates that exposing plants to stress during somatic embryogenesis form a type of transcriptional memory leading to improved stress adaptation phenotypes in sexually propagated progenies.

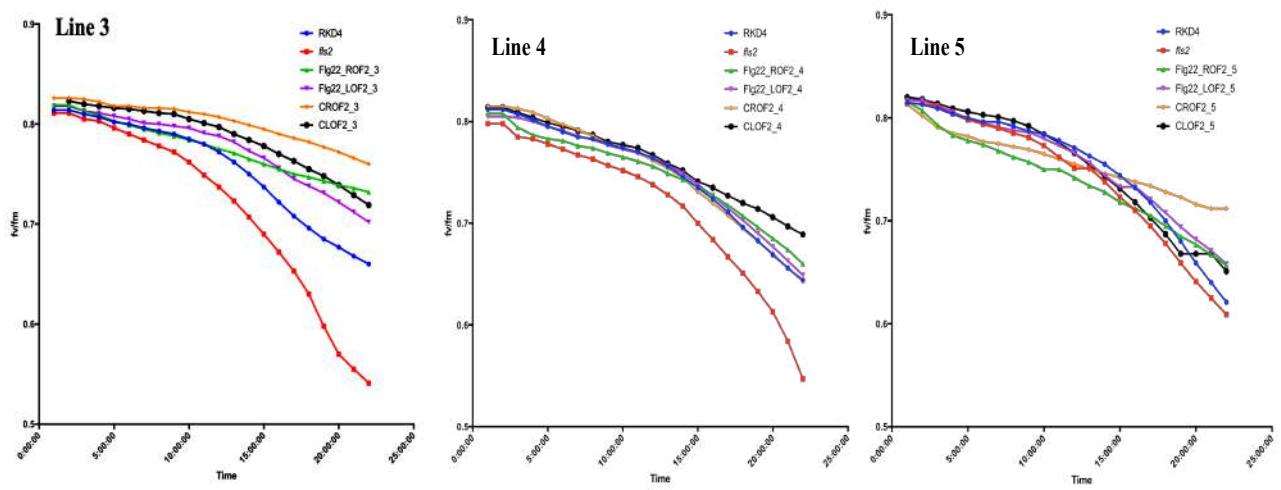
Whole-genome bisulphite sequencing revealed that regenerated plants treated with salt and cold stress have altered CG and CHG methylation in both RO and LO plants, with mostly CG and CHG hypermethylation in LO plants, and equal distribution between hyper- and hypomethylation of CG and CHG in the RO plants. However there are no changes in CHH methylation in the LO plants, and only small changes of CHH methylation are maintained in the RO plants. The methylation changes still persist in the second generation, indicating that these epigenetic imprints are faithfully maintained after meiosis. Intriguingly, these methylation changes are mostly found in the intergenic and genic regions in the salt-treated regenerated plants and cold-treated regenerated plants. The DMRs found the stress-treated regenerated plants may control the expression genes of flanking/neighbouring, although further experiment should be performed to test this hypothesis. However, the relative expression of these differentially methylated genes is not directional indicating that methylation is a dynamic process partly dependent on the genomic location. Interestingly, we found DMR associated with stress-related genes that may confer the stress adaptation phenotypes observed. However, further experiments are

needed to establish a direct link between methylation and the expression of stress-responsive genes.

Stress-induced during somatic regeneration can direct tissue-specific responses in the phenotype, transcriptome and methylome could be explained by the hypothesis that stress signals may activate specific transcriptional networks that permit the establishment of epigenetic changes that can be mitotically and meiotically propagated. However, this hypothesis will require investigation in future studies.

8. Appendix

Appendix for Chapter 4



Appendix 8.1 *Pst. DC3000* infiltration in line 3, 4, and 5.

Appendix for Chapter 5

Appendix 8.2 Salt LO differentially expressed genes (FDR 0.01). Base mean: mean of normalisation counts; log2foldchange: log fold change between control vs stress; lfcSE: standard error of log2fold change; stat: Wald statistic; pvalue: Wald test p-value between control vs stress; padj: adjusted p-values.

Gene ID	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Primary Gene Symbol
AT5G47350.1	55.8505333	-4.0427869	0.46291973	-8.7332352	2.47E-18	4.51E-14	
AT1G72260.1	38.0990847	-3.4157116	0.43694699	-7.817222	5.40E-15	4.92E-11	THIONIN 2.1 (THI2.1)
AT1G43640.1	3.91911488	-3.2413129	0.462553	-7.0074411	2.43E-12	1.47E-08	TUBBY LIKE PROTEIN 5 (TLP5)
AT5G42800.1	26.8271185	-2.7424216	0.45060436	-6.0860964	1.16E-09	4.22E-06	DIHYDROFLAVONOL 4-REDUCTASE (DFR)
AT2G47895.1	7.71070629	-2.6785232	0.46425651	-5.7694898	7.95E-09	2.07E-05	
AT4G12870.1	14.0799019	-2.4837715	0.39064653	-6.3581046	2.04E-10	9.31E-07	
AT5G07990.1	23.7583277	-2.396078	0.40546451	-5.9094641	3.43E-09	1.04E-05	TRANSPARENT TESTA 7 (TT7)
AT3G60900.1	9.06281617	-2.371178	0.44784957	-5.2945858	1.19E-07	0.00024153	FASCICLIN-LIKE ARABINOGALACTAN-PROTEIN 10 (FLA10)
AT1G10540.1	6.42698979	-2.3480039	0.45271441	-5.1865014	2.14E-07	0.00039049	NUCLEOBASE-ASCORBATE TRANSPORTER 8 (NAT8)
AT3G28830.1	10.8886358	-2.3311529	0.46589495	-5.0036019	5.63E-07	0.00085449	
AT4G00870.1	6.35191639	-2.1396718	0.46605356	-4.5910427	4.41E-06	0.00382715	BASIC HELIX-LOOP-HELIX 14 (BHLH14)
AT2G45840.1	2.1778926	-2.1155475	0.456615	-4.6331099	3.60E-06	0.00364676	
AT2G25540.1	3.77339288	-2.1117987	0.46296525	-4.5614628	5.08E-06	0.00420773	CELLULOSE SYNTHASE 10 (CESA10)
AT3G01270.1	9.57466389	-2.0937634	0.45576061	-4.5939982	4.35E-06	0.00382715	

AT3G13400.1	15.8773627	-2.0410981	0.44287757	-4.6087186	4.05E-06	0.00382715	SKU5 SIMILAR 13 (sks13)
AT2G47050.1	4.83401693	-2.0258095	0.45299831	-4.4720023	7.75E-06	0.0054312	
AT1G02790.1	11.4206606	-1.994931	0.44039617	-4.5298554	5.90E-06	0.0046765	POLYGALACTURONASE 4 (PGA4)
AT5G21150.1	3.25174737	-1.9698265	0.36847593	-5.3458755	9.00E-08	0.00020497	ARGONAUTE 9 (AGO9)
AT1G03050.1	2.20553027	-1.952952	0.43495508	-4.4900085	7.12E-06	0.00519139	
AT5G13930.1	621.367764	-1.9473436	0.44967287	-4.3305784	1.49E-05	0.00757052	TRANSPARENT TESTA 4 (TT4)
AT3G07850.1	6.39932196	-1.9286654	0.42709168	-4.5158112	6.31E-06	0.00478922	
AT3G62230.1	3.48582086	-1.836899	0.41884945	-4.3855829	1.16E-05	0.00658736	DUO1-ACTIVATED F-BOX 1 (DAF1)
AT5G56840.1	35.2116706	-1.8368602	0.36966838	-4.9689405	6.73E-07	0.00087626	
AT1G51140.1	241.685895	-1.7885294	0.40475437	-4.4188019	9.92E-06	0.00658736	FLOWERING BHLH 3 (FBH3)
AT2G27880.1	2.05617706	-1.6639861	0.35217987	-4.7248189	2.30E-06	0.0024689	ARGONAUTE 5 (AGO5)
AT1G62333.1	5.06693395	-1.6447981	0.32434525	-5.0711337	3.95E-07	0.00065512	
AT1G61720.1	20.7940151	-1.4986889	0.30117256	-4.9761801	6.49E-07	0.00087626	BANYULS (BAN)
AT1G78020.1	1338.01658	-1.4950584	0.33879515	-4.4128684	1.02E-05	0.00658736	
AT5G01870.1	3.29380286	-1.300683	0.29580709	-4.3970649	1.10E-05	0.00658736	
AT1G22160.1	215.053798	-1.2987541	0.299989	-4.329339	1.50E-05	0.00757052	
AT2G28350.1	145.146879	-0.8323006	0.19086762	-4.3606172	1.30E-05	0.00695133	AUXIN RESPONSE FACTOR 10 (ARF10)
AT2G17820.1	1002.44702	0.79958909	0.18150186	4.40540437	1.06E-05	0.00658736	HISTIDINE KINASE 1 (HK1)
AT4G15660.1	124.734526	1.62723162	0.37092595	4.38694463	1.15E-05	0.00658736	GLUTAREDOXIN 8 (GRXS8)
AT4G13494.1	54.5782903	1.6447913	0.33912509	4.85010214	1.23E-06	0.00149912	MICRORNA863A (MIR863A)
AT4G15690.1	196.128339	1.69913438	0.39538	4.29747177	1.73E-05	0.00850853	GLUTAREDOXIN 5(GRXS5)
AT4G15700.1	217.290941	1.88500673	0.39306308	4.79568501	1.62E-06	0.00184644	GLUTAREDOXIN 3 (GRXS3)
AT2G26020.1	98.7547874	2.03631762	0.4652373	4.37694402	1.20E-05	0.00664614	PLANT DEFENSIN 1.2B (PDF1.2b)

Appendix 8.3 Salt RO differentially expressed genes (FDR 0.01). Base mean: mean of normalisation counts; log2foldchange: log fold change between control vs stress; lfcSE: standard error of log2fold change; stat: Wald statistic; pvalue: Wald test p-value between control vs stress; padj: adjusted p-values.

