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Epigenetics of Stress Adaptation in Arabidopsis: the Case of Histone Modifications

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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EPIGENETICS OF STRESS ADAPTATION IN ARABIDOPSIS: THE CASE OF HISTONE MODIFICATIONS

(Thesis format: Monograph)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Changes in the epigenetic status of plants, which contribute to changes in gene expression in response to abiotic stress, are well documented. However, their stability and transmission to subsequent generations, and their incorporation into plant stress adaptation are still a matter for debate. Using chromatin immuno-precipitation and Next Generation Sequencing (ChIP-seq), I compared genome wide enrichment of two histone marks, H3K9ac and H3K4me2, in the progeny of Arabidopsis salt stressed and control plants. Data showed less enrichment of the H3K9ac in the chromatin of the progeny of salt stressed plants, but no changes were detected in the enrichment of the H3K4me2. The expression analysis of the genes, with significantly reduced enrichment of the H3K9ac, identified down-regulated genes in the salt stressed progeny including one known salt stress responsive gene, which suggests that histone modifications play a role in establishment of trans-generational stress memory. Moreover, these genes are candidates to be involved in salt stress adaptation.

Keywords

Epigenetic, histone modifications, abiotic stress, chromatin immuno-precipitation, next generation sequencing, epigenetic stress memory, stress adaptation.
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<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic Acid</td>
</tr>
<tr>
<td>AVT</td>
<td>AtGeneExpress Visualization Tools</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean Acid Metabolism</td>
</tr>
<tr>
<td>Cat #</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CG</td>
<td>Cytosine Guanine</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno Precipitation</td>
</tr>
<tr>
<td>ChIP-chip</td>
<td>ChIP Followed by Microarray</td>
</tr>
<tr>
<td>chr</td>
<td>Chromosome</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double-distilled Water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F1</td>
<td>First Filial</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>H</td>
<td>Histone</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone De-acetylase</td>
</tr>
<tr>
<td>HDM</td>
<td>Histone De-methylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone Methyltransferase</td>
</tr>
<tr>
<td>IGB</td>
<td>Integrated Genome Browser</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>miRNAs</td>
<td>micro-RNAs</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>nat-siRNAs</td>
<td>Natural Antisense Transcripts</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
</tr>
<tr>
<td>padj</td>
<td>P Adjusted Value</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Reverse Transcription qPCR</td>
</tr>
<tr>
<td>RdDM</td>
<td>RNA-directed DNA Methylation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per Minute</td>
</tr>
<tr>
<td>RSB</td>
<td>Resuspension Buffer</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>siRNAs</td>
<td>Small Interfering RNAs</td>
</tr>
<tr>
<td>smRNAs</td>
<td>Small RNAs</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA Buffer</td>
</tr>
<tr>
<td>TEs</td>
<td>Transposable Elements</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-methylamine</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<td>Vol</td>
<td>Volume</td>
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1 INTRODUCTION AND BACKGROUND

1.1 Plant Stress

With the increasing world population, it is necessary to adapt current agricultural biotechnology practices to satisfy the growing demand for food (Godfray et al., 2010). However, environmental stresses to which plants are exposed are the greatest factors limiting world crop yield.

Stress has negative effects on the growth, development and reproduction of a plant. Furthermore, stress limits the distribution of plant species in various regions while otherwise exerting strong evolutionary pressure on the plant population (Madlung and Comai, 2004; Boyko and Kovalchuk, 2008). Stresses can be either intrinsic, such as spontaneous gene mutations, or extrinsic, such as biotic and abiotic stresses. Biotic stresses include pathogen attack, competition, and herbivory, whereas abiotic stresses originate from unfavorable environmental conditions, such as excessive or inadequate light, water, salt, and temperature (Madlung and Comai, 2004).

All living organisms have developed mechanisms of protection and adaptation to minimize stress influences in order to survive under various environmental conditions. These mechanisms include tolerance, resistance, and avoidance. Briefly, tolerance mechanisms help the organism to withstand harsh conditions, resistance mechanisms involve the organism in an active counteraction against the stress, and avoidance mechanisms, especially escape, help the organism to minimize exposure to stress (Madlung and Comai, 2004). Plants being sessile organisms, cannot escape from unfavorable environmental conditions and therefore need more protection and/or adaptation strategies (Boyko and Kovalchuk, 2008).
1.2 Plant Response to Abiotic Stress

Abiotic stresses are the main cause of crop loss in the world and these stresses cause on average a reduction of more than 50% in the yield of major agricultural crops (Wang et al., 2003). Furthermore, according to reports from the Intergovernmental Panel on Climate Change (www.ipcc.ch), abiotic stress factors will become even more severe and frequent because of global climate change (Hirayama and Shinozaki, 2010). Therefore, understanding plant response to abiotic stress and exploiting it to improve crop yield in response to unfavorable conditions is one of the most important subjects in plant science research today (Hirayama and Shinozaki, 2010).

Abiotic stress leads to a complex series of molecular, biochemical, physiological, and morphological changes (Wang et al., 2001). Therefore, unlike the situation for most monogenic traits, such as resistance to herbicides and pests, producing plants that are tolerant to a complex trait such as abiotic stress, using genetic modification, is more challenging (Wang et al., 2003). The application of various molecular biology approaches, in different forward and reverse genetic pathways, has helped to characterize many genes and gene products, which are involved in plant abiotic stress response and tolerance (Hirayama and Shinozaki, 2010). In addition, the availability of the full genome sequences of some plants, such as Arabidopsis thaliana and Oryza sativa, has enabled global analysis of gene expression in response to abiotic stresses (Hirayama and Shinozaki, 2010). For example, global analyses of transcriptome changes in response to different abiotic stress conditions in various tissues and developmental stages of Arabidopsis (Kreps et al., 2002; Seki et al., 2002; Kilian et al., 2007; Matsui et al., 2008) have identified thousands of genes with altered transcript levels. A complete list of these genes along with details on the amount of altered expression in response to stress conditions is available in several online databases, such as www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp (Kilian et al., 2007). Global changes in the proteome and metabolome in response to different abiotic stresses such as dehydration, high salinity, cold, and heat treatment have also been analyzed in plants, especially Arabidopsis (Urano et al., 2010). The combination of these ‘omics’ data obtained from different levels of functional genomics have provided a more
comprehensive picture of plant abiotic stress responses (Urano et al., 2010). Progress in newly developing research fields, such as the discovery of functional small RNAs (smRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs), and assessing chromatin variation, have allowed for a better understanding of the various regulatory networks evolved in plants in response to abiotic stresses (Hirayama and Shinozaki, 2010).

Generally, plant response to abiotic stress could be controlled at several interdependent levels (Kilian et al., 2012). First, stress is sensed by primary receptors; this is followed by the activation of several signalling pathways such as oxidative signals and calcium-dependent signals that are common in many stressed plants (Reviewed in Kilian et al., 2012). These signalling pathways could result in altered expression of a set of stress-responsive genes (early-responsive genes), which are either common between stresses or specific to a particular stress. Some early-responsive genes encode transcription factors that control other stress-responsive genes (late-responsive genes). These latter genes are usually stress-specific genes which can be grouped into distinct categories with different functions, such as genes involved in protein turnover or mechanical defense (Reviewed in Kilian et al., 2012). All in all, this multi-level response allows the plant to survive or adapt to the changing environment (Kilian et al., 2012).

Typically high soil salinity, caused by NaCl, inhibits plant growth due to induction of hyperosmotic and ionic toxicity effects as well as overproduction of reactive oxygen species (ROS) which cause oxidative damage (Reviewed in Gong et al., 2001). ROS disrupt cellular components such as membrane lipids, photosynthetic pigments, proteins and nucleic acids and ultimately lead to cell death (Ghorbanli et al., 2004). Plant salt stress responses include induction of osmotic stress tolerance effectors (such as enzymes involved in the biosynthesis osmoprotectants e.g sugar alcohol), and proteins involved in membrane integrity, control water or ion homeostasis, and ROS scavenging (Gong et al., 2001). Several signal pathway intermediates and transcription factors (such as zinc finger transcription factors) posttranscriptionally activate and regulate effectors through interaction with promoters of osmotic-regulated genes (Gong et al., 2001). Thus salt stress induces changes in gene expression patterns and protein distribution profiles.
Regulation of the plant genome at the transcriptional level in response to various abiotic stresses results in the up-regulation or down-regulation of the affected genes (Kilian et al., 2007). The accessibility of DNA sequences for DNA-binding factors, which regulate gene expression, is influenced and controlled to a large extent by chromatin structure (Chinnusamy and Zhu, 2009). Regulation at the post-transcriptional, translational and post-translational levels also play significant roles in plant stress responses (Urano et al., 2010). In addition, alternative splicing and production of diverse polypeptides from a single gene also occur in response to various abiotic stresses, indicating the existence of complex multi-step regulation of genes in response to abiotic stress (Iida et al., 2004).

1.3 Chromatin Structure Changes Associated with Plant Abiotic Stress Responses

Genome-wide alterations induced by abiotic stresses include transposon activation, transposition, and chromatin structural changes (Madlung and Comai, 2004). Chromatin structure, which greatly influences gene expression level, is controlled mostly by processes associated with epigenetic modifications (Chinnusamy and Zhu, 2009).

Epigenetic modification is defined as changes in gene activity without changes in the original DNA sequence. These changes can be transferred to the cell’s progeny during mitosis or meiosis (Chen et al., 2010b). Furthermore, these changes can be mediated at several interdependent levels, including DNA methylation, histone post transaltional modifications, expression of histone variants (Chinnusamy and Zhu, 2009), and non-coding RNAs (Chen et al., 2010b). Physiological and developmental stimuli as well as environmental stress can induce these epigenetic modifications (Boyko and Kovalchuk, 2008). Many studies have revealed that changes in gene expression are coordinated with changes in epigenetic status during plant stress responses. Some of these studies are discussed in the following sections.

In general, nucleosome occupancy is negatively associated with transcriptional activation. Therefore, when a genomic region is activated transcriptionally, nucleosome density at that region decreases and the chromatin structure relaxes (Kim et al., 2010). Changes in chromatin structure through epigenetic modifications depend on the
involvement of several chromatin-related proteins (Verbsky and Richards, 2001). These chromatin-associated proteins such as linker histone 1, high-mobility group (HMG) proteins, histone modification enzymes, and components of the chromatin remodeling complex, change the higher-order structure of chromatin through chromatin assembly and nucleosome packing in order to regulate the corresponding genes (Bianchi and Agresti, 2005; Grasser et al., 2007; Jerzmanowski, 2007). The chromatin-related factors are influenced by abiotic stresses (Reviewed in Kim et al., 2010). For example, AtCHR12, a chromatin remodeling protein, acts to arrest growth under stress conditions (Mlynarova et al., 2007), and histone 3 levels decrease in response to cold stress on the promoters of cold-responsive genes COR15A and ATGOLS3, resulting in increased transcription of the two genes (Kwon et al., 2009).

1.4 Changes in Histone Modifications Associated with Abiotic Stress Conditions

Histone modifications represent one of the important levels in epigenetic regulation. Chromatin is composed of nucleosomal subunits which consist of ~146 base pair (bp) of DNA wrapped twice around an octamer of core histones. The histone octamer contains two copies of each histone 2A (H2A), histone 2B (H2B), histone 3 (H3), and histone 4 (H4) proteins (Figure 1, Marks et al., 2001). Each core histone has an N-terminal tail which protrudes from the nucleosome and is rich in lysine (Marks et al., 2001). Amino acids on H3 and H4 tails are easier to modify chemically than other histone amino acids (Chen et al., 2010b). Various modifications, such as acetylation, methylation, phosphorylation, ubiquitination, biotinylation, and ribosylation, require different enzymes, change the nucleosome arrangement and chromatin structure allowing activators or inhibitors to bind to DNA sequences (Chen et al., 2010b). Histone modification enzymes, such as histone methyltransferase (HMT), histone de-methylase (HDM), histone acetyltransferase (HAT), and histone de-acetylase (HDAC) are fundamental factors of chromatin remodeling (Kim et al., 2010).

Histone tail modification is a key control point of chromatin structure and therefore gene regulation (Zhang et al., 2007a; Zhu et al., 2008; Chen et al., 2010b). In general,
Acetylation, phosphorylation, and ubiquitination of histone tails are associated with active transcription, while de-acetylation and biotinylation result in gene repression (Chen et al., 2010b). In total, 28 histone modification sites have been recognized in Arabidopsis; some of them are conserved in mammalian and yeast cells, while some are unique to plants (Zhang et al., 2007a; Kim et al., 2008). Each histone modification according to its position can be used as a mark to understand the status of target genes. For example, acetylation of H3 Lys9 (H3K9ac) and mono/di/tri-methylation of H3 Lys4 (H3K4me3/2/1) are often positive marks associated with transcriptionally active genes. On the other hand, H3K9 de-acetylation, H3K9me2/3, H3K27me2/me3 are generally negative marks associated with transcriptionally repressed genes (Zhou, 2009). Furthermore, the euchromatin state is correlated with hyper-acetylation of H3 and H4 along with methylation of H3K4, whereas the heterochromatin structure is dependent on hypo-acetylation of H3 and H4, de-methylation of H3K4, and methylation of H3K9 residues (Bender, 2004). It is also suggested that the deposition of two independent H3 variants differing in sequence and post-translational modifications, particularly in the enrichment of methylated K9 and K27, result in different methylation levels of H3 and transcriptional activity of their corresponding genes (Zilberman and Henikoff, 2005). Investigations of diverse histone modifications on a global scale have also disclosed that, in plants, one histone modification can interact with another histone modification or DNA methylation and these diverse modifications function jointly in gene regulation (Zhang et al., 2009).

Histone modifications play a critical role in both plant development and plant responses to stress (Chen et al., 2010). There are many reports on dynamic alterations of histone tail modifications in response to abiotic stresses in plants. For example, exposure of tobacco and Arabidopsis cells to salinity, cold and Abscisic Acid (ABA) treatment resulted in a rapid and transient increase in the global enrichment level of H3 Ser10 phosphorylation (H3S10ph), H3 Ser10 phospho-acetylation (H3S10ph-ac), and H4 Lys14 acetylation (H4K14ac). The dynamic changes in these histone modifications were followed by the up-regulation of stress specific genes (Sokol et al., 2007). Using chromatin immune-
Figure 1: Schematic structure of nucleosome and histone post transcriptional modifications. The nucleosomes consist of octamer histone core (two copies of each H2A, H2B, H3 and H4) and the DNA (black) is wraps around it (a). The amino-terminal tails of core histones are usually modified with different group recruiting histone modification enzymes such as histone acetyltransferases (HATs) and histone de-acetylases (HDACs) (b). A, acetyl; C, carboxyl terminus; E, glutamic acid; M, methyl; N, amino terminus; P, phosphate; S, serine; Ub, ubiquitin (from Mark et al., 2001 with permission from Nature Publishing Group, see Appendix B ).
precipitation (ChIP) assays, Kim et al (2008) showed an increase in the enrichment of H3K4me3 and H3K9ac marks on the coding regions of Arabidopsis drought stress-responsive genes (RD29A, RD29B, RD20, and RAP2.4), as well as an increase in the enrichment level of H3K23ac and H3K27ac on the promoters and coding regions of RD29B and RAP2.4 during dehydration stress. These changes were associated with activation of the genes (Kim et al., 2008). Exposure to UV-B triggered an increase in the enrichment of H3K9/14ac on the promoter of ELIP1 in Arabidopsis and wheat (Cloix and Jenkins, 2008). Chen et al. (2010) also showed that gene expression induced by ABA and salt stress is associated with the induction of gene activation marks, such as H3K9/14ac and H3K4me3, and the reduction of gene repression marks, such as H3K9me2, at ABA and abiotic stress-responsive genes. They also showed that HDA6, a histone de-acetylase, is involved in ABA and abiotic stress responses, and in ABA- and salt stress-induced gene expression in Arabidopsis. Further, they suggested that histone acetylation and methylation may mutually interact and affect each other in order to regulate gene activity in response to stress conditions (Chen et al., 2010a). Taken together, these studies prove that histone acetylation/de-acetylation and methylation/de-methylation play important roles in gene regulation, plant stress response, and tolerance to abiotic stresses (Cloix and Jenkins, 2008; Kim et al., 2008; Chen et al., 2010a).

1.4.1 Lysine acetylation and methylation of histones

Among various histone modifications, histone acetylation directly loosens the histone association with DNA resulting in transcriptional activation; in contrast, histone methylation assists in recruiting other effector proteins and their complexes, which act either on activation or repression of gene expression (Rice and Allis, 2001).

HMTs belong to SET (Su(var)3-9, E(Z) and Trithorax) domain proteins; Arabidopsis encodes 41 SET domain proteins that catalyze lysine methylation at different targets of histone N-terminal tails (Liu et al., 2010). The expression of SUVH2, SUVH5 and SUVH6 genes encoding histone methyltransferases decreased in the progeny of salt stressed plants, suggesting a role for these genes in plant stress adaptation (Bilichak et al., 2012).
Histone methylation is a dynamic changes and two types of HDMs, namely LSD1 (lysine-specific de-methylase1) and JHDM (JmjC domain–containing histone de-methylases), de-methylate lysine at histone N-terminal tails (Liu et al., 2010).

In Arabidopsis there are 15 HAT proteins that are classified into several families (Chen et al., 2010b). Several studies showed involvement of specific HATs in gene activation through lysine acetylation of the histone N-terminal tails (Fukuda et al., 2006; Earley et al., 2007). Arabidopsis GENERAL CONTROL NON-REPRESSIBLE 5 (AtGCN) gene, which encodes a HAT protein, is associated with response to environmental cues, such as light and cold (Stockinger et al., 2001; Benhamed et al., 2006). In addition, there are 18 HDACs in Arabidopsis that are generally involved in repression of gene activity through lysine de-acetylation of histone N-terminal tails (Kim et al., 2010). HDA6 and HDA19 act in ABA and salt stress responses (Chen and Wu, 2010), HOS15, a de-acetylase-like protein, mediates H4 de-acetylation and represses gene expression associated with cold stress tolerance (Zhu et al., 2008), and AtHD2C, a plant-specific HD2-type HDAC, is suppressed by ABA, while overexpression of this gene confers salt and drought tolerance (Sridha and Wu, 2006).

1.5 Changes in DNA Methylation Associated with Abiotic Stress Conditions

In addition to histone modification, DNA methylation also regulates gene expression by hindering transcription (Zilberman et al., 2007). DNA methylation, for the most part, occurs at the fifth carbon position of a cytosine ring (Chen et al., 2010b). In plants, cytosine methylation occurs both symmetrically (mCG and mCHG where H is adenine, cytosine or thymine) and asymmetrically (mCHH). The maintenance of symmetric CG methylation is mediated by METHYLTRANSFERASE1 (MET1), the counterpart of Mammalian DNA METHYLTRANSFERASE1 (DNMT1). The plant-specific DNA methyltransferase, CHROMOMETHYLASE3 (CMT3) maintains DNA methylation at CHG sites. Further, de novo methyltransferases, DOMAINS REARRANGED METHYLASE 1 and 2 (DRM 1/ 2) catalyze new cytosine methylation at CHH sites (Reviewed inChen et al., 2010b). It was also suggested that the maintenance of
symmetric methylation at CG and CHG sites may be important for \textit{de novo} methylation by DRM1 and DRM2, while MET1 and CMT3 may also have a role in \textit{de novo} methylation (Lister et al., 2008; Zhu, 2008). RNA-directed DNA methylation (RdDM), as a \textit{de novo} DNA methylation system, directs methylation through small interfering RNAs (siRNA) to specific sites that are in need of genome modification, but mainly at non-CG sites which are much more flexible than methylation at CG sites (Chen et al., 2010b). On the other hand, DNA glycosylase REPRESSOR OF SILENCING 1 (ROS1) actively performs DNA de-methylation by a base excision repair mechanism and can work against the RdDM pathway (Agius et al., 2006). It was also suggested that the putative DNA de-methylation may also be directed by small RNAs (smRNAs) or the RdDM pathway (Zheng et al., 2008). Furthermore, a maternal gene encoding DNA glycosylase DEMETER (DME) directly de-methylates the CG sites of the endosperm genome, and regulates “parent-of-origin-specific” (imprinted) gene expression (Hsieh et al., 2009).

In plants, heterochromatin, regions producing siRNAs, transposable elements (TEs), and pseudogenes are heavily methylated (Zhang et al., 2006). Methylation in repeat sequences, such as TEs, prevent these sequences to interfere with normal genome functioning (Chan et al., 2005). Although the density of methylcytosines in genes is much lower than repeat sequences, over one third of Arabidopsis expressed genes contain methylation at their transcribed regions, mainly at the CG sites (Zhang et al., 2006). Only ~5% of expressed genes contain methylation at their promoter regions (Zhang et al., 2006). Methylation at transcribed regions moderately suppresses gene expression, while genes methylated at promoter regions show tissue specific expression (Zhang et al., 2006; Zilberman et al., 2007).