Gene ID	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	All Gene Symbols
AT1G36922.1	8.24089753	-2.2157197	0.43698974	-5.0704157	3.97E-07	0.0002609	
AT1G36920.1	10.3676168	-2.1235331	0.44224773	-4.8016823	1.57E-06	0.00066069	
AT1G47950.1	5.79890108	-2.1101789	0.45129371	-4.6758438	2.93E-06	0.00093506	
AT3G26460.1	4.68725537	-1.953872	0.45539449	-4.2905043	1.78E-05	0.00266819	
AT3G55500.1	141.268722	-1.9278592	0.42941121	-4.489541	7.14E-06	0.00156377	EXPANSIN A16 (EXPA16)
AT2G47770.1	21.6851557	-1.8747076	0.43753259	-4.2847268	1.83E-05	0.00271163	TSPO(OUTER MEMBRANE TRYPTOPHAN-RICH SENSORY PROTEIN)-RELATED (TSPO)
AT4G15990.1	35.6612885	-1.8583876	0.46142944	-4.0274578	5.64E-05	0.00535539	
AT1G29230.1	4.2830205	-1.8255269	0.45365934	-4.0240038	5.72E-05	0.00537236	CBL-INTERACTING PROTEIN KINASE 18 (CIPK18)
AT3G13400.1	15.8773627	-1.8035164	0.45101802	-3.9987678	6.37E-05	0.00583361	SKU5 SIMILAR 13 (sks13)
AT4G19430.1	39.8124546	-1.7676855	0.46555234	-3.796964	0.00014648	0.00986442	
AT3G61890.1	237.268363	-1.7588664	0.37146697	-4.73492	2.19E-06	0.00083239	ARABIDOPSIS THALIANA HOMEOBOX 12 (ATHB12)
AT5G66052.1	1068.70674	-1.7494604	0.40930465	-4.2742255	1.92E-05	0.00273536	
AT5G66080.1	62.2517781	-1.699336	0.36465499	-4.6601201	3.16E-06	0.00095331	ARABIDOPSIS PP2C CLADE D 9 (APD9)
AT4G21060.1	6.55077675	-1.6725737	0.42849837	-3.9033374	9.49E-05	0.00735503	GALACTOSYLTRANSFERASE 2 (GALT2)
AT3G52310.1	88.945056	-1.6488224	0.31197894	-5.2850439	1.26E-07	0.00011175	ATP-BINDING CASSETTE G27 (ABCG27)
AT4G40040.2	15.718132	-1.5833352	0.32799003	-4.8273881	1.38E-06	0.00061818	HISTONE 3.3 (H3.3)
AT3G14360.1	36.2359949	-1.5083691	0.37767834	-3.9937929	6.50E-05	0.0058861	

AT5G60910.1	18.191824	-1.442349	0.3561042	-4.0503565	5.11E-05	0.00501999	FRUITFULL (FUL);AGAMOUS-LIKE 8 (AGL8)
AT1G30820.1	684.577473	-1.4214468	0.34677458	-4.0990514	4.15E-05	0.00444769	
AT5G37500.1	82.786948	-1.3857008	0.21667691	-6.3952396	1.60E-10	1.21E-06	GATED OUTWARDLY-RECTIFYING K+ CHANNEL (GORK)
AT4G16750.1	44.7029593	-1.3839726	0.35997805	-3.8446028	0.00012075	0.00857562	
AT5G62950.3	27.8607629	-1.3596622	0.35098919	-3.8738009	0.00010715	0.00797932	
AT2G47880.1	241.833548	-1.342152	0.25603717	-5.2420203	1.59E-07	0.00012637	CEP DOWNSTREAM 2 (CEPD2)
AT5G28300.1	564.317828	-1.3069083	0.31673922	-4.1261334	3.69E-05	0.00414663	GT-2LIKE PROTEIN (GT2L)
AT5G49990.1	228.376968	-1.1950092	0.19196122	-6.2252639	4.81E-10	2.42E-06	
AT1G72850.1	16.7719106	-1.1909098	0.29184555	-4.0806165	4.49E-05	0.00471529	
AT3G28150.1	19.7501164	-1.1716414	0.2986069	-3.9236917	8.72E-05	0.0070873	ALTERED XYLOGLUCAN 4-LIKE (AXY4L);TRICHOME BIREFRINGENCE-LIKE 22 (TBL22)
AT1G07090.1	292.12184	-1.0342848	0.25964681	-3.9834298	6.79E-05	0.00600505	LIGHT SENSITIVE HYPOCOTYLS 6 (LSH6)
AT2G25964.1	135.076839	-1.0303035	0.24858464	-4.1446789	3.40E-05	0.00398773	
AT3G17160.1	137.15888	-1.0150053	0.21838831	-4.6477089	3.36E-06	0.00097575	
AT2G39570.1	1576.60309	-1.0096956	0.25050359	-4.0306631	5.56E-05	0.00532154	ACT DOMAIN REPEATS 9 (ACR9)
AT1G47970.1	808.815579	-0.9744217	0.2415635	-4.0338117	5.49E-05	0.00528414	
AT3G52490.1	93.3401344	-0.9679024	0.19896616	-4.8646581	1.15E-06	0.00059767	SMAX1-LIKE 3 (SMXL3)
AT3G48740.1	2962.41864	-0.9454602	0.23659145	-3.9961722	6.44E-05	0.00586238	SWEET11
AT1G09960.1	62.5798209	-0.9209126	0.21224755	-4.3388609	1.43E-05	0.00227849	SUCROSE TRANSPORTER 4 (SUT4);
AT2G28350.1	145.146879	-0.9084161	0.18625677	-4.8772245	1.08E-06	0.00058087	AUXIN RESPONSE FACTOR 10 (ARF10)
AT4G20060.1	112.120417	-0.8998943	0.18077882	-4.9778748	6.43E-07	0.00037378	DEFECTIVE IN SNRNA PROCESSING 1 (DSP1);EMBRYO DEFECTIVE 1895 (EMB1895)

AT4G31820.1	1554.93923	-0.8625371	0.22055141	-3.9108209	9.20E-05	0.00728148	ENHANCER OF PINOID (ENP);MACCHI-BOU 4 (MAB4); (ENP);NAKED PINS IN YUC MUTANTS 1 (NPY1)
AT1G44760.1	89.8588268	-0.8586261	0.22031884	-3.8971977	9.73E-05	0.00750545	
AT1G05160.1	263.4499	-0.8286866	0.20814068	-3.9813773	6.85E-05	0.00602194	ENT-KAURENOIC ACID OXYDASE 1 (KAO1); (ATKAO1);"CYTOCHROME P450, FAMILY 88, SUBFAMILY A, POLYPEPTIDE 3" (CYP88A3)
AT4G10690.1	116.646749	-0.8092038	0.18107808	-4.4688114	7.87E-06	0.00162881	
AT3G22440.1	5353.03422	-0.8011139	0.21143354	-3.7889629	0.00015128	0.00994288	
AT2G17650.1	105.530431	-0.7946162	0.2042243	-3.8908996	9.99E-05	0.00762517	
AT2G41760.1	207.183902	-0.7256382	0.19135045	-3.7921952	0.00014932	0.00990041	
AT1G19980.1	77.7358864	-0.7208104	0.1835722	-3.9265773	8.62E-05	0.0070873	
AT2G15292.1	286.606973	-0.7092753	0.14240293	-4.9807631	6.33E-07	0.00037378	
AT5G24830.1	135.881202	-0.6500717	0.15436534	-4.211254	2.54E-05	0.00322611	
AT5G06360.1	880.318548	-0.6480213	0.12196855	-5.3130199	1.08E-07	0.00010187	
AT1G35115.1	720.94725	-0.6176763	0.13591075	-4.5447203	5.50E-06	0.001414	
AT3G43700.1	128.63104	-0.6024065	0.15578381	-3.8669391	0.00011021	0.00805647	BTB-POZ AND MATH DOMAIN 6 (BPM6);BTB-POZ AND MATH DOMAIN 6 (ATBPM6)
AT3G01890.1	154.737964	-0.5962632	0.15683666	-3.80181	0.00014364	0.00975576	SWP73A (SWP73A)
AT3G14400.1	599.8418	-0.591133	0.14969996	-3.9487854	7.85E-05	0.00659679	UBIQUITIN-SPECIFIC PROTEASE 25 (UBP25)
AT1G68490.1	642.155829	-0.5612597	0.1253257	-4.478409	7.52E-06	0.00161954	
AT4G26720.1	349.604943	-0.5109161	0.11695837	-4.3683587	1.25E-05	0.00212445	PP4 CATALYTIC (PP4C) SUBUNIT 1 (PP4-1);PROTEIN PHOSPHATASE X 1 (PPX1);PROTEIN PHOSPHATASE X-1 (PPX-1)
AT5G24690.1	2499.88124	-0.4776676	0.11828347	-4.0383295	5.38E-05	0.00522836	

AT2G38770.1	661.912884	-0.4694635	0.1200143	-3.9117297	9.16E-05	0.00728148	MOS4-ASSOCIATED COMPLEX 7 (MAC7);EMBRYO DEFECTIVE 2765 (EMB2765)
AT3G07660.1	895.334426	-0.4652317	0.09857877	-4.7193908	2.37E-06	0.00085142	
AT5G26360.1	1569.50491	-0.4648416	0.10229703	-4.5440377	5.52E-06	0.001414	
AT4G19600.1	392.147564	-0.4585161	0.11591837	-3.9555087	7.64E-05	0.00644981	CYCLIN-T1-4 (CYCT1;4)
AT5G55190.1	1468.11874	-0.4512512	0.11038777	-4.0878732	4.35E-05	0.0046346	RAN GTPASE 3 (ATRAN3);RAN GTPASE 3 (RAN3)
AT3G13772.1	859.906556	-0.4500497	0.11107537	-4.0517506	5.08E-05	0.00501999	TRANSMEMBRANE NINE 7 (TMN7)
AT1G73100.1	221.958687	-0.4445176	0.1137857	-3.9066212	9.36E-05	0.00729323	SET DOMAIN PROTEIN 19 (SDG19);SU(VAR)3-9 HOMOLOG 3 (SUVH3)
AT1G76810.1	2072.23026	-0.3561443	0.08174831	-4.3565955	1.32E-05	0.00217062	EUKARYOTIC TRANSLATION INITIATION FACTOR B1 (EIF5B1)
AT1G71440.1	252.125995	0.40541611	0.1062217	3.81669761	0.00013525	0.00937876	TUBULIN-FOLDING COFACTOR E (TFC E);PFIFFERLING (PFI)
AT4G22310.1	718.666166	0.46041238	0.09690955	4.75094947	2.02E-06	0.00080543	
AT3G12290.1	1594.05033	0.48925032	0.1027404	4.76200533	1.92E-06	0.00078314	METHYLENETETRAHYDROFOLATE DEHYDROGENASE/METHENYLtetrahydrofolate cyclohydrolase (MTHFD1)
AT4G17050.1	644.424381	0.52391136	0.12907809	4.05887149	4.93E-05	0.00496951	UREIDOGLYCINE AMINOHYDROLASE (UGLYAH)
AT1G54090.1	500.903748	0.53373286	0.1177666	4.53212435	5.84E-06	0.00144017	EXOCYST SUBUNIT EXO70 FAMILY PROTEIN D2 (EXO70D2);EXOCYST SUBUNIT EXO70 FAMILY PROTEIN D2 (ATEXO70D2)
AT3G57870.1	1424.96358	0.5466181	0.09744994	5.6092195	2.03E-08	3.66E-05	SUMO CONJUGATION ENZYME 1 (ATSCE1)
AT2G01110.1	2355.19755	0.55255776	0.12912554	4.27922914	1.88E-05	0.00272428	ALBINO AND PALE GREEN 2 (APG2);TWIN-ARGININE TRANSLOCATION C (TATC);(PGA2);UNFERTILIZED EMBRYO SAC 3 (UNE3)