Changes in DNA methylation contribute greatly to the plant’s ability to respond to stresses (Boyko and Kovalchuk, 2008). There are many examples indicating that the level of DNA methylation increases or decreases in response to stresses. For example, reversible DNA hyper-methylation was induced in the heterochromatic loci in tobacco cell-suspension culture in response to osmotic stress (Kovarik et al., 1997). On the other hand, infection of tobacco plants with tobacco mosaic virus resulted in transcriptional activation and a decrease in the methylation level of the pathogen-responsive gene
Also, exposure of tobacco to aluminum, salt, or cold stress led to hypo-methylation of the coding sequence of \textit{NtGDPL} encoding a glycerophosphodiesterase-like protein, which correlated with induced expression of this gene in response to abiotic stresses (Choi and Sano, 2007). TEs, which make up a significant portion of plant genomes and are maintained in an inactivated condition by DNA methylation, may be activated by environmental factors via DNA de-methylation (Chinnusamy and Zhu, 2009). For example, cold stress-induced hypo-methylation of the \textit{Ac/Ds} retrotransposon-like sequence contained in \textit{ZmMI1} is responsible for its cold-induced expression in maize roots (Steward et al., 2002). This cold-induced hypo-methylation was not reset to basal level even after seven days of recovery (Steward et al., 2002). Hypo-methylation and transposition of the \textit{Antirrhinum majus} Tam-3 transposon by cold stress (Hashida et al., 2006), and salt stress-induced nuclear genome methylation at CHG sites and specific satellite DNA in \textit{Mesembryanthemum crystallinum}, which induced a change in the photosynthesis mode from C3 to the crassulacean acid metabolism (CAM) pathway (Dyachenko et al., 2006), are additional examples.

### 1.6 Changes in Non-Coding RNAs Associated with Abiotic Stress Conditions

Various smRNAs and miRNAs are changed in their levels in response to abiotic stresses such as dehydration, salt, and cold stress, (Sunkar and Zhu, 2004; Zhou et al., 2008). For example, miR319c level increased in response to cold stress, but not in response to dehydration and salt stresses (Sunkar and Zhu, 2004). In addition to smRNAs and miRNAs, siRNAs, which are derived from natural antisense transcripts (nat-siRNAs) are up-regulated in response to abiotic stress conditions (Borsani, et al., 2005). This is nicely illustrated by the P5CDH-SRO5 regulatory system in Arabidopsis. Here, high salt conditions induce the expression of \textit{SRO5} (\textit{SIMILAR TO RCD ONE 5}), which in turn leads to the production of a 24-nucleotide SRO5-P5CDH nat-siRNA from the overlapping mRNAs of \textit{SRO5} and \textit{P5CDH} (\textit{DELTA1-PYRROLINE-5-CARBOXYLATE DEHYDROGENASE}) genes. This nat-siRNA down-regulates the expression of \textit{P5CDH}, a gene involved in proline catabolism, through mRNA cleavage resulting in the accumulation of proline. Proline being an osmoprotectant helps confer salt tolerance in
Arabidopsis (Borsani et al., 2005). Another specific nat-siRNA induced by *Pseudomonas syringae* infection in Arabidopsis, mediates resistance against this pathogen (Katiyar-Agarwal et al., 2006). Since there are several natural antisense overlapping genes with potential to produce nat-siRNAs, this mechanism may play a critical role in stress-mediated regulation of genes (Borsani et al., 2005). Other types of non-coding RNAs, such as some cytoplasmic RNA-containing granules, also appear to play a role in controlling gene expression during plant stress response (Urano et al., 2010).

Full transcriptome analysis of plants using Whole-Genome Tiling Arrays or High-Throughput DNA Sequencing has enabled the detection of additional un-annotated, non-coding RNAs in response to salinity, heat, dehydration, cold, and osmotic stresses, as well as ABA treatment (Matsui et al., 2008; Zeller et al., 2009). Nearly 80% of previously un-annotated transcripts up-regulated in response to abiotic stresses have been identified as nat-siRNAs, including nat-siRNA of the stress-responsive genes such as *RD29A* and *CYP707A1* (Matsui et al., 2008). Furthermore, the accumulation of transposons or pseudogene transcripts, which are sources of siRNAs and nat-siRNA, in response to abiotic stress support the view that pseudogenes and transposons have regulatory roles in plant abiotic stress responses (Zeller et al., 2009). However, so far, only a few identified stress-responsive non-coding RNAs have been functionally characterized (Borsani et al., 2005).

1.7 **Interplay between Various Epigenetic Factors Regulating Gene Expression**

Evidence suggests that epigenetic regulation is a complex mechanism involving many chromatin remodelling factors (Bilichak et al., 2012). For example, a close link between changes in DNA methylation level and post-translational histone modifications was revealed in mutants deficient in DNA methyltransferase and histone methyltransferase. The *DECREASE IN DNA METHYLATION1 (DDM1)* gene is responsible for maintaining DNA methylation level, as well as gene and transposon silencing (Gendrel et al., 2002). Mutant plants deficient in *DDM1* gene showed a decrease in the DNA methylation level at heterochromatin regions, which was associated with an increase in the enrichment
level of the H3K4me2 mark and a decrease in the enrichment level of the H3K9me2 mark (Gendrel et al., 2002). Also, a loss of methylation level at CG sites in heterochromatin regions occurred in the met1 mutant, which resulted in a decrease in the enrichment level of H3K9me2 (Tariq et al., 2003). Further, enrichment level of H3K9me2 mark was decreased in the mutant of the KRYPTONITE gene, which encodes a histone methyltransferase; this was accompanied by a decrease in the DNA methylation level, and a reduction in gene silencing (Jackson et al., 2004). There is also a link among DNA methylation, histone modifications, and non-coding RNAs, especially siRNA, in epigenetic regulation. The involvement of siRNAs in the RdDM pathway was proven by genetic analysis of mutants impaired in genes for siRNA biogenesis or functioning in Arabidopsis (Pontes et al., 2006). In fact, siRNAs mediate methylation of almost one-third of methylated loci (Lister et al., 2008). In contrast, ROS3, an important regulator of de-methylation, encodes a RNA recognition motif-containing protein, which binds to small RNAs, and may guide de-methylation of specific sequences by ROS1 (Zheng et al., 2008). Moreover, it was shown that HDA6 is also involved in the RdDM pathway (Aufsatz et al., 2002).

Many studies have proved that stress-induced changes in one epigenetic factor are correlated to changes in other epigenetic factors as well. For example, Song et al. (2012) showed that DNA methylation level of several salt-responsive transcription factors decreased in response to salt stress and this change was accompanied by changes in the level of several histone modifications as well as gene expression changes in soybean. In addition, Bilichak et al. (2012) also revealed that hypo-methylation level of several loci in the progeny of Arabidopsis salt stress plants is correlated to changes in their H3 acetylation and methylation levels.

1.8 Stability of Stress Induced Epigenetic Modification

To date there have been a wealth of reports that various stresses cause epigenetic changes at different levels, however the stability of these modifications is still a subject for debate. In rice, flooding resulted in a decrease in the enrichment level of the H3K4me2 mark and
an increase in the enrichment level of H3K4me3 on the 5′- and 3′-coding regions of submergence-inducible genes (ADH1 and PDC1), while the H3 acetylation enrichment level was gradually increased throughout these genes resulting in increased expression levels. These histone modifications were reset to the basal levels during re-aeration (Tsuji et al., 2006). Using ChIP assays, Kwon et al. (2009) also showed that the enrichment level of the H3K27me3 mark was gradually decreased in the two Arabidopsis cold-responsive genes, COR15A and AtGOLS3, during exposure to cold stress. This decrease was maintained for up to 3 days after returning to normal growth temperature, while the transcription level of these genes was reduced to the initial level at 1 day after removal of cold stress. Therefore, the decrease in the enrichment level of the H3K27me3 mark can be inherited somewhat through cell divisions but it does not influence the transcription level anymore (Kwon et al., 2009). In addition, cold, heat, and UV-B stresses induced release of gene silencing in the transcriptionally silenced β-glucuronidase transgene (TS–GUS) as well as in several silent endogenous TEs, which was correlated with a less condensed chromatin conformation (higher histone occupancy) and an increase in the enrichment level of H3 acetylation, but was not associated with pronounced alterations in DNA methylation level in these loci (Lang-Mladek et al., 2010). The transcriptional reactivation of the transgene was heritable, but was limited to only two progeny generations and confined to a small number of cells, and could be reset by seed aging (Lang-Mladek et al., 2010). Similarly, other reports showed that exposure of Arabidopsis plants to a special temperature stress resulted in release of transcriptional gene silencing at several heterochromatin regions and this destabilized status was also verified at the genome-wide level by transcriptomic analyses (Pecinka et al., 2010; Tittel-Elmer et al., 2010). However, this transcriptional activation was transient and silencing was re-established a few days after removing the stress (Pecinka et al., 2010; Tittel-Elmer et al., 2010). Pecinka et al. (2010) determined that the transient release of silencing and its restoration was related to temporary changes in nucleosome density. It was generally concluded that most of the stress-induced epigenetic modifications are transient and restore to initial levels when the stress is removed, however some of the modifications might be stable and inherited across mitotic or even meiotic cell divisions (Chinnusamy and Zhu, 2009).
1.9 Trans-generational Transmission of Epigenetic Stress Memory

Epigenetic changes, which occurred rapidly and reversibly with the potential to maintain the “memory of stress” via several cell divisions, can be a potential mechanism for describing the flexibility of plant response to environmental conditions (Bruce et al., 2007), and perhaps can be utilized to improve plant stress tolerance. This holds true especially since plants, unlike animals, establish their germ-line late during development; therefore they could sense stresses during their life and memorize them, perhaps by epigenetic mechanisms, in cell lineages that later form the germ-line and pass them into their progeny (Mirouze and Paszkowski, 2011). It is speculated that epigenetic stress memory may help plants cope more efficiently with future stresses (Chinnusamy and Zhu, 2009; Boyko and Kovalchuk, 2011) while providing more genome diversity (Lang-Mladek et al., 2010; Boyko and Kovalchuk, 2011). In the following sections I will present evidence supporting trans-generational epigenetic stress memory.

1.9.1 Trans-generational genome instability induced by stress and its impact on stress adaptation

Increased genome instability, which is measured by homologous recombination frequency (HRF), is well documented in plants after their exposure to various stresses (Molinier et al., 2006; Boyko et al., 2007; Pecinka et al., 2009; Boyko et al., 2010). These reports have shown higher HRF in response to various biotic and abiotic stresses, including pathogen attacks, flagellin (the bacterial elicitor of plant defense), salt, heat, cold, flood, and UV-C. Since the majority of tested stresses cause an increase in HRF, it was suggested that genome instability is part of a general response to stress in plants (Boyko and Kovalchuk, 2011). In addition, some evidence demonstrates that a stress-induced increase in HRF can be transmitted to the following generation(s) (Molinier et al., 2006; Boyko et al., 2007). For example, one of the most important reports in this field is by Molinier et al (2006) who showed that plants treated with UV-C or flagellin had a higher somatic HRF of the GUS contained transgene. This trait was transmitted in the absence of the stresses as a dominant trait in reciprocal crosses (through both gametes) to at least the fourth generation. Since the whole population showed the acquired higher
HRF phenotypes, the authors proposed that the mechanisms responsible for this trans-generational stress-induced effect were likely to be epigenetic, because a mutation would be expected to affect only a few plants. Although the mechanism responsible for homologous recombination is not well known, through the use of Arabidopsis mutants affecting chromatin remodeling it was shown that chromatin structure plays an important role in regulating homologous recombination (Endo et al., 2006; Kirik et al., 2006). Therefore, it was speculated that changes in chromatin could provide the basis for transmission of the stress memory to subsequent generation(s) (Bond and Finnegan, 2007). Similarly, infection of tobacco plants with the tobacco mosaic virus (TMV) caused a high somatic and meiotic HRF at several loci with homology to the leucine-rich repeat (LRR) region of the TMV resistance gene. Furthermore, the non-infected progeny of these plants (F1) showed a higher HRF, which was accompanied by a lower DNA methylation level at the LRR-containing loci (Boyko et al., 2007). Thus, they hypothesized that the observed changes might be an early attempt to develop novel resistance (R) genes corresponding to a given pathogen (Boyko et al., 2007). This is consistent with a previous suggestion that the evolution of plant R genes was through gene duplication and recombination events (Meyers et al., 2005). However, another report suggested that trans-generational changes in genome stability were not a general response in Arabidopsis, and happen in a somewhat low and stochastic manner (Pecinka et al., 2009). Although they observed increased HRF after all 10 abiotic stresses tested, only a few abiotic stresses resulted in trans-generational effects on HRF (Pecinka et al., 2009). Similarly, Boyko et al., (2010) showed that maintaining increased HRF in the F1 progeny of heat, cold, flood, UV-C or salt stressed plants and transferring it to the second generation (F2) required further exposure of the F1 plants to the stress condition. If the progeny of stressed plants were propagated for more than one generation without stress, the effect diminished and HRF returned back to the basal level. The authors argued that this may be more probable from an evolutionary point of view (Boyko and Kovalchuk, 2011). Stress induces acclimation response at least partially through epigenetic changes. These new and potentially advantageous epigenetic states could be transiently established and passed on to the immediate progeny (F1), as a stress memory to cope with expected future stress. If the stress condition exists during the life span of this F1 progeny, the
newly established epigenetic state will be maintained and possibly fixed for many generations; otherwise it will return to the original state which was the best for the existing environmental conditions (Boyko and Kovalchuk, 2011).

1.9.2 Trans-generational stress memory and adaptive responses

There is evidence showing that maintenance of stress memory is beneficial to the progeny of exposed plants. For example, increased tolerance to freezing stress was seen in the progeny of Arabidopsis plants exposed to low temperatures (Blodner et al., 2007). They showed that exposure of plants to cold stress during flowering and seed development resulted in improved photosynthetic yield recovery in their progeny in response to chilling conditions (Blodner et al., 2007). Similarly, higher survival rates of seeds and enhanced germination was observed in the progeny of Campanulastrum americanum plants grown in the maternal light environment, understory indirect light versus light gap conditions (Galloway and Etterson, 2007). Further, Arabidopsis plants grown in heat stress conditions for two generations (parent and F1) caused an increased fitness (seed production efficiency) to elevated temperature in the non-stressed F3 generation, even though the F2 generation rose in a normal temperature (Whittle et al., 2009). The molecular mechanisms that maintain the memory of stress and induce the adaptive responses over one unexposed generation have not been characterized yet. Nonetheless, genome instability and epigenetic modifications are discussed more and more as some of the possible mechanisms of the adaptive responses (Boyko and Kovalchuk, 2011). The experimental evidence supporting this idea is quite limited, which I will cite in the following section. Interestingly, there are a few studies on naturally adapted plant populations, which were associated with epigenetic modifications. For example, Kovalchuk et al (2003) showed a correlation between stress adaptation of Pinus silvestris trees with higher levels of global DNA methylation and higher genome stability in response to radioactive contamination in the Chernobyl zone. Also, distinct morphological changes of two populations of Laguncularia racemosa (mangrove tree species) inhabiting different environmental conditions were related to variation in
methylation levels at CG sites rather than to genetic variations (Lira-Medeiros et al., 2010).

1.9.3 Trans-generational epigenetic modifications induced by stress

Experimental evidence of stress induced trans-generational epigenetic changes in plants is rare and mostly limited to studies on changes in DNA methylation levels in the progeny of stressed plants. For example, the progeny of tobacco plants infected with TMV inherited increased HRF at the disease resistance gene-like loci which was accompanied by hypo-methylation at these loci despite having global genome hyper-methylation (Boyko et al., 2007). Another report from the same group showed that progeny of Arabidopsis salt stressed plants had a higher tolerance to salt stress, as well as a higher HRF and a higher global DNA methylation level (Boyko et al., 2010). These trans-generational effects of salt stress, however, were not maintained in following generations without further stress condition (Boyko et al., 2010). They also showed a correlation between trans-generational changes of the DNA methylation level and stress tolerance in the progeny of plants exposed to salt stresses. Although, they did not find a link between the level of DNA methylation and the level of transgene HRF, they provided the first experimental proof establishing a correlation between stress acclimation and adaptation with changes in epigenetic modifications. Interestingly, they reported that while the genome of F1 stressed plant was hyper-methylated at the global level, some loci were hypo-methylated; mostly genes involved in signaling and DNA repair. Genes that were hyper-methylated included TEs and genes involved in signaling, transcription, protein metabolism, histone modifications (such as heterochromatin formation), and abiotic and biotic stress responses (Boyko et al., 2010). Verhoeven et al. (2010) also reported that changes in DNA methylation level observed at several loci in a population of asexual dandelion, upon exposure to several abiotic and biotic stresses, were faithfully transmitted to the offspring of these plants. Further, more recently, changes in the enrichment levels of H3K9ac and H3K4me2 marks at several hyper-methylated loci were disclosed in the progeny of Arabidopsis salt-stressed plants (Bilichak et al., 2012). This hyper-methylation correlated with an increase in the
enrichment level of the H3K9me2 mark and a decrease in the enrichment level of the H3K9ac mark, as well as a lower gene expression level of the selected loci in the F1 salt-stressed plants (Bilichak et al., 2012). To date no more data on changes of histone modifications in the progeny of stressed plants exist.

1.9.4 Epigenetically determined traits (an evolutionary perspective)

Epigenetic modifications could cause the single genome to form multiple epigenomes (different epigenetic states) which are different in transcriptional activities in response to developmental and environmental stimuli (Zhu, 2008). The new epigenome state can be transmitted through mitoses and sometimes meiosis (Chinnusamy and Zhu, 2009). Propagation of this alternative epigenetic state across generations may result in the formation of epialleles, heritable epigenetic state variants (Mirouze and Paszkowski, 2011). Epialleles that are produced in response to environmental changes might have an important role in plant acclimation and also might be the primary mechanisms of stress-induced genome variability in the population (Lang-Mladek et al., 2010; Boyko and Kovalchuk, 2011). This belief is consistent with the sedentary lifestyle of plants, growing in a fluctuating environment, which their adaptation to the environment cannot be satisfied only by Mendelian inheritance (Boyko and Kovalchuk, 2011). Therefore, besides Mendelian inheritance, that relies on rare genetic mutations followed by selection, a fast and flexible inheritance mechanism, possibly through epigenetic changes, needs to exist in plants (Boyko and Kovalchuk, 2011). Although the role of epigenetic mechanisms in short-term acclimation is clear; so far, there is no well-documented evidence of heritable epigenetically determined traits induced by stress in plants (Mirouze and Paszkowski, 2011; Pecinka and Mittelsten Scheid, 2012).

Interestingly, involvement of epigenetic regulation in acquired traits through genetic elements such as TEs in response to stress conditions is also proposed (Mirouze and Paszkowski, 2011). Transposons are maintained in a repressed and inactivated state through epigenetic regulations in plant genomes; however, there are many examples of transposon activation and transposition triggered by environmental stress (Reviewed in
Grandbastien, 1998; Slotkin and Martienssen, 2007). Retrotransposons, which are abundant in plant genomes, could be especially important in acquired stress adaptive characteristic through epigenetic regulation and are known as “fast drivers of evolution” (Mirouze and Paszkowski, 2011).

1.10 Trans-generational Histone Modifications in Response to Abiotic Stress

There are many reports on changes in histone modification in response to abiotic stresses. However it is important to note that most available data so far have described histone modifications induced by stress only at individual plant genes, and so they are only isolated examples of histone modification during stress responses.

Progress in techniques such as ChIP, microarray (whole genome Tilling Array), as well as high-resolution sequencing (Next-Generation Sequencing, NGS) have facilitated the analysis of changes in histone modification on a whole genome basis (Urano et al., 2010). The whole genomic distribution patterns of H3 methylation and acetylation at several lysine residues such as H3K4me1/2/3, H3K9me2, H3K9ac, and H3K27me3 have been determined in Arabidopsis (Zhang et al., 2007b; Zhang et al., 2009; Zhou et al., 2010). Importantly, Zhou et al. (2010) correlated the genome-wide distribution of H3K9ac and H3K9me2 marks with levels of gene expression. To date, however, only a few groups have studied the dynamic changes of histone modification at the genome wide level in response to stresses, i.e., light and dehydration stresses (Charron et al., 2009; van Dijk et al., 2010). I am unaware of any studies that have assessed these histone modification changes on a global scale in the progeny of stressed plants, in order to find out the role of histone modifications in establishment of the trans-generational stress memory, and also in plant stress adaptation.

1.11 Scope of the Research and Objectives

In this research, I analyzed global changes of histone modifications in the progeny of Arabidopsis plants exposed to salt stress conditions in order to assess the role of histone
modifications in memorizing and maintaining the parental salt stress memory, and further, to identify the genes which are involved in plant salt stress adaptation through heritable histone modifications.