AT2G04700.1	2016.91824	0.56880804	0.14345275	3.96512475	7.34E-05	0.00626523	
AT2G39930.1	1027.31442	0.5833256	0.15173253	3.84443326	0.00012083	0.00857562	ARABIDOPSIS THALIANA ISOAMYLASE 1 (ATISA1);ISOAMYLASE 1 (ISA1)
AT4G25840.1	211.800231	0.58475845	0.14523778	4.02621435	5.67E-05	0.00535539	GLYCEROL-3-PHOSPHATASE 1 (GPP1)
AT1G28240.1	723.64135	0.58727625	0.13799953	4.25563943	2.08E-05	0.00286471	
AT3G29400.1	399.439595	0.59646672	0.14007395	4.25822724	2.06E-05	0.00285773	EXOCYST SUBUNIT EXO70 FAMILY PROTEIN E1 (EXO70E1);EXOCYST SUBUNIT EXO70 FAMILY PROTEIN E1 (ATEXO70E1)
AT5G47840.1	1627.33908	0.59657218	0.13499446	4.41923449	9.91E-06	0.0018717	ADENOSINE MONOPHOSPHATE KINASE (AMK2)
AT1G73740.1	300.150342	0.61783715	0.14601511	4.23132346	2.32E-05	0.00304578	
AT2G43945.1	1034.28048	0.63282825	0.14960568	4.22997474	2.34E-05	0.00304578	
AT1G65960.2	1026.12238	0.65727124	0.15156324	4.33661369	1.45E-05	0.00227849	GLUTAMATE DECARBOXYLASE 2 (GAD2)
AT5G56260.1	772.100724	0.66076664	0.17013107	3.8838682	0.00010281	0.00773206	
AT5G43830.1	2878.2538	0.6621846	0.17072921	3.87856643	0.00010507	0.00786337	
AT3G15360.1	5214.75197	0.69921148	0.15436137	4.52970501	5.91E-06	0.00144017	ARABIDOPSIS THALIANA MYOSIN 4 (ATM4);THIOREDOXIN M-TYPE 4 (TRX-M4);ARABIDOPSIS THIOREDOXIN M-TYPE 4 (ATM4);(ATHM4)
AT5G59250.1	1202.99835	0.70071381	0.15947717	4.39381886	1.11E-05	0.00202224	(HP59)
AT3G56440.1	94.682078	0.71184743	0.18456675	3.85685637	0.00011486	0.00826789	HOMOLOG OF YEAST AUTOPHAGY 18 (ATG18) D (ATATG18D);HOMOLOG OF YEAST AUTOPHAGY 18 (ATG18) D (ATG18D)
AT1G69830.1	3424.4345	0.71666227	0.18587187	3.85567904	0.00011541	0.00826841	ALPHA-AMYLASE-LIKE 3 (AMY3);ALPHA-AMYLASE-LIKE 3 (ATAMY3)

AT1G13750.1	369.63084	0.72836626	0.16272809	4.47597136	7.61E-06	0.00161954	ARABIDOPSIS THALIANA PURPLE ACID PHOSPHATASE 1 (ATPAP1)
AT3G29320.1	5360.5127	0.73760398	0.18535061	3.9795066	6.91E-05	0.00602653	ALPHA-GLUCAN PHOSPHORYLASE 1 (PHS1)
AT5G49555.1	407.315909	0.78450449	0.13953481	5.62228523	1.88E-08	3.66E-05	
AT5G61660.1	764.674866	0.79786727	0.20249748	3.94013432	8.14E-05	0.00680148	
AT2G37470.1	283.074907	0.84864434	0.20432627	4.15337859	3.28E-05	0.00390156	
AT5G10750.1	221.747104	0.86088898	0.202938	4.24212797	2.21E-05	0.00301537	
AT2G36310.1	469.386119	0.89379149	0.16343357	5.46883656	4.53E-08	5.71E-05	NUCLEOSIDE HYDROLASE 1 (NSH1);URIDINE-RIBOHYDROLASE 1 (URH1)
AT3G60200.1	107.652923	0.89857778	0.22510859	3.99175248	6.56E-05	0.00590164	
AT4G18950.1	475.292965	0.90030815	0.22976178	3.91844175	8.91E-05	0.00716636	
AT3G57030.1	310.616822	0.90979639	0.22098644	4.11697841	3.84E-05	0.00423576	
AT2G16500.1	2090.42613	0.91447925	0.18862043	4.84825128	1.25E-06	0.00061818	ARGININE DECARBOXYLASE 1 (ADC1)
AT5G18600.1	1134.87656	0.94856856	0.2281272	4.1580686	3.21E-05	0.00388144	
AT4G28550.1	78.3923673	0.9755515	0.226143	4.31387008	1.60E-05	0.00247458	
AT1G57770.1	502.854936	1.00070613	0.1908195	5.24425516	1.57E-07	0.00012637	
AT2G06520.1	21678.1148	1.00073892	0.22759659	4.39698558	1.10E-05	0.00202224	PHOTOSYSTEM II SUBUNIT X (PSBX)
AT4G14930.1	530.699356	1.00098149	0.26417177	3.78913122	0.00015118	0.00994288	
AT1G61380.1	554.393269	1.00869796	0.21820968	4.62260872	3.79E-06	0.00106083	IPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE);S-DOMAIN-1 29 (SD1-29)
AT3G44720.1	613.145637	1.0155961	0.21042596	4.82638208	1.39E-06	0.00061818	AROGENATE DEHYDRATASE 4 (ADT4)
AT1G74640.1	750.420099	1.01821099	0.2451596	4.15325773	3.28E-05	0.00390156	
AT3G23400.1	2989.30473	1.02260244	0.24556917	4.16421342	3.12E-05	0.00387062	FIBRILLIN 4 (FIB4)
AT1G49840.1	181.141296	1.0300273	0.26954049	3.82141961	0.00013269	0.00928615	
AT1G18060.1	1069.31365	1.03266416	0.26530377	3.89238402	9.93E-05	0.00761712	
AT1G32220.1	1199.31382	1.03645902	0.26406369	3.92503416	8.67E-05	0.0070873	

AT3G10060.1	1133.58656	1.04762465	0.24089658	4.34885647	1.37E-05	0.0022008	
AT3G15760.1	177.054032	1.04838064	0.27645431	3.79223832	0.0001493	0.00990041	
AT1G78590.1	214.419845	1.07398646	0.24150613	4.44703602	8.71E-06	0.00175485	ARABIDOPSIS THALIANA NADH KINASE 3 (ATNADK-3);NAD(H) KINASE 3 (NADK3)
AT3G53800.1	990.826041	1.07526893	0.23362049	4.602631	4.17E-06	0.00112618	FES1B (Fes1B)
AT2G40840.1	3070.31404	1.09455466	0.27512105	3.97844753	6.94E-05	0.00602653	DISPROPORTIONATING ENZYME 2 (DPE2)
AT1G72900.1	390.160737	1.10894612	0.26252478	4.22415796	2.40E-05	0.0030988	
AT5G16400.1	1583.61433	1.11661893	0.28859162	3.86920085	0.00010919	0.00805647	THIOREDOXIN F2 (TRXF2)
AT3G16560.1	297.330564	1.12055345	0.29166555	3.84191231	0.00012208	0.00858361	
AT5G52540.1	786.573209	1.12532066	0.2677427	4.20299286	2.63E-05	0.00331829	
AT3G25597.1	45.532432	1.12979744	0.26280929	4.29892503	1.72E-05	0.00259451	
AT1G51110.1	1030.4543	1.13385024	0.23548544	4.81494835	1.47E-06	0.00063594	
AT5G42530.1	14148.703	1.15385666	0.29883867	3.86113568	0.00011286	0.00816327	
AT3G11650.1	143.255646	1.16547747	0.24103862	4.83523119	1.33E-06	0.00061818	NDR1/HIN1-LIKE 2 (NHL2)
AT1G25530.1	40.1473687	1.18000228	0.291142	4.05301288	5.06E-05	0.00501999	
AT3G23150.1	86.911085	1.18519681	0.30238899	3.91944433	8.88E-05	0.00716636	ETHYLENE RESPONSE 2 (ETR2)
AT2G25480.1	238.157495	1.20075882	0.2601942	4.6148562	3.93E-06	0.00108119	
AT2G26440.1	791.268877	1.23177614	0.29593006	4.16238942	3.15E-05	0.00387062	PECTIN METHYLESTERASE 12 (PME12)
AT3G18080.1	6124.9042	1.24140665	0.29443536	4.21622823	2.48E-05	0.00318254	B-S GLUCOSIDASE 44 (BGLU44)
AT3G56880.1	793.31252	1.2433623	0.2848709	4.36465188	1.27E-05	0.00212445	
AT3G21510.1	51.7504299	1.25196062	0.22441855	5.57868608	2.42E-08	3.66E-05	HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 1 (AHP1)
AT2G22860.1	68.7829352	1.27976255	0.3351433	3.8185533	0.00013424	0.0093514	PHYTOSULFOKINE 2 PRECURSOR (ATPSK2);PHYTOSULFOKINE 2 PRECURSOR (PSK2)
AT4G27270.1	58.1347678	1.28578201	0.26638947	4.82669993	1.39E-06	0.00061818	

AT4G34930.1	29.9424051	1.28735211	0.32269524	3.98937439	6.62E-05	0.00592585	
AT1G02360.1	80.2800159	1.28822224	0.33942697	3.79528548	0.00014747	0.00986442	
AT1G22410.1	1289.41281	1.28906041	0.3112789	4.14117506	3.46E-05	0.00399642	
AT1G72940.1	217.792287	1.31503074	0.31066643	4.23293475	2.31E-05	0.00304578	
AT2G33530.1	612.618237	1.32614411	0.29914631	4.4330953	9.29E-06	0.00182366	SERINE CARBOXYPEPTIDASE-LIKE 46 (scpl46)
AT3G62780.1	28.1776063	1.3322281	0.30911791	4.30977331	1.63E-05	0.0024954	
AT5G45750.1	426.233648	1.34708339	0.29854037	4.51223191	6.41E-06	0.00149881	RAB GTPASE HOMOLOG A1C (RABA1c);RAB GTPASE HOMOLOG A1C (AtRABA1c)
AT1G25550.1	1444.46357	1.36379661	0.29042264	4.69590329	2.65E-06	0.00089167	HRS1 HOMOLOG3 (HHO3)
AT1G19450.1	1083.40163	1.39304785	0.3563224	3.90951521	9.25E-05	0.00728148	
AT2G30230.1	150.908798	1.395338	0.31861348	4.3794067	1.19E-05	0.00211643	
AT1G70820.1	2920.75038	1.40922504	0.31623339	4.45628163	8.34E-06	0.00170359	
AT1G09390.1	214.699202	1.40957564	0.33026525	4.26801076	1.97E-05	0.00276059	
AT4G37540.1	263.894634	1.41062539	0.35184536	4.0092198	6.09E-05	0.00561538	LOB DOMAIN-CONTAINING PROTEIN 39 (LBD39)
AT3G55840.1	68.3482695	1.4115444	0.36117429	3.90820839	9.30E-05	0.00728303	
AT3G17390.1	5736.86443	1.41358074	0.36353109	3.88847272	0.00010088	0.0076631	METHIONINE OVER-ACCUMULATOR 3 (MTO3)
AT4G03400.1	1285.80879	1.41554291	0.37296844	3.79534236	0.00014744	0.00986442	DWARF IN LIGHT 2 (DFL2); (GH3-10)
AT5G67300.1	1847.43681	1.41587781	0.36627253	3.86564014	0.0001108	0.00805647	MYB DOMAIN PROTEIN R1 (MYBR1);ARABIDOPSIS THALIANA MYB DOMAIN PROTEIN 44 (ATMYB44)
AT5G65660.1	676.248742	1.41832281	0.34820336	4.07325999	4.64E-05	0.00473794	
AT1G25560.1	1376.54219	1.42736161	0.37430101	3.81340567	0.00013707	0.00941823	TEMPRANILLO 1 (TEM1);ETHYLENE RESPONSE DNA BINDING FACTOR 1 (EDF1); (ATTEM1)
AT2G16660.1	6523.70942	1.465857	0.38143993	3.8429564	0.00012156	0.00858361	
AT5G56230.1	39.9127754	1.46821224	0.295535	4.96798095	6.77E-07	0.00037879	PRENYLATED RAB ACCEPTOR 1.G2 (PRA1.G2)