Because of the extensive genomic resources already available for Arabidopsis, including genome wide distribution of some histone marks, I carried out my research using this model species for proof-of-concept. Also, since salt stress is known to be a positive model in establishment of the epigenetic stress memory (Boyko et al., 2010), I used the progeny of Arabidopsis salt stressed plants, which were adapted to salt stress, for this research.

Of the numerous possible histone modifications, I chose the two transcriptionally positive marks, H3K9ac and H3K4me2, since their global genome wide distribution patterns have been reported in unstressed Arabidopsis (Zhang et al., 2009; Zhou et al., 2010). Therefore, the target genes for these modifications are known, and provided a reference point for my research. Furthermore, the acetylation and methylation states of histones have been associated with stress tolerance in plants (Stockinger et al., 2001; Zhu et al., 2008; Song et al., 2012).

So far most of the genome wide studies on Arabidopsis histone modifications have relied on ChIP-chip, but recent developments in NGS techniques and the possibility of analyzing ChIP products directly by sequencing prompted me to use ChIP-seq by Illumina/Solexa sequencing technology. ChIP-seq has a number of advantages over ChIP-chip, including higher resolution, specificity, sensitivity, and simplicity (Schones and Zhao, 2008; Kaufmann et al., 2010).

My hypothesis is that salt stress induces trans-generational changes in the enrichment level of histone acetylation/methylation at stress responsive/adaptive genes in Arabidopsis, and thus histone modification has a critical role in the establishment of salt stress memory and plant adaptation to salt stress through heritable epialleles.

The objectives of this study are:
To investigate global changes in the enrichments of two histone marks, H3K9ac and H3K4me2, in the progeny of salt stressed plants.

To investigate the effects of changes in the enrichments of H3K9ac and H3K4me2 marks on the expression of the corresponding genomic loci.

To identify genes which could be involved or co-regulated in plant adaptation to salt stress through transmitted histone modifications.
2 MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

The progeny of Arabidopsis salt stressed plants were used in this study to examine the possibility of stress-induced trans-generational changes in histone modifications. The F1 progeny of salt stressed and F1 control seeds were obtained from my collaborator, Dr. Igor Kovalchuk (the University of Lethbridge). His group used the transgenic Arabidopsis recombination reporter line (ecotype C24, containing the GUS homologous recombination substrate), to produce these seeds (Boyko et al., 2010). Seeds of this transgenic line were germinated and grown on sterile MS media supplemented with 0.25 mM NaCl. After three weeks, the plants were transferred into soil and the seeds of 20 plants were pooled together (Boyko et al., 2010).

I used these F1 progeny of salt stressed and F1 control seeds, obtained from the University of Lethbridge, to propagate them to the next generation under normal conditions. First, the seeds were sown on moistened soil in 4-inch plastic pots and sprayed with water. After that the pots were covered with a plastic dome and placed at 4º C in the dark for 3-4 days to be stratified. Following stratification, the pots were transferred to a growth room set to 22ºC, 18 h day/6 h night, light intensity of 120 µmol/m²/sec and 70% humidity for germination and growth of the seedlings. The plastic domes were removed one week after germination, and seedlings were watered three times a week, on every alternate day. Three-week-old shoots were used for subsequent experiments.

2.2 Genome Wide Chromatin Immuno-Precipitation (ChIP) Assay

The genome-wide ChIP assay was performed according to the protocol described by Gendrel (2005), which was developed for Arabidopsis plants, with some modifications. Three-week-old wild type Arabidopsis shoots were used to adapt the protocol after which
three-week-old shoots of the progeny of salt stressed and control shoots were used for the ChIP assay.

The entire ChIP procedure took approximately 3-4 days to complete, and the major steps are described, with special emphasis on modifications made to the original protocol by Gendrel (2005). To crosslink the proteins to DNA, 1.7 g of shoots from healthy three-week-old seedlings (6-8 shoots) were harvested, rinsed twice with ddH₂O and fixed immediately with 1% formaldehyde under vacuum (Scienceware®, Cat. # 420220000) for 20 min at room temperature (RT). The fixation was stopped by applying 125 mM glycine to the fixation mixture under vacuum for 5 min. The cross-linked tissues were rinsed twice with ddH₂O, dried by blotting between paper towels, and flash frozen in liquid nitrogen.

For the extraction of chromatin from the frozen tissues, three different extraction buffers (Table 1) were used. All three buffers were freshly made and pre-cooled by incubating on ice. The tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle, and the powder was suspended in 30 ml of Extraction Buffer 1. After suspension, the solution was filtered through two layers of Miracloth (Millipore) on ice. The filtered solution was centrifuged at 3000g at 4°C for 20 min (Eppendorf, Centrifuge 5810R). The pellet was then re-suspended in 1 ml of Extraction Buffer 2 in a 1.5-ml microfuge tube and centrifuged at 12000g at 4°C for 10 min (Eppendorf, Centrifuge 5417R). The chlorophyll overlay was discarded and the white pellet was re-suspended in 300 µl of Extraction Buffer 3 by gentle vortexing. The re-suspended pellet was layered on 300 µl of Extraction Buffer 3 in a fresh microfuge tube and was centrifuged at 16000g at 4°C for 1 h. The chromatin pellet was then re-suspended in 300 µl of fresh cold Nuclei Lysis Buffer (Table 1) by pipetting the solution or by gentle vortexing while keeping it cold. The re-suspended chromatin was sonicated (Fisher Scientific, Sonic Dismembrator, model 100) three times for 15 s each at power 3 and the sample was placed on ice for 1 min between sonication treatments. The sonication efficiency was assessed by running a 5 µl aliquot of each extracted chromatin sample, before and after sonication, on an agarose gel. Before running the agarose gel, the samples were incubated at 65°C overnight in the presence of 200 mM NaCl to reverse the cross-linking of the
corresponding DNAs and their proteins. The rest of the sonicated chromatin was stored at -80°C.

For immuno-precipitation using specific antibodies, the debris was removed from each sonicated chromatin sample by centrifugation at 12000g at 4°C for 5 min. A 10 µl aliquot of each sample was set aside and stored at -20°C to serve as the ‘input DNA control’ (sheared chromatin that was not subjected to immuno-precipitation) in the following steps. The rest of the sheared chromatin samples were diluted to 3 ml in ChIP Dilution Buffer (Table 1), so that the final concentration of SDS, which is present in the Nuclei Lysis Buffer, was reduced to 0.1% in order to prevent denaturation of the antibody. Further, the diluted chromatin solution for each sample was divided equally among three tubes; 2 tubes were used for the immuno-precipitation reactions, each with a different antibody, and the third tube was used as the negative control. According to this protocol, each immuno-precipitation reaction had 8 times more chromatin than the input DNA control. The diluted chromatin was pre-cleared using 40 µl of rinsed agarose beads (Protein A Agarose/Salmon Sperm DNA, Millipore, Cat # 16-157) and incubation at 4°C for approximately 1-2 h with gentle rotation. The beads were then collected by centrifugation at 3800g at 4°C for 30 s and the supernatant was transferred to fresh tubes. 5 ul of the desired antibody was added to each pre-cleared chromatin sample, except in the case of the no-antibody negative control. I used three ChIP grade antibodies against H3K9ac (Millipore, Cat. # 06-942), H3K4me2 (Millipore, Cat. # CS200547) and H3K27me3 (Millipore, Cat # 07-449) histone marks for immuno-precipitation. The H3K27me3 antibody was only used during adaptation of the ChIP protocol using wild type Arabidopsis shoots. A no-antibody negative control was used to measure the non-specific binding of DNA to the protein A-agarose beads. After adding the antibodies, the reactions were incubated at 4°C overnight with gentle shaking. The following day, the antibody–protein/DNA complex (the immuno complexes) were precipitated by incubating each reaction with 50 µl of protein A-agarose beads for at least 2 h at 4 ºC with gentle rotation followed by recovery of the beads by centrifugation (3800g at 4°C for 30 s). The immuno complexes were then washed using buffers in the following sequence: Low-Salt Wash Buffer, High-Salt Wash Buffer, LiCl Wash Buffer (Millipore, Cat # 20-156), and Tris-EDTA (TE) Buffer (Table 1). Each wash was performed with 1
ml of the appropriate buffer followed by incubation at 4°C for 10 min. After the last wash, the immuno complexes were eluted by briefly vortexing the beads in 250 µl of fresh Elution Buffer. The mix was then incubated at 65°C for 15 min in a shaker incubator (800-100 rpm). The beads were then separated by centrifugation (3800g at RT for 2 min) followed by carefully transferring the eluate of each sample to a fresh tube. This step was repeated with fresh Elution Buffer (250 µl), and the two eluates were combined for each sample. Subsequently, the combined eluate of each sample was incubated at 65°C overnight in the presence of 200 mM NaCl to reverse the cross-linking of the corresponding DNA and their proteins. The input samples were also included; these were diluted to 500 µl with Elution Buffer and cross-linked in the presence of 200 mM NaCl.

On the last day, the samples were incubated at 45°C for 1 h in the presence of proteinase K (40 ng), 100 mM EDTA and 40 mM Tris-HCl (pH 6.5) to digest the proteins. The DNA from each sample was purified using phenol/chloroform (1:1; vol/vol) and precipitated using anhydrous ethanol in the presence of 0.3 M sodium acetate (NaOAc, PH 5.2) and 20 µg of glycogen carrier. Finally, the DNA pellet was washed with 70% ethanol and re-suspended in 18 µl of sterile Milli-Q (Millipore) water.

After adaptation of the protocol, the ChIP assay was performed on three-week-old shoots of the progeny of salt stressed and control plants in three independent experiments. The DNA obtained from these ChIP assays was quantified using a NanoVue (GE Healthcare) and used either for quantitative sequencing by NGS or for quantitative amplification by qPCR.

2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used in this study for assessing the sonication efficiency in the ChIP protocol and also for separating DNA fragments after amplification of the ChIP-DNA using specific primers. In both cases, 1% agarose gel (weight/vol) prepared in 0.5X TBE (Tris/Borate/EDTA) buffer containing 1.5 µg/mL ethidium bromide was used.
**Table 1: The Buffers used in the ChIP assay**

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Reagents used at final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extraction Buffer 1</strong></td>
<td>0.4 M sucrose, 10 mM Tris-HCl (pH 8), 10 mM Magnesium Chloride (MgCl₂), 5 mM β-mercaptoethanol (β-ME), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 Protease inhibitor (PI) Complete tablets (Roche, Cat. # 04693132001) in 100 ml of the buffer</td>
</tr>
<tr>
<td><strong>Extraction Buffer 2</strong></td>
<td>0.25 M sucrose, 10 mM Tris-HCl (pH 8), 10 mM MgCl₂, 1% Triton, 5 mM β-ME, 0.1mM PMSF 1/5 Complete tablet of PI in 10 ml of the buffer</td>
</tr>
<tr>
<td><strong>Extraction Buffer 3</strong></td>
<td>1.7 M sucrose, 10 mM Tris-HCl (pH 8), 2 mM MgCl₂, 0.15% Triton, 5 mM β-ME, 0.1mM PMSF, 1/5 Complete tablet of PI in 10 ml of the buffer</td>
</tr>
<tr>
<td><strong>Nuclei Lysis Buffer</strong></td>
<td>50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% SDS 1/5 Complete tablet of PI in 10 ml of the buffer</td>
</tr>
<tr>
<td><strong>ChIP Dilution Buffer</strong></td>
<td>1.1% Triton, 1.2 mM EDTA, 16.7 Mm Tris-HCl (pH 8), 167 mM NaCl, Tris-HCl (pH 8)</td>
</tr>
<tr>
<td><strong>Low-Salt Immune Complex</strong></td>
<td>150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)</td>
</tr>
<tr>
<td><strong>High-Salt Immune Complex</strong></td>
<td>500mMNaCl,0.1%SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)</td>
</tr>
<tr>
<td><strong>Wash Buffer</strong></td>
<td>Tris-HCl (pH 8)</td>
</tr>
<tr>
<td><strong>TE Buffer</strong></td>
<td>10 mM Tris-HCl (pH 8), 1 mM EDTA 1 M Tris-HCl (pH 6.5)</td>
</tr>
<tr>
<td><strong>Elution Buffer</strong></td>
<td>1% SDS, 0.1 M NaHCO₃</td>
</tr>
</tbody>
</table>
The samples were loaded along with a DNA ladder on the gel in the presence of 1X DNA loading dye and electrophoresed in 0.5X TBE buffer for 30-40 min at 100-120 V. The DNA smear or bands were visualized using the UV trans-illuminator on the Gel-Doc instrument (Gel Doc, BioRad).

2.4 Assessing the Success of ChIP Analysis Using PCR Amplification

ChIP-DNA samples from three-week old wild type Arabidopsis shoots were assessed by PCR amplification. For this purpose, the ACTIN2 gene, which is known to be targeted by H3K4me2 mark and not by H3K27me3 mark (Zhang et al., 2009; Zhang et al., 2007), was amplified as a positive and a negative control gene in Chip-DNA samples of the corresponding histone modifications. The specific primers designed for this gene are listed in Appendix A. This control gene was also amplified in the input DNA samples and the no-antibody samples, which are the positive and negative controls for ChIP analysis, respectively.

A 2 µl aliquot of 10-fold diluted ChIP-DNA was used as a template for amplifying the control gene in the presence of Taq DNA polymerase (Fermentas, 0.6 U), 0.2 mM dNTPs, 0.5µM of each forward and reverse primers, and 2mM of MgCl₂ in a 20 µl reaction. PCR was carried out in a thermo cycler (Eppendorf, Mastercycler® pro) under the following parameters: initial denaturation step at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 30 sec, annealing at 60 °C for 30 s and extension at 70°C for 30 sec, and a final extension at 72°C for 7 min. PCR products were separated on 1% agarose gels and visualized using UV light on the Gel-Doc instrument.

2.5 Preparation of Multiplexed End-Repaired Library of ChIP-DNA Samples

ChIP-DNA samples from three-week-old progeny of salt stressed and control Arabidopsis shoots in triplicate experiment was used for sequencing. For this goal, the multiplexed paired-end library of each ChIP-DNA sample was prepared for subsequent cluster generations and DNA sequencing according to the Low Throughput protocol of the Illumina TruSeq DNA Sample Prep Kit v2 guide with minor changes. For instance,
the protocol recommended using 1 μg of DNA for each sample; however, since the DNA yield after the ChIP assay resulted in much less DNA, the protocol was performed with less amounts of DNA. Also, the first step of the protocol, which was fragmentation of DNA, was skipped since the DNA was sheared during the sonication step in the ChIP assay, resulting in fragments of sizes ranging from 300 to 500 base pair (bp).

Library preparation processes included: i) End repair of the ChIP-DNA fragments, ii) Adenylation of 3' ends of the ChIP-DNA fragments, iii) Ligation of adapters (index sequences) to the ChIP-DNA fragments, iv) Purification of the ligation products of the ChIP-DNA samples, v) Enrichment of the DNA fragments of the ChIP-DNA samples, vi) Quantification of the ChIP-DNA library samples, and vii) Normalization and pooling of the ChIP-DNA library samples. These steps are described below in detail.

2.5.1 End repair of the ChIP-DNA fragments

The ChIP-DNA fragments were end repaired by removing the 3' overhangs and filling in the 5' overhangs using 3' to 5' exonuclease and 3' to 5' polymerase activity of the Klenow DNA polymerase. For this aim, a 40 μl aliquot of End Repair Mix, which contained Klenow DNA polymerase, was added to each sample containing the fragmented DNA, and the reaction volume was brought to 100 μl using Resuspension Buffer (RSB). After mixing the DNA by gentle pipetting, the mixture was incubated at 30°C for 30 min. The End Repair Mix also contains phosphate kinase to add 5'-phosphate groups that are required for downstream ligation. After incubation, 160 μl of well dispersed Agencourt AMPure XP beads (Beckman Coulter Inc.) were added to each sample to collect the DNA fragments. After the beads were added, the samples were mixed thoroughly by gentle pipetting and incubated at RT for 15 min for maximum recovery. Next, the sample plate was placed on a magnetic stand at RT for 5 min or until the liquid appeared clear. The supernatant was removed and discarded from each well, while the plate was kept on the magnetic stand, and the beads in the wells were then washed twice with freshly prepared 80% ethanol (EtOH) for 30 s at RT without disturbing the beads. After air drying for at least 15 min, the plate was removed from the magnetic stand and the DNA
was eluted from the beads by gently pipetting with 17.5 μl of RSB. To maximize DNA recovery during elution, the DNA-bead mix was incubated at RT for 5-10 min. After that, the beads were collected on the magnetic stand at RT for 5 min or until the liquid appeared clear. While still on the magnetic stand, 15 μl of the clear supernatant (end-repaired DNA fragments) from each well was transferred to the corresponding wells of a fresh 0.3 ml PCR plate.

2.5.2 Adenylation of 3’ ends of the ChIP-DNA fragments
A-tailing of the 3’ ends of the blunt ended fragments was performed to prevent self-ligation during the adapter ligation process. A corresponding single ‘T’ nucleotide on the 3’ end of the adapter provides a complementary overhang and makes the fragments compatible with the adapters. The adapters were then ligated to the fragments. This strategy prevents the formation of chimera (concatenated template). For this purpose, 12.5 μl of A-Tailing Mix was added to each well and the volume was brought to 30 μl by adding RSB. After the reaction was mixed, the plate was incubated at 37°C for 30 min. The next steps were carried out immediately after this.

2.5.3 Ligation of adapters (Index Sequences) to the ChIP-DNA fragments
This step involves ligation of the universal adapter sequences to the ends of the ChIP-DNA fragments, thus allowing for their hybridization to the flow cell. The Ligation Mix and specific diluted Adapter Index (2.5 μl of each) were added individually to each well of the plate that contained the samples. Since the concentration of my ChIP-DNA samples was lower than the recommended amount of DNA, diluted adapters (1:10) were used. The reaction volume in each well was brought to 37.5 μl with RSB and mixed thoroughly by gentle pipetting. The plate was then incubated at 30°C for 15 min and finally, the ligation was inactivated by adding 5 μl of Stop Ligation Buffer to each well. Next, the indexed end-pair fragments of each sample were recovered by using well dispersed AMPure XP Beads and re-suspended in RSB as described in 2.5.1. This
recovery step was repeated twice, and 20 μl of clear supernatant from each well was transferred to the corresponding well of a fresh new 0.3 ml PCR plate.

2.5.4 Purification of the ligation products of the ChIP-DNA samples

This step involves separation of the ligation reaction products on a gel to remove un-ligated or self-ligated adapters, and to provide a size-range selection of the fragments required for cluster generation. For this goal, an agarose gel (2%) was prepared using 1 X TAE (Tris-cetate EDTA) buffer and 1X SyBr Gold. A total of 20 μl of each sample was loaded on the gel lanes flanked on either side with a DNA ladder to help locate the gel area to be excised. The gel was run in 1 X TAE buffer at 120 V for 120 min and then the bands viewed using a UV trans-illuminator. The bands ranging in size from 400-500 bp were excised. Since the adapters added approximately 120 bp to each fragment, the actual fragment sizes (insert size) were approximately 300–400 bp. After excision of the bands, the MinElute Gel Extraction Kit (QIAGEN) was used to purify each sample according to the manufacturer’s instructions with minor changes. Finally, 20 μl of each sample was eluted using Elution Buffer of the kit and transferred into a 0.3 ml PCR plate.

2.5.5 Enrichment of the DNA fragments of the ChIP-DNA samples

In this step, the DNA fragments that had been ligated with adapters on either end are selectively enriched in each ChIP-DNA sample. Using a PCR primer cocktail that anneals to the ends of the adapters, random PCR amplification was performed and the DNA fragments in each sample were amplified. Fragments without adapters do not hybridize to surface bound primers in the flow cell and thus cannot generate clusters. Also fragments, with only one adapter cannot participate in cluster generation. For this aim, a mix of 5 μl of PCR Primer Cocktail, and 25 μl of PCR Master Mix were added to each sample. After mixing thoroughly by gentle pipetting and sealing the plate using a Microseal ‘B’ adhesive seal, the PCR plate was placed in the thermal cycler, and amplification was performed by using the following parameters: initial denaturation at
98°C for 30 sec, denaturation at 98°C for 10 sec, annealing at 60°C for 30 s and extension at 72°C for 30 s for 18 cycles, and final extension at 72°C for 5 min and hold at 10°C. PCR was performed in 18 cycles as recommended for ChIP samples and this resulted in a robust yield of the library in each sample. After PCR, DNA purification was performed as previously described in 2.5.1 using 50 μl of well-mixed AMPure XP Beads and finally, the recovered PCR product was dissolved in 30 μl of RSB.