AT3G25600.1	176.038445	1.46888489	0.26330639	5.57861477	2.42E-08	3.66E-05	
AT3G07195.1	127.678786	1.48604646	0.36201713	4.10490649	4.04E-05	0.00439892	
AT5G24210.1	1584.80616	1.48992808	0.35930117	4.14673874	3.37E-05	0.00398291	
AT4G29905.1	1434.77932	1.4996634	0.3648263	4.11062309	3.95E-05	0.00432251	
AT3G23450.1	732.022896	1.51609222	0.39737791	3.8152403	0.00013605	0.00939121	
AT1G32920.1	490.393747	1.53007821	0.32858907	4.65650974	3.22E-06	0.00095331	
AT3G28340.1	128.797022	1.53090915	0.40272834	3.80134445	0.00014391	0.00975576	GALACTINOL SYNTHASE 8 (GolS8);GALACTURONOSYLTRANSFERASE-LIKE 10 (GATL10)
AT1G15820.1	46630.4323	1.53155748	0.37101949	4.12797036	3.66E-05	0.00414663	LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 6 (LHCB6); (CP24)
AT1G12090.1	11560.2366	1.53936991	0.3269928	4.70765697	2.51E-06	0.00087901	EXTENSIN-LIKE PROTEIN (ELP)
AT2G30040.1	95.4368885	1.54588455	0.35355844	4.37235939	1.23E-05	0.00212267	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 14 (MAPKKK14)
AT2G28630.1	1225.66408	1.5575415	0.35464029	4.39189104	1.12E-05	0.00202224	3-KETOACYL-COA SYNTHASE 12 (KCS12)
AT5G52810.1	244.363212	1.56395925	0.35361643	4.42275613	9.74E-06	0.00186474	SAR DEFICIENT 4 (SARD4)
AT4G13494.1	54.5782903	1.5645747	0.30766629	5.08529777	3.67E-07	0.0002609	MICRORNA863A (MIR863A)
AT4G27970.1	17.0029718	1.57424601	0.41406077	3.80196852	0.00014355	0.00975576	SLAC1 HOMOLOGUE 2 (SLAH2)
AT1G66400.1	26.8635761	1.57780022	0.40065918	3.93801087	8.22E-05	0.00682424	CALMODULIN LIKE 23 (CML23)
AT1G73540.1	382.666577	1.5780731	0.40206205	3.92494919	8.67E-05	0.0070873	NUDIX HYDROLASE HOMOLOG 21 (NUDT21);NUDIX HYDROLASE HOMOLOG 21 (atnudt21)
AT1G66180.1	758.364325	1.5941222	0.35322989	4.51298786	6.39E-06	0.00149881	
AT5G06320.1	2244.72015	1.59423289	0.290678	5.48453237	4.15E-08	5.70E-05	NDR1/HIN1-LIKE 3 (NHL3)
AT1G24530.1	213.665748	1.60417437	0.35559394	4.51125335	6.44E-06	0.00149881	
AT3G52748.1	10.6820338	1.60740252	0.39507634	4.06858714	4.73E-05	0.00479879	SHORT OPEN READING FRAME 4 (SORF4)

AT5G61600.1	572.992798	1.6077349	0.36259842	4.43392692	9.25E-06	0.00182366	ETHYLENE RESPONSE FACTOR 104 (ERF104)
AT1G04250.1	142.25735	1.6184032	0.39727263	4.07378476	4.63E-05	0.00473794	AUXIN RESISTANT 3 (AXR3);INDOLE-3-ACETIC ACID INDUCIBLE 17 (IAA17)
AT4G11521.1	87.9317671	1.63169922	0.30060185	5.42810773	5.70E-08	6.62E-05	
AT1G18300.1	144.943886	1.64694076	0.41510938	3.96748623	7.26E-05	0.00623874	NUDIX HYDROLASE HOMOLOG 4 (NUDT4);NUDIX HYDROLASE HOMOLOG 4 (atnudt4)
AT5G64310.1	173.412178	1.65109098	0.37772037	4.37119918	1.24E-05	0.00212267	ARABINOGLACTAN PROTEIN 1 (AGP1)
AT4G15690.1	196.128339	1.65121068	0.38997859	4.23410596	2.29E-05	0.00304578	GLUTAREDOXIN 5 (GRXS5)
AT4G39830.1	219.687912	1.66511327	0.38895633	4.28097739	1.86E-05	0.00272428	
AT1G69900.1	82.0310425	1.66835537	0.33418215	4.99235333	5.96E-07	0.00037378	
AT1G72920.1	90.1369659	1.68874188	0.41823282	4.03780331	5.40E-05	0.00522836	
AT2G16060.1	28.1790296	1.69319275	0.35999234	4.70341322	2.56E-06	0.00087901	CLASS I HEMOGLOBIN (GLB1);HEMOGLOBIN 1 (AHB1);HEMOGLOBIN 1 (HB1); (NSHB1); (ATGLB1); (ARATH GLB1)
AT2G43150.1	773.429751	1.70481574	0.31932189	5.3388628	9.35E-08	0.00010099	
AT1G30730.1	43.6564862	1.70763383	0.41394523	4.12526516	3.70E-05	0.00414663	ARABIDOPSIS THALIANA BERBERINE BRIDGE ENZYME 11 (ATBBE11)
AT1G32928.1	63.6305156	1.71289191	0.38919874	4.40107267	1.08E-05	0.00201032	
AT1G07610.1	103.405793	1.72140928	0.39579038	4.34929546	1.37E-05	0.0022008	METALLOTHIONEIN 1C (MT1C)
AT3G10930.1	58.9298791	1.72389803	0.43571506	3.95648026	7.61E-05	0.00644981	IDA-LIKE7 (IDL7)
AT1G77760.1	1560.96431	1.72623028	0.42380944	4.07312844	4.64E-05	0.00473794	NITRATE REDUCTASE 1 (NIA1);NITRATE REDUCTASE 1 (NR1); (GNR1)
AT2G44080.1	239.649368	1.73117058	0.37341329	4.63607112	3.55E-06	0.00101282	ARGOS-LIKE (ARL)
AT4G15700.1	217.290941	1.73496567	0.38637229	4.49039876	7.11E-06	0.00156377	GLUTAREDOXIN 3 (GRXS3)
AT3G26960.1	120.816255	1.75144092	0.40136679	4.36369164	1.28E-05	0.00212445	

AT5G25190.1	541.48686	1.75450229	0.41074789	4.27148213	1.94E-05	0.00274336	ETHYLENE AND SALT INDUCIBLE 3 (ESE3)
AT2G29170.1	50.5324158	1.76214867	0.44192194	3.98746595	6.68E-05	0.00593856	
AT5G44390.1	57.9010003	1.76877268	0.43656616	4.05155697	5.09E-05	0.00501999	ARABIDOPSIS THALIANA BERBERINE BRIDGE ENZYME 25 (ATBBE25)
AT4G33960.1	110.973167	1.76879179	0.34865851	5.07313527	3.91E-07	0.0002609	
AT5G59820.1	136.838522	1.76946961	0.40954293	4.3205961	1.56E-05	0.00242508	RESPONSIVE TO HIGH LIGHT 41 (RHL41); (ATZAT12); (ZAT12)
AT4G15670.1	103.494416	1.76976073	0.44121629	4.01109563	6.04E-05	0.00560513	MONOTHIOL GLUTAREDOXIN-S7 (GRXS7)
AT1G72910.1	391.104041	1.77485164	0.39473775	4.49628048	6.92E-06	0.00156377	
AT1G35140.1	440.181988	1.79917384	0.46544135	3.86552214	0.00011085	0.00805647	EXORDIUM LIKE 7 (EXL7);EXORDIUM LIKE 1 (EXL1);PHOSPHATE-INDUCED 1 (PHI-1)
AT1G02820.1	57.2112488	1.80183933	0.46596964	3.8668599	0.00011025	0.00805647	(ATLEA3);LATE EMBRYOGENESIS ABUNDANT 3 (LEA3)
AT4G16880.1	185.880751	1.80250371	0.4422435	4.07581732	4.59E-05	0.00473794	
AT5G37770.1	327.377292	1.80359214	0.39595	4.55510075	5.24E-06	0.00138866	CALMODULIN-LIKE 24 (CML24); (ATCML24);TOUCH 2 (TCH2)
AT1G27730.1	598.546196	1.80747676	0.43454951	4.15942652	3.19E-05	0.00388144	(ZAT10);SALT TOLERANCE ZINC FINGER (STZ)
AT1G73600.2	52.2012838	1.80900995	0.46567115	3.88473697	0.00010244	0.00773206	N-MYRISTOYL TRANSFERASE (NMT)
AT1G18350.1	4.96326154	1.81266382	0.45086673	4.02039827	5.81E-05	0.00542158	MAP KINASE KINASE 7 (ATMKK7)
AT1G11185.1	4.13750993	1.81353095	0.45672913	3.97069253	7.17E-05	0.00619054	SHORT OPEN READING FRAME 2 (SORF2)
AT4G30280.1	42.609534	1.81649713	0.46449681	3.9106773	9.20E-05	0.00728148	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 18 (XTH18)
AT2G04515.1	8.46386114	1.82740067	0.44732636	4.08516208	4.40E-05	0.00465625	
AT4G11280.1	392.062158	1.8293557	0.39239725	4.66199923	3.13E-06	0.00095331	1-AMINO CYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6); (ATACS6)

AT2G27660.1	84.7762587	1.83493888	0.40867142	4.49001027	7.12E-06	0.00156377	
AT3G50800.1	41.5083084	1.83692885	0.44792941	4.10093376	4.11E-05	0.00444317	
AT5G54490.1	75.4298215	1.84250873	0.38928897	4.73301041	2.21E-06	0.00083239	PINOID-BINDING PROTEIN 1 (PBP1)
AT1G36370.1	1459.53776	1.84555224	0.44630406	4.13519033	3.55E-05	0.00406167	MORE SULPHUR ACCUMULATION1 (MSA1);SERINE HYDROXYMETHYLTRANSFERASE 7 (SHM7)
AT3G57450.1	270.504456	1.8595202	0.3604656	5.15866205	2.49E-07	0.000188	
AT5G01180.1	57.2354308	1.86031549	0.27497514	6.76539521	1.33E-11	2.01E-07	PEPTIDE TRANSPORTER 5 (PTR5); (ATNPF8.2);NRT1/ PTR FAMILY 8.2 (NPF8.2);ARABIDOPSIS THALIANA PEPTIDE TRANSPORTER 5 (ATPTR5)
AT5G51190.1	106.358593	1.8801586	0.43957275	4.27724108	1.89E-05	0.00272428	ETHYLENE RESPONSE FACTOR 105 (ERF105)
AT1G07135.1	548.948342	1.90605171	0.43592278	4.3724526	1.23E-05	0.00212267	
AT1G30370.1	8.97040438	1.90736953	0.46064468	4.14065243	3.46E-05	0.00399642	DAD1-LIKE ACYLHYDROLASE (DLAH)
AT1G21910.1	334.006149	1.91440908	0.46491708	4.11774308	3.83E-05	0.00423576	DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 26 (DREB26)
AT4G24570.1	421.219051	1.91462081	0.45941091	4.16755627	3.08E-05	0.0038465	DICARBOXYLATE CARRIER 2 (DIC2)
AT1G33760.1	38.4282869	1.97300743	0.46607808	4.23321227	2.30E-05	0.00304578	ETHYLENE RESPONSE FACTOR022 (ERF022)
AT3G49940.1	458.925822	1.98282063	0.34236377	5.79156089	6.97E-09	2.11E-05	LOB DOMAIN-CONTAINING PROTEIN 38 (LBD38)
AT5G24030.1	265.043444	2.01552983	0.44409577	4.53850266	5.67E-06	0.00142743	SLAC1 HOMOLOGUE 3 (SLAH3)
AT5G26220.1	157.557726	2.02093811	0.45627777	4.42918383	9.46E-06	0.00183323	GAMMA-GLUTAMYL CYCLOTRANSFERASE 2;1 (GGCT2;1); (ATGGCT2;1)
AT3G46090.1	120.209441	2.04726572	0.43724825	4.6821587	2.84E-06	0.00093288	ZINC FINGER OF ARABIDOPSIS THALIANA 7 (ZAT7)

AT5G48850.1	226.849539	2.05168356	0.45872885	4.47254093	7.73E-06	0.00162289	SULPHUR DEFICIENCY-INDUCED 1 (SDI1);SULPHUR DEFICIENCY-INDUCED 1 (ATSDI1)
AT2G37430.1	11.057873	2.05517701	0.434601	4.72888242	2.26E-06	0.00083239	ZINC FINGER OF ARABIDOPSIS THALIANA 11 (ZAT11)
AT1G78090.1	16.2747158	2.08554982	0.44630263	4.67294989	2.97E-06	0.00093506	TREHALOSE-6-PHOSPHATE PHOSPHATASE B (TPPB);ARABIDOPSIS THALIANA TREHALOSE-6-PHOSPHATE PHOSPHATASE B (ATTPPB)
AT5G44568.1	291.161209	2.08655116	0.39211697	5.32124671	1.03E-07	0.00010187	
AT4G27280.1	390.542416	2.31018348	0.39513985	5.84649579	5.02E-09	1.90E-05	
AT3G50060.1	305.371808	2.38835861	0.41954857	5.69268686	1.25E-08	3.15E-05	MYB DOMAIN PROTEIN 77 (MYB77)

Appendix 8.4 Cold LO differentially expressed genes (FDR 0.01). Base mean: mean of normalisation counts; log2foldchange: log fold change between control vs stress; lfcSE: standard error of log2fold change; stat: Wald statistic; pvalue: Wald test p-value between control vs stress; padj: adjusted p-values.

Gene ID	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Primary Gene Symbol
AT3G13400	15.8773627	-2.3525305	0.45088389	-5.2175971	1.81E-07	0.00011925	SKU5 SIMILAR 13 (sks13)
AT3G28830	10.8886358	-2.1794845	0.46462261	-4.6908705	2.72E-06	0.00104054	
AT3G01270	9.57466389	-2.0934741	0.44574607	-4.6965622	2.65E-06	0.00104054	
AT5G47350	55.8505333	-2.0923412	0.45346944	-4.6140732	3.95E-06	0.00132534	
AT1G43640	3.91911488	-2.0910502	0.46519139	-4.495032	6.96E-06	0.0017046	TUBBY LIKE PROTEIN 5 (TLP5)
AT5G04180	3.83407016	-2.0758771	0.46273855	-4.4860691	7.25E-06	0.0017046	ALPHA CARBONIC ANHYDRASE 3 (ACA3)
AT3G05610	3.93318114	-2.002566	0.44259214	-4.5246307	6.05E-06	0.00160494	
AT1G02790	11.4206606	-1.9260337	0.42563502	-4.5250828	6.04E-06	0.00160494	POLYGALACTURONASE 4 (PGA4)
AT1G56650	23.7212023	-1.9218562	0.45856222	-4.1910479	2.78E-05	0.00466002	PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1)
AT1G18710	183.176256	-1.8962998	0.40694037	-4.659896	3.16E-06	0.00113116	MYB DOMAIN PROTEIN 47 (MYB47)
AT4G35010	5.51820434	-1.8663971	0.45072818	-4.1408484	3.46E-05	0.00542005	BETA-GALACTOSIDASE 11 (BGAL11)
AT5G14380	8.62956213	-1.8155815	0.44467056	-4.082981	4.45E-05	0.00643731	ARABINOGLACTAN PROTEIN 6 (AGP6)
AT1G52000	1340.09577	-1.8077059	0.42609223	-4.2425226	2.21E-05	0.00408621	
AT2G26450	2.52860883	-1.8067224	0.44592741	-4.0516066	5.09E-05	0.00690907	
AT2G47050	4.83401693	-1.7942532	0.44378146	-4.0431009	5.27E-05	0.00690907	
AT4G15530	8.27281972	-1.7908616	0.44318412	-4.0408976	5.32E-05	0.00690907	PYRUVATE ORTHOPHOSPHATE DIKINASE (PPDK)
AT1G56600	832.749395	-1.7887926	0.44525783	-4.0174309	5.88E-05	0.00711527	GALACTINOL SYNTHASE 2 (GolS2)

AT3G01700	2.96138421	-1.7788148	0.43145271	-4.12285	3.74E-05	0.00575205	ARABINOGALACTAN PROTEIN 11 (AGP11)
AT5G07430	4.53217574	-1.7721606	0.41559374	-4.2641658	2.01E-05	0.00383732	
AT1G03050	2.20553027	-1.772081	0.42671735	-4.1528215	3.28E-05	0.00519347	
AT2G47040	16.6267055	-1.7648136	0.4185268	-4.2167277	2.48E-05	0.00447996	VANGUARD1 (VGD1)
AT5G43840	13.9578818	-1.7519178	0.44293079	-3.9552857	7.64E-05	0.00849501	HEAT SHOCK TRANSCRIPTION FACTOR A6A (HSFA6A)
AT2G47030	6.13492609	-1.7351609	0.44219371	-3.9239837	8.71E-05	0.00895299	VANGUARD 1 HOMOLOG 1(VGDH1)
AT5G67620	11.9536108	-1.6894359	0.40202545	-4.2023108	2.64E-05	0.00461578	
AT3G07820	7.69956738	-1.6838936	0.39712197	-4.2402429	2.23E-05	0.00408621	
AT3G07850	6.39932196	-1.668054	0.4109295	-4.0592218	4.92E-05	0.00674828	
AT3G01240	3.89738423	-1.6502029	0.41349623	-3.9908536	6.58E-05	0.00758765	
AT1G75410	8.0496177	-1.6198657	0.41476617	-3.9054915	9.40E-05	0.00926095	BEL1-LIKE HOMEO DOMAIN 3 (BLH3)
AT3G61890	237.268363	-1.6157328	0.3937411	-4.1035412	4.07E-05	0.00608351	HOMEobox 12 (HB-12)
AT1G27200	733.236124	-1.6100151	0.35136564	-4.5821643	4.60E-06	0.0013999	
AT3G62230	3.48582086	-1.5846482	0.40404341	-3.9219752	8.78E-05	0.00897189	DUO1-ACTIVATED F-BOX 1 (DAF1)
AT5G07410	6.51043855	-1.5600657	0.38653853	-4.0359902	5.44E-05	0.00690907	PECTIN METHYLESTERASE 48 (PME48)
AT1G51090	185.886032	-1.5356839	0.39084799	-3.929108	8.53E-05	0.0088753	HEAVY METAL ASSOCIATED DOMAIN 1 (ATHMAD1)
AT2G35950	23.3795935	-1.5094684	0.38819559	-3.8884223	0.0001009	0.00981934	EMBRYO SAC DEVELOPMENT ARREST 12 (EDA12)
AT1G22160	215.053798	-1.4989744	0.32234464	-4.6502228	3.32E-06	0.00116031	
AT1G78070	1451.02883	-1.4491793	0.28676882	-5.0534757	4.34E-07	0.0002303	
AT1G08230	121.987526	-1.4264443	0.34983128	-4.0775207	4.55E-05	0.00645382	