2.5.6 Quantification of the ChIP-DNA library samples

Before sequencing, it is important that each DNA library sample is quantified accurately in order to create optimum cluster densities in every lane of the flow cell so that the highest quality of data can be achieved on the Illumina sequencing platforms. The libraries were quantified using qPCR and known standard concentrations, according to the Illumina Sequencing Library qPCR Quantification Guide.

2.5.7 Normalization and pooling of the ChIP-DNA library samples (for multiplexed paired-end libraries)

In this step, the ChIP-DNA library samples were prepared for cluster generation. For this purpose, the concentration of each library sample was normalized to 10 nM using 10 mM Tris-HCl (pH 8.5) and 0.1% Tween 20. For multiplexed paired-end libraries, equal volumes (10 μl) of each normalized library sample, with a different index to be pooled, were combined together. For example, ChIP-DNA samples of the same antibody were multiplexed and sequenced in the same lane. I then had a pool of all 6 ChIP-DNA libraries using the antibody against H3K9ac on the F1 progeny of salt stressed and control plants as well as a pool of all 6 libraries using the antibody against H3K4me2. The entire volume of each pool was mixed by gentle pipetting and this cluster template was diluted to 1 nM and then to 6-8 pM using NaOH. After mixing by gentle pipetting, 1 ml of this diluted multiplex library was allocated in the same lane of the flow cell. Next, the protocol for cluster generation was carried out as described in the Illumina Cluster Generation User Guide.
2.6 Next Generation Sequencing of ChIP-DNA Library Samples

This bioinformatics section was conducted by my collaborator in the University of Lethbridge, and so here I just mention the softwares and procedure which they used. Each pool was sequenced according to the manufacturer’s protocols (Illumina, San Diego, CA). Short read data (QSEQ files format) obtained by the CASAVA 1.6 pipeline were converted to FASTQ format and their quality was assessed using FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/).

2.6.1 Alignment of the short read data of each library sample and converting of file formats

After data quality assessment, short read data of each library sample were aligned to the Arabidopsis genome (TAIR10) using the Bowtie Aligner (Langmead et al., 2009). Since many samples displayed a significant drop in base quality near the end of the reads, 21 nucleotides onwards were excluded from the alignment. The following command was used for running the Bowtie:

```
bowtie -n 2 -l 21 -m 1 -3 20 -S -p 6 <ebwt> <reads.fastq> <aligned_reads.sam>
```

Using this command, read lengths of 21 nucleotides with a maximum of 2 base mismatches to the genome were retained. The Bowtie output in SAM format files were converted to binary BAM format files using SAMTools (Li et al., 2009). Duplicated reads were removed from the aligned retained reads using the SAMTools. Finally, duplicate-free aligned files were converted to the BED format (http://genome.ucsc.edu/FAQ/FAQformat.html#format1) using BEDTools (Quinlan and Hall, 2010).
2.6.2 Calling peaks with MACS and SICER

Two different softwares, MACS (Feng et al., 2011) and SICER (Zang et al., 2009), were used to determine the genomic regions significantly enriched with specific histone marks (peak) in each ChIP-DNA library samples.

In the method based on MACS software, peaks were determined for each sample according to the basic protocol 2 (Running MACS Program to Profile Histone Modification Status) as described in Feng et al. (2011). For this, the previously obtained BAM files were used as the input files to MACS software version 1.4.1 with the following command:

```
macs14 -t <reads.bam> -g 0.1118e9 --nomodel --nolambda -w -n <prefix> --space=30
```

The analyzed output files contained four different kinds of files as follows:

(i) sample_peaks.xls: these files present the data for each found peak, for example, information on the genomic position of each peak (e.g. start position and end position), the location and height of the summits (highest point of the peak), and the number of reads in each peak.

(ii) sample_peak.bed and sample_summit.bed: The first file contains genomic position and p-values for each found peak, and the second one has location and height information for the summits. These files can be visualized into a genome browser.

(iii) sample.wig: These files, which are built for every chromosome in Wiggle format (http://genome.ucsc.edu/FAQ/FAQformat.html#format6), are used for quantitative visualization of each peak and can be uploaded into a genome browser.

Calling peaks based on the SICER program (Zang et al., 2009) was performed as described in the program’s README manual (section 3.2. Running SICER without control library: SICER-rb.sh) using the following options: Species, TAIR10; Redundancy threshold 1, 1; Window size bp, 200; Fragment size, 200; Effective genome fraction, 0.71; Gap size (bp), 200; E-value, 100. The program gives several output files such as:

- sample-W200.graph: This file contains summary graph file in BedGraph format.
- sample-W200-normalized.wig: The previous file normalized by library size per million and converted into wig format. This file quantitatively display peak data and can be uploaded in a genome browser.

All possible peaks related to specific histone modification were identified in each library sample using these programs. Next, regions with differential peaks were determined between the progeny of salt stressed samples and the progeny of control samples as described in the following section.

2.6.3 Determining differential peaks using DESeq based approach

In order to determine the differential peaks, a common set of peak boundaries was assembled from all of the MACS and SICER peaks in different samples of each specific histone mark using IntersectBed and BedMerge programs from BedTools. This common set that contained all the found peaks were used as a template by the DESeq program, so that the peaks of each sample could be compared with it. Subsequently, the number of reads located within each merged peak boundary was counted using the following command in BedTools:

```
intersectBed -a reads.bed -b regions.bed -f 1.0 | coverageBed -a Ac_CT_2.bed -b merged_peaks.bed | cut -f 4,5 > outfile
```

The DESeq program disregards peaks with a low number of reads through the analysis. Finally, the differential peaks between the progeny of salt stressed and the progeny of control samples were estimated using the DESeq R software (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). This program determine the differences in the number of the short reads between libraries and it is appropriate in cases where I want compare differences in the number of the short reads between genomic intervals of the different samples, especially where the biological replicates are available. The analysis was performed as described in the DESeq user guide under Section 3.1 (Standard Comparison between Two Experimental Conditions). The result file of DESeq analysis, named ct_salt.txt, contained differential peaks found
between the F1 progeny of salt stressed and F1 control samples from all repeats of the same histone mark. This file includes the following information:

- Id: differential peak name;
- baseMean: the mean of the counts divided by the size factors for the counts for the both conditions;
- baseMean CT: The mean of the counts divided by the size factors for the counts for the progeny of control samples;
- baseMean SALT: The mean of the counts divided by the size factors for the counts for the progeny of salt stress samples;
- foldChange: the ratio of mean CT/ mean SALT;
- log2FoldChange: the logarithm (to basis 2) of the fold Change;
- pval: p-value for the statistical significance of the fold change;
- padj: adjusted p-value for multiple testing (Benjamini-Hochberg method);

2.6.4 Peak annotation

The differential peaks obtained by DESeq analysis were annotated using Seqmonk software by the corresponding gene, and distance cut-off of 2000 bp, (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/). The output file, named ct_salt_annotated.txt, has the following information besides the above mentioned data:

- chr: the chromosome number in which the differential peak is placed;
- start: the genomic position of the start point of the peak;
- end: the genomic position of the end point of the peak;
- feature: corresponding gene of the differential peak (proximal gene area: +/- 2000 bp from the transcription start site);
- feature description: short description of the corresponding gene;
- orientation: direction related to the corresponding gene;
- distance: distance of the differential peak to the corresponding gene;
- ct (RPM): number of the reads (per million) of the differential peak in the library of the progeny of control samples;
exp (RPM): number of the reads (per million) of the differential peak in the library of the progeny of salt stressed samples;

2.7 Primer Design

The summit regions of the statistically significant differential peaks, with the most differences in the number of short reads, were selected for designing the primers. These primers were used for subsequent qPCR analysis. These primers were also located on the cDNA sequences of the corresponding gene, and therefore the same primer pairs were used for the reverse transcription qPCR (qRT-PCR) analysis. All of the primers were designed using Lasergene 6 (DNASTAR) software with the following parameters: product size ranged 80-250 bp, melting temperature between 50-65°C, primer length ranged 18-24 bp, and the GC content between 40-60%. The primers were blasted using Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) before final selection. All primers which were used are listed in Appendix A.

2.8 Real Time qPCR

Statistically significant differential peaks identified by ChIP-seq analysis were confirmed using ChIP assay followed by qPCR analysis. These analyses were performed using four biological and two technical replicates.

For qPCR analysis, the summit region of the differential peak was amplified with PerfeCta® SYBR® Green FastMix® (Quanta Biosciences, cat# 95072-012). One µl of 10-fold diluted ChIP-DNA (100 pg to 100 ng) was used as a template, and 100 nM of each forward and reverse primer were used in each 10 µl reaction. After all of the components were added, the samples were vortexed gently to mix the contents and centrifuged briefly to collect all the components. The fast 2-step cycling program recommended by the kit (95°C for 30s for one cycle, and 95°C for 5s and 60°C for 30s for 45 cycles) was used for the quantitative amplification of the ChIP-DNA samples.
(Bio-Rad, CFX96™). Melt-curve analysis was also performed (65-95°C for 5 s, with a 0.5°C increment) for each primer.

The relative amount of each region in the ChIP-DNA samples was normalized against the ACTIN7 gene (AT5G09810) using the $2^{-\Delta\Deltact}$ method as described by the machine manufacturer (Bio-Rad, CFX96™). An average of the relative amount of each region was then calculated for all four independent biological replicates. Further, the relative amounts of each region in the different biological replicates were compared between the F1 progeny of salt stressed and the F1 control ChIP-DNA samples using the T-test.

2.9 RNA isolation and RT-qPCR analysis

Total RNA was extracted from frozen three-week-old Arabidopsis shoots from the progeny of stressed and control plants using the RNeasy Mini kit (QIAGEN). For this purpose, 2-3 shoots were ground to a fine powder using liquid nitrogen, and 100 mg of the powder was used for RNA extraction. The extraction was performed according to the manufacturer’s protocol, and in the final step, the RNA was dissolved in 50µl of RNase-free water. The experiment was performed in four biological replicates.

To remove the DNA contamination from each RNA sample, DNase treatment was performed after RNA extraction. For this, 1 µl of the TURBO DNase (Ambion, Cat. # AM2238), and 5.5 µl of its 10X buffer were added to each RNA samples (50 µl); the samples were then incubated at 37 ºC for 30 min. Next, the DNase was inactivated by incubation at 75 ºC for 10 min in the presence of 15mM EDTA. After that, the concentration of RNA in each sample was determined using a spectrophotometer (NanoVue, GE Healthcare).