AT2G07718	28.3635713	-1.3995342	0.32796864	-4.2672806	1.98E-05	0.00382867	
AT5G03640	37.5022588	-1.3858379	0.30341549	-4.5674591	4.94E-06	0.0013999	
AT3G11080	139.401455	-1.2061258	0.29573125	-4.0784523	4.53E-05	0.00645382	RECEPTOR LIKE PROTEIN 35 (RLP35)
AT4G01910	32.1987191	-1.1957635	0.29097806	-4.109463	3.97E-05	0.005984	
AT3G14067	4958.1641	-1.1592837	0.27737869	-4.1794258	2.92E-05	0.00485512	SENESCENCE-ASSOCIATED SUBILISIN PROTEASE (SASP)
AT1G78670	786.439789	-1.1460815	0.26267931	-4.3630446	1.28E-05	0.00270458	GAMMA-GLUTAMYL HYDROLASE 3 (GGH3)
AT1G55110	613.351071	-1.1304139	0.21291992	-5.3091035	1.10E-07	7.88E-05	INDETERMINATE(ID)-DOMAIN 7 (IDD7)
AT1G80310	593.369722	-1.1113082	0.26626977	-4.1736178	3.00E-05	0.00493082	MOLYBDATE TRANSPORTER 2 (MOT2)
AT1G12240	2491.18071	-1.1019694	0.2737132	-4.0260002	5.67E-05	0.00696111	BETA-FRUCTOFURANOSIDASE 4 (ATBETAFRUCT4)
AT4G23320	106.55699	-1.0070405	0.25196166	-3.9968007	6.42E-05	0.00758765	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 24 (CRK24)
AT4G15540	779.018123	-1.0062036	0.25912732	-3.8830474	0.00010316	0.00997999	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 38 (UMAMIT38)
AT3G54680	163.817722	-0.997963	0.2440413	-4.0893199	4.33E-05	0.00635324	
AT3G18490	4970.64552	-0.9589387	0.24278209	-3.9497918	7.82E-05	0.00851968	ASPARTIC PROTEASE IN GUARD CELL 1 (ASPG1)
AT5G39030	541.100854	-0.9446551	0.23985648	-3.9384182	8.20E-05	0.00871139	
AT2G37200	321.076883	-0.8984097	0.21351809	-4.2076513	2.58E-05	0.004613	CASP-LIKE PROTEIN 5A1 (CASPL5A1)

AT3G26180	253.537958	-0.8724949	0.19226562	-4.5379661	5.68E-06	0.0015694	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 20 (CYP71B20)
AT1G29060	129.382422	-0.8491219	0.17338804	-4.8972344	9.72E-07	0.00045673	ARABIDOPSIS QC-SNARE 12 (ATSFT12)
AT3G27020	1406.17411	-0.7901982	0.17583632	-4.4939418	6.99E-06	0.0017046	YELLOW STRIPE LIKE 6 (YSL6)
AT3G16940	1176.7841	-0.7119214	0.17759701	-4.0086338	6.11E-05	0.00727852	
AT4G23630	2478.2552	-0.6830069	0.17398115	-3.9257524	8.65E-05	0.00894335	VIRB2-INTERACTING PROTEIN 1 (BTI1)
AT2G05710	5608.17077	-0.6186904	0.1580615	-3.9142382	9.07E-05	0.009095	ACONITASE 3 (ACO3)
AT5G58590	353.634308	-0.5598003	0.14034611	-3.9887126	6.64E-05	0.00758765	RAN BINDING PROTEIN 1 (RANBP1)
AT3G07660	895.334426	-0.4298739	0.10921944	-3.9358733	8.29E-05	0.00871139	
AT3G57870	1424.96358	0.48948159	0.10850806	4.51101613	6.45E-06	0.00164656	SUMO CONJUGATION ENZYME 1 (SCE1)
AT3G53580	988.508998	0.57691186	0.12615491	4.57304335	4.81E-06	0.0013999	
AT5G54800	535.553989	0.65746143	0.12759749	5.15262057	2.57E-07	0.00015647	GLUCOSE 6-PHOSPHATE/PHOSPHATE TRANSLOCATOR 1 (GPT1)
AT4G14870	662.226526	0.67311268	0.16025516	4.20025575	2.67E-05	0.00461578	(SECE1)
AT5G50210	730.325849	0.67498923	0.15219305	4.43508574	9.20E-06	0.00201828	QUINOLINATE SYNTHASE (QS)
AT2G29560	277.004679	0.74103957	0.18573838	3.9896954	6.62E-05	0.00758765	CYTOSOLIC ENOLASE (ENOC)
AT5G57460	593.502928	0.77526407	0.16957743	4.57174086	4.84E-06	0.0013999	
AT1G31180	608.183248	0.78606172	0.20152354	3.90059498	9.60E-05	0.00939403	ISOPROPYLMALATE DEHYDROGENASE 3 (IMD3)
AT1G45180	178.234669	0.79079669	0.20095904	3.93511376	8.32E-05	0.00871139	
AT1G52260	137.333531	0.80619228	0.20201872	3.99068111	6.59E-05	0.00758765	PDI-LIKE 1-5 (PDIL1-5)

AT2G26540	481.738918	0.8565892	0.20918946	4.09480097	4.23E-05	0.00626069	UROPORPHYRINOGEN III (HEMD)
AT5G14910	2183.74094	0.86208545	0.21744425	3.96462742	7.35E-05	0.00828103	
AT2G37470	283.074907	0.88125389	0.22525739	3.91220862	9.15E-05	0.00911621	
AT1G35780	190.536609	0.97071592	0.24113951	4.02553657	5.68E-05	0.00696111	
AT2G20680	102.090443	0.9796442	0.24278879	4.0349648	5.46E-05	0.00690907	MANNOSIDASE 5-2(MAN5-2)
AT2G34680	1069.4407	0.9829998	0.20893756	4.70475388	2.54E-06	0.00104054	AUXIN-INDUCED IN ROOT CULTURES 9 (AIR9)
AT5G61660	764.674866	1.00175043	0.22326529	4.4868167	7.23E-06	0.0017046	
AT4G27435	67.7527077	1.02310994	0.26105274	3.91916948	8.89E-05	0.00902094	
AT1G27500	95.1050552	1.07153846	0.25730337	4.16449439	3.12E-05	0.00507384	KINESIN LIGHT CHAIN-RELATED 3 (KLCR3)
AT3G20670	181.922722	1.11166788	0.23993923	4.63312266	3.60E-06	0.00123418	HISTONE H2A 13 (HTA13)
AT3G01450	95.9280843	1.11987564	0.24354673	4.59819625	4.26E-06	0.00134791	
AT1G48280	71.5688473	1.12570989	0.26550281	4.23991699	2.24E-05	0.00408621	
AT1G64510	2720.31592	1.13817481	0.29107955	3.91018478	9.22E-05	0.00913755	
AT1G23090	814.083009	1.13991416	0.28957523	3.9365044	8.27E-05	0.00871139	SULFATE TRANSPORTER 3.3 (SULTR3;3)
AT2G24395	106.973082	1.1649676	0.28948122	4.02432883	5.71E-05	0.00696111	
AT5G52970	741.941871	1.214666	0.30403034	3.99521316	6.46E-05	0.00758765	
AT3G27160	2922.59831	1.24326368	0.28651307	4.33929136	1.43E-05	0.00293873	GLUCOSE HYPERSENSITIVE 1 (GHS1)
AT4G11080	45.6995096	1.26667939	0.3235625	3.91479051	9.05E-05	0.009095	3XHIGH MOBILITY GROUP-BOX1 (3xHMG-box1)
AT1G14890	178.413499	1.27437289	0.32217498	3.95553029	7.64E-05	0.00849501	
AT4G18670	635.546536	1.29497005	0.32174122	4.02488079	5.70E-05	0.00696111	
AT5G44030	181.12648	1.31068946	0.2318896	5.65221326	1.58E-08	1.74E-05	CELLULOSE SYNTHASE A4 (CESA4)

AT1G25530	40.1473687	1.33895146	0.33121378	4.04255967	5.29E-05	0.00690907	
AT3G14240	1095.56188	1.36712108	0.2911798	4.69510963	2.66E-06	0.00104054	
AT5G04680	27.3441394	1.39465387	0.34636127	4.02658725	5.66E-05	0.00696111	
AT2G41830	213.498142	1.39869285	0.34390871	4.06704694	4.76E-05	0.00667267	
AT3G15520	553.37219	1.4102885	0.35126026	4.01493893	5.95E-05	0.00713838	
AT1G23460	110.257796	1.42989235	0.30173323	4.73892902	2.15E-06	0.00092991	
AT1G08340	14.5081552	1.43524349	0.3641119	3.94176486	8.09E-05	0.00869479	
AT4G09950	35.4187522	1.45189668	0.34610293	4.19498528	2.73E-05	0.00465372	
AT2G17880	241.79782	1.45511592	0.35245399	4.12852736	3.65E-05	0.00566481	DNA J PROTEIN C24 (DJC24)
AT2G38080	148.216796	1.47272156	0.32465342	4.53628853	5.73E-06	0.0015694	IRREGULAR XYLEM 12 (IRX12)
AT2G44040	164.790386	1.48292816	0.3381807	4.38501714	1.16E-05	0.00247723	
AT5G48060	25.9863428	1.4859317	0.34378267	4.32229955	1.54E-05	0.00313531	
AT1G08160	65.5929498	1.49314859	0.36925236	4.04370764	5.26E-05	0.00690907	
AT4G03190	484.221848	1.49495558	0.31520459	4.74281034	2.11E-06	0.00092991	GRR1-LIKE PROTEIN 1 (GRH1)
AT5G46530	20.4941964	1.49503551	0.36623648	4.08215893	4.46E-05	0.00643731	
AT3G17380	22.2069769	1.49610909	0.37065924	4.03634641	5.43E-05	0.00690907	
AT3G16180	382.73377	1.50705361	0.37212685	4.04983845	5.13E-05	0.00690907	NITRATE TRANSPORTER 1.12 (NRT1.12)
AT4G18290	49.8631889	1.51013637	0.32277869	4.67855039	2.89E-06	0.00105594	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2 (KAT2)
AT1G53250	79.2016557	1.52349716	0.38637992	3.94300294	8.05E-05	0.00869479	
AT2G39900	306.109396	1.53457442	0.33327835	4.60448279	4.13E-06	0.00133803	WLIM2A (WLIM2a)
AT3G01680	464.659451	1.57013068	0.24463897	6.41815434	1.38E-10	3.78E-07	SIEVE-ELEMENT-OCCLUSION-RELATED 1 (SEOR1)
AT1G62380	2248.12502	1.57063944	0.30543688	5.14227182	2.71E-07	0.00015944	ACC OXIDASE 2 (ACO2)

AT3G10680	14.6945947	1.57954241	0.39972413	3.95158129	7.76E-05	0.00851258	SIEVE ELEMENT-LINING CHAPERONE 1 (SLI1)
AT3G42660	89.5874165	1.58167192	0.3892096	4.06380504	4.83E-05	0.00667267	ENHANCER OF LHP1 (EOL1)
AT1G52700	28.0334092	1.58675758	0.36562451	4.3398556	1.43E-05	0.00293873	
AT5G56720	16.1988156	1.59238705	0.38254773	4.16258398	3.15E-05	0.00507384	CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 3 (c-NAD-MDH3)
AT4G02100	87.6988261	1.5931163	0.37164501	4.2866613	1.81E-05	0.00358997	
AT1G74670	3468.6533	1.61119292	0.35088912	4.59174375	4.40E-06	0.00136404	GA-STIMULATED ARABIDOPSIS 6 (GASA6)
AT5G51890	168.42375	1.61150468	0.2956751	5.45025504	5.03E-08	4.60E-05	PEROXIDASE 66 (PRX66)
AT1G63310	48.658132	1.61883275	0.36364777	4.45165037	8.52E-06	0.00194652	
AT3G62160	33.158041	1.6346995	0.36796482	4.44254294	8.89E-06	0.00200297	
AT5G60660	18.1732367	1.63599386	0.40527856	4.03671458	5.42E-05	0.00690907	PLASMA MEMBRANE INTRINSIC PROTEIN 2;4 (PIP2;4)
AT3G01670	488.580719	1.6366682	0.27676487	5.91356907	3.35E-09	5.01E-06	SIEVE ELEMENT OCCLUSION A (SEOA)
AT1G32100	76.7069083	1.64280507	0.34809023	4.71948059	2.36E-06	0.00099714	PINORESINOL REDUCTASE 1 (PRR1)
AT1G50240	29.8018038	1.64712962	0.36699155	4.48819501	7.18E-06	0.0017046	FUSED (FU)
AT4G18780	74.0594216	1.65294083	0.37094976	4.45596952	8.35E-06	0.00193461	IRREGULAR XYLEM 1 (IRX1)
AT5G07080	59.8842253	1.66436223	0.39116531	4.25488195	2.09E-05	0.00395407	
AT1G09890	16.3631637	1.67501217	0.42377781	3.95257166	7.73E-05	0.00851258	
AT1G77700	19.2232985	1.68944161	0.37466573	4.50919704	6.51E-06	0.00164656	
AT1G06830	92.1004468	1.69431795	0.42692382	3.9686658	7.23E-05	0.00819811	CEP DOWNSTREAM 1 (CEPD1)
AT5G04310	64.4099524	1.69814725	0.38263884	4.43798979	9.08E-06	0.00201816	

AT5G05940	24.1261732	1.70473501	0.41939709	4.06472779	4.81E-05	0.00667267	ROP (RHO OF PLANTS) GUANINE NUCLEOTIDE EXCHANGE FACTOR 5 (ROPGEF5)
AT1G63300	113.524286	1.71416304	0.40874905	4.19368077	2.74E-05	0.00465372	
AT2G27140	36.3603489	1.73897354	0.34694725	5.01221308	5.38E-07	0.00027655	
AT3G20570	124.594111	1.73988023	0.29353546	5.92732558	3.08E-09	5.01E-06	EARLY NODULIN-LIKE PROTEIN 9 (ENODL9)
AT3G16920	70.5836999	1.74518267	0.3081735	5.66298753	1.49E-08	1.74E-05	CHITINASE-LIKE PROTEIN 2 (CTL2)
AT3G25100	20.5012381	1.75973264	0.42743684	4.1169419	3.84E-05	0.0058468	CELL DIVISION CYCLE 45 (CDC45)
AT4G00890	12.4268854	1.76111473	0.41106656	4.28425684	1.83E-05	0.00358997	
AT4G24430	14.6740279	1.76280389	0.42395044	4.15804234	3.21E-05	0.0051255	
AT5G17420	79.4150762	1.76351348	0.33516484	5.26163028	1.43E-07	9.78E-05	IROQUOIS HOMEOBOX 3 (IRX3).
AT5G55820	28.028558	1.76463294	0.42007435	4.20076336	2.66E-05	0.00461578	WYRD (WYR)
AT4G35970	22.9349424	1.77090493	0.35044019	5.05337283	4.34E-07	0.0002303	ASCORBATE PEROXIDASE 5 (APX5)
AT1G06490	39.0003825	1.79398189	0.38967645	4.60377287	4.15E-06	0.00133803	CALLOSE SYNTHASE 7 (CalS7)
AT5G04890	41.1345426	1.79814939	0.33351642	5.39148679	6.99E-08	5.63E-05	RESTRICTED TEV MOVEMENT 2 (RTM2)
AT1G29520	100.75377	1.79940977	0.31522321	5.70836707	1.14E-08	1.44E-05	
AT3G05620	13.0127941	1.8211172	0.40301973	4.51868004	6.22E-06	0.0016245	
AT2G46630	164.055032	1.84219831	0.41936586	4.39281897	1.12E-05	0.00242139	
AT3G20450	11.2428179	1.86874967	0.40899322	4.56914584	4.90E-06	0.0013999	
AT5G57390	17.9239497	1.88238768	0.38292407	4.91582498	8.84E-07	0.00042767	AINTEGUMENTA-LIKE 5 (AIL5)
AT5G60020	89.7071034	1.88299282	0.33572282	5.60877217	2.04E-08	2.09E-05	LACCASE 17 (LAC17)
AT5G04200	39.1957617	1.89209828	0.38089357	4.96752493	6.78E-07	0.00033798	METACASPASE 9 (MC9)
AT1G70500	12.8225934	1.9235213	0.41088285	4.68143483	2.85E-06	0.00105594	
AT4G19050	41.9520601	1.94390064	0.33949251	5.72590146	1.03E-08	1.41E-05	

AT2G43590	45.2658664	1.99009303	0.46304802	4.29781132	1.72E-05	0.00345975	
AT1G26820	37.3118845	2.04071887	0.4186067	4.87502677	1.09E-06	0.00049704	RIBONUCLEASE 3 (RNS3)
AT1G43790	95.869322	2.05671808	0.37827458	5.43710368	5.42E-08	4.69E-05	TRACHEARY ELEMENT DIFFERENTIATION-RELATED 6 (TED6)
AT1G20850	313.370071	2.08639077	0.28417123	7.34201973	2.10E-13	2.29E-09	XYLEM CYSTEINE PEPTIDASE 2 (XCP2)
AT5G45530	22.8507435	2.11078032	0.39424028	5.35404525	8.60E-08	6.43E-05	
AT3G21550	70.252233	2.11951778	0.33882471	6.25549952	3.96E-10	9.31E-07	DUF679 DOMAIN MEMBRANE PROTEIN 2 (DMP2)
AT5G60720	21.9888468	2.17137127	0.39486244	5.49905757	3.82E-08	3.69E-05	
AT5G48920	32.6391702	2.20857237	0.41003496	5.38630254	7.19E-08	5.63E-05	TRACHEARY ELEMENT DIFFERENTIATION-RELATED 7 (TED7)
AT5G27925	385.574903	2.32822772	0.35967741	6.47309972	9.60E-11	3.16E-07	
AT3G16670	318.537372	2.34446668	0.45787261	5.12034706	3.05E-07	0.00017296	
AT5G28335	20.9920876	2.38158672	0.46126038	5.16321544	2.43E-07	0.00015355	
AT4G33810	25.035728	2.38940179	0.39472902	6.05327122	1.42E-09	2.92E-06	
AT1G02335	90.6541896	2.45947812	0.34098969	7.2127639	5.48E-13	3.01E-09	GERMIN-LIKE PROTEIN SUBFAMILY 2 MEMBER 2 PRECURSOR (GL22)
AT3G05730	427.518788	2.52845152	0.42192805	5.99261306	2.06E-09	3.77E-06	
AT5G03260	95.0515404	2.55136552	0.34928984	7.30443662	2.78E-13	2.29E-09	LACCASE 11 (LAC11)
AT1G58370	70.1529127	2.58604592	0.38013794	6.80291452	1.03E-11	4.22E-08	XYLAN ENDOHYDROLASE 12 (RXF12)

Appendix 8.5 Cold RO differentially expressed genes (FDR 0.01). Base mean: mean of normalisation counts; log2foldchange: log fold change between control vs stress; lfcSE: standard error of log2fold change; stat: Wald statistic; pvalue: Wald test p-value between control vs stress; padj: adjusted p-values.

Gene ID	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Primary Gene Symbol
AT3G13400.1	15.8773627	-2.6426565	0.44983896	-5.8746723	4.24E-09	3.04E-05	SKU5 SIMILAR 13 (sks13)
AT1G43590.1	15.4797779	-1.9300344	0.44706571	-4.3171159	1.58E-05	0.00877622	
AT4G32430.1	20.2637117	-1.3148537	0.30373186	-4.3289952	1.50E-05	0.00863075	GROWING SLOWLY 1 (GRS1)
AT1G17630.1	28.7420368	-1.2862895	0.27521734	-4.6737226	2.96E-06	0.00328685	CELL WALL MAINTAINER 1 (CWM1)
AT3G16870.1	68.453518	-1.2856734	0.27936034	-4.6022044	4.18E-06	0.00331178	GATA TRANSCRIPTION FACTOR 17 (GATA17)
AT4G16835.1	36.6501723	-1.2723198	0.29730567	-4.2795006	1.87E-05	0.00910634	
AT2G36730.1	38.7667192	-1.2489852	0.23589753	-5.2946092	1.19E-07	0.00030925	
AT4G15215.1	74.3697391	-1.1320033	0.26340725	-4.2975405	1.73E-05	0.00895584	ATP-BINDING CASSETTE G41 (ABCG41)
AT5G06620.1	32.1349486	-1.1265311	0.25160281	-4.4774188	7.56E-06	0.00489728	SET DOMAIN PROTEIN 38 (SDG38)
AT1G56130.1	41.5480161	-1.0623343	0.24422723	-4.3497782	1.36E-05	0.00815398	
AT3G60050.1	78.717592	-0.7845417	0.17091565	-4.5902278	4.43E-06	0.00331178	
AT3G53630.1	360.497141	-0.6827539	0.14496569	-4.7097622	2.48E-06	0.00321519	
AT5G24830.1	135.881202	-0.6670902	0.15798278	-4.2225504	2.42E-05	0.00988988	
AT5G06360.1	880.318548	-0.5678999	0.12287208	-4.6218787	3.80E-06	0.00331178	
AT1G28240.1	723.64135	0.65930444	0.13938459	4.73011012	2.24E-06	0.00321519	
AT1G54090.1	500.903748	0.65948856	0.12044861	5.47526929	4.37E-08	0.00013592	EXOCYST SUBUNIT EXO70 FAMILY PROTEIN D2 (EXO70D2)
AT1G73740.1	300.150342	0.68366405	0.14903462	4.58728347	4.49E-06	0.00331178	
AT1G52140.1	101.515471	0.83408264	0.17841451	4.67497088	2.94E-06	0.00328685	

AT4G22840.1	149.856096	0.88642351	0.19235868	4.60818056	4.06E-06	0.00331178	BILE ACID SODIUM SYMPORTER 6 (BASS6)
AT4G14010.1	63.6338768	1.05073829	0.24884082	4.22253187	2.42E-05	0.00988988	RALF-LIKE 32 (RALFL32)
AT4G14930.1	530.699356	1.23219976	0.26513203	4.64749484	3.36E-06	0.00331178	
AT1G73500.1	652.591899	1.28179406	0.29921346	4.28387839	1.84E-05	0.00910634	MAP KINASE KINASE 9 (MKK9)
AT5G40230.1	18.4040143	1.2840615	0.30068892	4.2703984	1.95E-05	0.00919862	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 37 (UMAMIT37)
AT1G76800.1	127.935719	1.3269934	0.30115381	4.4063643	1.05E-05	0.00654141	VACUOLAR IRON TRANSPORTER-LIKE 2 (VTL2)
AT3G21510.1	51.7504299	1.37306246	0.23588784	5.82082764	5.86E-09	3.04E-05	HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 1 (AHP1)
AT1G25530.1	40.1473687	1.47588711	0.30439286	4.84862588	1.24E-06	0.00241755	
AT2G28630.1	1225.66408	1.503637	0.35482472	4.23768956	2.26E-05	0.00980078	3-KETOACYL-COA SYNTHASE 12 (KCS12)
AT3G55840.1	68.3482695	1.55076092	0.36602766	4.23673154	2.27E-05	0.00980078	
AT2G30230.1	150.908798	1.5934999	0.32149955	4.95646081	7.18E-07	0.00159546	
AT2G12190.1	38.97943	1.62823697	0.34509052	4.71828944	2.38E-06	0.00321519	
AT4G27280.1	390.542416	1.67855452	0.3950504	4.24896299	2.15E-05	0.00980078	
AT3G24450.1	14.0500566	1.83370194	0.40142566	4.56797392	4.92E-06	0.00333097	
AT5G62280.1	385.877181	1.89606322	0.39474447	4.80326738	1.56E-06	0.00269823	
AT3G50060.1	305.371808	1.94757521	0.41961935	4.64129026	3.46E-06	0.00331178	MYB DOMAIN PROTEIN 77 (MYB77)
AT5G26220.1	157.557726	1.9672384	0.45648515	4.30953429	1.64E-05	0.00877622	GAMMA-GLUTAMYL CYCLOTTRANSFERASE 2;1 (GGCT2;1)
AT1G18350.1	4.96326154	2.07999352	0.4542965	4.57849334	4.68E-06	0.00331178	MAP KINASE KINASE 7 (MKK7)
AT2G44080.1	239.649368	2.11138504	0.37486534	5.63238269	1.78E-08	6.91E-05	ARGOS-LIKE (ARL)

AT3G23150.1	86.911085	2.14427663	0.31231969	6.86564653	6.62E-12	1.03E-07	ETHYLENE RESPONSE 2 (ETR2)
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8.3 Appendix for Chapter 6

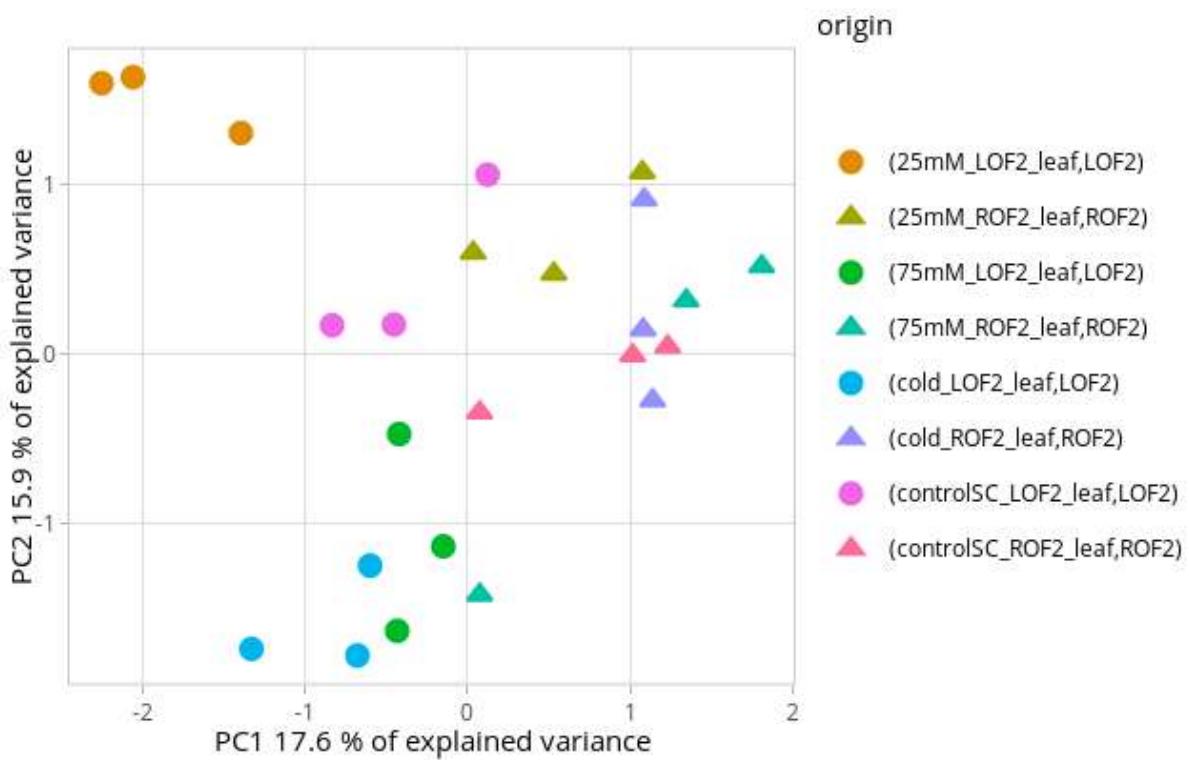
Appendix 8.6 Bisulfite sequencing information for each sample

Sample Name	Avg. GC	Insert Size	≥ 30X	Coverage	% Aligned	% mCpG	% mCHG	% mCHH	M C's	% Dups	% Aligned
controlSC_ROF2_leaf_3.R1.L4	19%	338bp	8.50%	19.0X	100.00%	20.40%	6.00%	1.90%	432.7	7.30%	81.90%
controlSC_ROF2_leaf_2.R1.L2	19%	264bp	2.40%	6.0X	100.00%	33.10%	12.40%	4.20%	138.8	43.80%	88.50%
controlSC_ROF2_leaf_1.R1.L2	19%	273bp	5.10%	9.0X	100.00%	31.40%	11.70%	3.80%	212.4	52.20%	88.20%
controlSC_LOF2_leaf_3.R1.L4	19%	350bp	73.10%	35.0X	100.00%	21.70%	6.30%	1.90%	815.9	12.40%	81.50%
controlSC_LOF2_leaf_2.R1.L3	19%	277bp	2.30%	6.0X	100.00%	32.30%	12.00%	4.10%	137.5	50.20%	88.30%
controlSC_LOF2_leaf_1.R1.L3	19%	277bp	3.80%	8.0X	100.00%	30.10%	11.20%	3.80%	194.6	43.40%	88.10%
cold_ROF2_leaf_3.R1.L4	19%	354bp	7.30%	18.0X	100.00%	21.80%	6.70%	2.20%	420.5	7.80%	79.20%
cold_ROF2_leaf_2.R1.L2	19%	269bp	6.70%	9.0X	100.00%	32.20%	12.30%	4.20%	223.5	59.30%	88.70%
cold_ROF2_leaf_1.R1.L2	19%	266bp	4.50%	8.0X	100.00%	33.10%	12.90%	4.50%	179.3	55.10%	88.50%
cold_LOF2_leaf_3.R1.L3	19%	354bp	73.20%	36.0X	100.00%	21.50%	6.20%	2.00%	805.3	9.90%	81.30%
cold_LOF2_leaf_2.R1.L3	19%	277bp	1.60%	5.0X	100.00%	32.60%	12.40%	4.30%	125	48.90%	88.50%
cold_LOF2_leaf_1.R1.L3	19%	279bp	4.10%	7.0X	100.00%	32.60%	12.60%	4.50%	181.9	53.20%	88.60%
75mM_ROF2_leaf_4.R1.L4	19%	353bp	1.80%	15.0X	100.00%	20.00%	6.00%	1.90%	357.5	6.40%	79.80%
75mM_ROF2_leaf_3.R1.L4	19%	353bp	3.60%	17.0X	100.00%	21.30%	6.40%	2.20%	387.9	7.00%	79.90%
75mM_ROF2_leaf_2.R1.L4	19%	351bp	9.80%	19.0X	100.00%	21.90%	6.70%	2.30%	446.4	6.90%	79.90%
75mM_LOF2_leaf_3.R1.L2	19%	355bp	14.1%	21.0X	100.00%	20.30%	6.00%	1.80%	488.4	8.60%	81.00%

75mM_LOF2_leaf_2.R1.L5	19%	355bp	95.2%	57.0X	100.00%	20.20%	5.90%	1.80%	1308. 4	15.80%	81.40%
75mM_LOF2_leaf_1.R1.L1	19%	359bp	61.5%	32.0X	100.00%	20.70%	5.80%	1.90%	752.5	11.00%	80.10%

Appendix 8.7 Bisulfite sequencing total read from each sample.

Name	Total Sequences	Sequence Length	%GC
75mM_LOF2_leaf_1.R1.L1	22437922	35-151	19
75mM_LOF2_leaf_1.R2.L1	22437922	35-151	19
75mM_LOF2_leaf_2.R1.L5	40481105	35-151	19
75mM_LOF2_leaf_2.R2.L5	40481105	35-151	19
75mM_LOF2_leaf_3.R1.L2	14023987	35-151	19
75mM_LOF2_leaf_3.R2.L2	14023987	35-151	19
75mM_ROF2_leaf_2.R1.L4	12905739	151	19
75mM_ROF2_leaf_2.R2.L4	12905739	151	20
75mM_ROF2_leaf_3.R1.L4	11126702	151	19
75mM_ROF2_leaf_3.R2.L4	11126702	151	20
75mM_ROF2_leaf_4.R1.L4	10188222	151	19
75mM_ROF2_leaf_4.R2.L4	10188222	151	20
cold_ROF2_leaf_1.R1.L2	18991469	101	19
cold_ROF2_leaf_1.R2.L2	18991469	101	19
cold_ROF2_leaf_2.R1.L2	26363850	101	19
cold_ROF2_leaf_2.R2.L2	26363850	101	19
cold_ROF2_leaf_3.R1.L4	12430895	151	19
cold_ROF2_leaf_3.R2.L4	12430895	151	20
cold_LOF2_leaf_1.R1.L3	18967860	101	20
cold_LOF2_leaf_1.R2.L3	18967860	101	20
cold_LOF2_leaf_2.R1.L3	11851792	101	20
cold_LOF2_leaf_2.R2.L3	11851792	101	20
cold_LOF2_leaf_3.R1.L3	23307449	35-151	19
cold_LOF2_leaf_3.R2.L3	23307449	35-151	19
controlSC_LOF2_leaf_1.R1.L3	15754904	101	19
controlSC_LOF2_leaf_1.R2.L3	15754904	101	19
controlSC_LOF2_leaf_2.R1.L3	13996244	101	20
controlSC_LOF2_leaf_3.R1.L4	24267912	35-151	19
controlSC_LOF2_leaf_3.R2.L4	24267912	35-151	19
controlSC_ROF2_leaf_1.R1.L2	20719479	101	19
controlSC_ROF2_leaf_1.R2.L2	20719479	101	19
controlSC_ROF2_leaf_2.R1.L2	12429056	101	20
controlSC_ROF2_leaf_2.R2.L2	12429056	101	21
controlSC_ROF2_leaf_3.R1.L4	12270459	151	19
controlSC_ROF2_leaf_3.R2.L4	12270459	151	19



Appendix 8.8 Principal Component (PC) Analysis of methylome data.

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