Reverse transcription was performed in a total volume of 20 µl with 4 µl of 5X qScript cDNA SuperMix (Quanta Biosciences, US) and 850 ng of the RNA sample. After all the components were added, the contents were gently vortexed and briefly centrifuged. The samples were then incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. After cDNA synthesis, a 3X dilution of the cDNA products was made by adding sterile
MilliQ water before they were used for qRT-PCR. qRT-PCR was performed in the same manner as qPCR which is described in Section 2.8.

For qRT-PCR data normalization, different reference genes, ACTIN2, PP2A, UBQ5, and UBQ10, were assessed to select the best fit for my experimental conditions. Since the expression of the PP2A gene remained constant between the two different conditions (F1 progeny of salt stressed and F1 control samples), it was selected as a reference gene. The expression of the tested genes in the different samples was normalized against the expression of the PP2A gene using the $2^{(-\Delta\Delta Ct)}$ method. An average of the relative expression of each gene was then calculated for all four independent biological replicates. Further, the relative expression levels of each gene in the different biological replicates were compared between the F1 progeny of salt stressed and the F1 control samples using the T-test.
3 RESULTS

3.1 ChIP Assay and Adaptation of the Protocol

The ChIP assay is regarded as the most accurate and reliable technique to analyze histone modifications in plants that have been subjected to various stress conditions (Haring et al., 2007; Urano et al., 2010). The ChIP protocol adapted for this study was developed for high resolution analysis of histone modifications in the entire genome of various tissues of Arabidopsis (Gendrel et al., 2005). I used three-week-old wild type Arabidopsis shoots to adapt the protocol. Cross-linking of chromatin (protein-DNA and protein-protein) in the cells, which helps to preserve the chromatin structure, is a critical step in the ChIP assay (Gendrel et al., 2005; Haring et al., 2007). Vacuum infiltration of 1% formaldehyde for 20 min provided sufficient penetration of the fixative into the cells, which was manifested by the translucent appearance of the tissues (data not shown). High resolution ChIP protocols are also dependent on optimal fragmentation (shearing) of the chromatin (Haring et al., 2007). The modified sonication step described in Section 2.2 produced the desired chromatin fragmentation sizes ranging from 300 bp to 500 bp (Figure 2). Reverse crosslinking of the sonicated and unsonicated chromatin prior to running them on the gel (Haring et al., 2007) allowed separation and clear assessment of the DNA fragments in the gel.

The ChIP experiment is validated by the amplification of the sequences known to be targets for specific histone modifications. For example, ACTIN2; a known target for H3K4me2 mark but not H3K27me3 mark (Zhang et al., 2007b; Zhang et al., 2009), was amplified by regular PCR from the ChIP-DNA samples as well as the input DNA control (sheared chromatin that was not subjected to immuno-precipitation) and the no-antibody control (sheared chromatin without antibody passed through the beads). An expected 198 bp band was produced in ChIP-DNA with antibody against the H3K4me2 mark as well as in the input control lane (Figure 3, lanes 1 and 4). A faint band was also seen for the no-antibody ChIP sample (lane 3) and in lane 2 representing the ChIP-DNA with antibody against the H3K27me3 mark. Although bands in lanes 2 and 3 were unexpected, some
**Figure 2:** Chromatin extracted samples before and after sonication. Lane 1: DNA ladder (HyperLadder II); lanes 2, 4, 6: samples of chromatin before sonication; lanes 3, 5, 7: samples of chromatin after sonication.

**Figure 3:** Amplification of the *ACTIN2* gene in the different DNA samples obtained from the ChIP assay. Lane 1: ChIP-DNA subjected to antibody against H3K4me2; Lane 2: ChIP-DNA subjected to antibody against H3K27me3; Lane 3: ChIP negative control (no-antibody DNA); Lane 4: ChIP positive control (input DNA); Lane 5: PCR negative control; Lane 6: DNA HyperLadder II.
background signal is often observed in ChIP experiments (Haring et al., 2007). Based on these results the ChIP protocol was considered to be reasonably adapted, and was used in subsequent experiments.

### 3.2 ChIP-seq Assay on the Progeny of Salt-Stressed and Control Plants

The optimized ChIP protocol was next performed on the progeny of salt stressed and control (non-stressed) plants. According to Boyko et al. (2010), salt stress induces a higher HRF, higher tolerance to salt stress, as well as global hypo-methylation in the progeny of Arabidopsis plants. Therefore, salt stress was taken as a positive model of epigenetic transmission. Since trans-generational stress effects, such as increased HRF, were more obvious and prominent in plants subjected to 25 mM NaCl as compared to 100 mM NaCl (Boyko et al., 2010), in the present study the progeny of plants exposed to 25 mM NaCl concentrations were used for the ChIP assay.

Three-week-old shoots of the progeny of salt-stressed and control plants were subjected to ChIP assay in triplicate experiments using antibodies against histone H3K4me2 and H3K9ac marks. To analyze genome-wide changes of these histone marks, first the libraries were made from the ChIP-DNA fragments of different samples (Section 2.5), and then the libraries were sequenced on the Illumina platform (San Diego, CA). During library preparation, one of the ChIP-DNA replicates for the progeny of salt stressed plants enriched for the H3K9ac mark was miss-handled, and thus it was omitted from further analysis. The procedure was continued using the remaining two replicates of this sample. In order to have reliable ChIP-seq data, it is necessary to have at least two libraries for each ChIP-DNA sample (Beta Cell Biology Consortium, 2011). In fact, some ChIP-seq experiments were performed using only one library of each sample (van Dijk et al., 2010).

After sequencing, the quality of the data obtained for each library was assessed using FastQC software. All of the ChIP-DNA library samples passed the quality control assessment and were used for downstream bioinformatics analysis.
The short reads in each library were aligned to the Arabidopsis genome sequence. The total number of sequenced reads, and the total number of reads aligned to the Arabidopsis genome, with two or less mismatches, is presented in Tables 2 and 3 for H3K4ac and H3K4me2, respectively. The ChIP-DNA samples enriched by H3K9ac and H3K4me2 marks obtained from the F1 progeny of salt stressed plants are referred to as ac_salt and me_salt, respectively; and the ChIP-DNA samples enriched by H3K9ac and H3K4me2 marks from F1 control plants are referred to as ac_ct and me_ct, respectively.

The total numbers of aligned reads were inconsistent among the libraries (Tables 2 and 3), which may be due to contamination of the ChIP-DNA fragments with DNA carrier beads (salmon sperm DNA), and/or contamination of the libraries during the PCR reactions of the library preparation steps. For example, in the case of ac_salt2, me_salt3 and me_ct2 samples, the total number of uniquely aligned reads is quite low (lower than a million); however, since all of them passed quality control, they were used for bioinformatics analysis.

In the next step, the regions in the genome that were significantly enriched for specific histone marks and that had a high number of short reads (peaks) in the ChIP-DNA samples were identified using the MACS and SICER software. Although there was considerable overlap between peaks identified by the MACS and SICER programs, the use of two softwares greatly reduced the probability of missing any peaks, providing a more comprehensive dataset for the subsequent bioinformatics steps. All of the identified peaks of each sample with the Bedgraph file format can be visualized using a genome browser, such as the Integrated Genome Browser (IGB, http://bioviz.org/igb/download.html), against the Arabidopsis genome (TAIR10). An example of a Bedgraph file of histone H3K9ac peaks in the representative length of chromosome 1 in one ac_salt sample and one ac_ct sample is shown in Figure 4.
Table 2: The total number of sequenced reads, and the total number of aligned reads onto the Arabidopsis genome (containing 2 or less mismatches) for each library sample related to the H3K4me2 mark.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of sequenced reads</th>
<th>Total number of aligned reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>me_salt1</td>
<td>2,362,944</td>
<td>952,523</td>
</tr>
<tr>
<td>me_salt2</td>
<td>3,604,115</td>
<td>1,850,663</td>
</tr>
<tr>
<td>me_salt3</td>
<td>719,936</td>
<td>98,286</td>
</tr>
<tr>
<td>me_ct1</td>
<td>3,686,154</td>
<td>1,247,969</td>
</tr>
<tr>
<td>me_ct2</td>
<td>4,732,580</td>
<td>569,056</td>
</tr>
<tr>
<td>me_ct3</td>
<td>2,055,643</td>
<td>1,096,089</td>
</tr>
</tbody>
</table>

Table 3: The total number of sequenced reads and the total number of aligned reads onto the Arabidopsis genome (containing 2 or less mismatches) for each library sample related to the H3K9ac mark.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of sequenced reads</th>
<th>Total number of aligned reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>ac_salt1</td>
<td>7,874,223</td>
<td>1,012,631</td>
</tr>
<tr>
<td>ac_salt2</td>
<td>1,701,220</td>
<td>247,263</td>
</tr>
<tr>
<td>ac_ct1</td>
<td>4,134,339</td>
<td>2,465,547</td>
</tr>
<tr>
<td>ac_ct2</td>
<td>10,722,139</td>
<td>8,297,178</td>
</tr>
<tr>
<td>ac_ct3</td>
<td>5,632,672</td>
<td>2,807,371</td>
</tr>
</tbody>
</table>
Figure 4: IGB browser view of the all found peaks related to the H3K9ac mark on the 1,000,000 bp length of chromosome 1 in a library of the F1 progeny of salt stressed plants (red peaks) and control plants (green peaks) (A). A representative, 30,000 bp close-up of the region is also shown (B). The heights of the peaks represent the number of short reads that were recovered in each region. Along with the peaks, there is an indication of the genes and their direction at the bottom of the picture.
3.3 Differential Peaks on the Chromatin of the Progeny of Salt Stressed and Control Plants

The DESeq program was used for finding the differential peaks in the F1 salt and control ChIP-DNA Libraries for each specific histone mark. The output file after annotation, named “ct_vs_salt_annotated.txt”, contained all the information related to each differential peak as well as the information related to the corresponding gene of each differential peak. This output annotated file was used as the main reference for the subsequent steps, including the selection of significant differential peaks throughout the chromatin of the progeny of salt stressed plants compared to those of control plants. This file is available in the online supplementary file. In the following section, I will present analysis of the identified differential peaks related to each histone mark.

3.3.1 Genomic regions with differential enrichment level of the H3K4me2 mark

According to DESeq analysis, there were no significant differences in enrichment levels of H3K4me2 mark between the F1 progeny of salt stressed and control plants. Although the program identified some differential peaks between the samples, none of them had a \( P \) adjusted (padj) value of less than 0.2, indicating that the peaks were quite similar between the two sets of samples. These results are illustrated in Figure 5, which shows the enrichment level of the H3K4me2 mark in library samples of the F1 progeny of salt stressed and control plants. As shown in this figure, the genome wide enrichment level of the H3K4me2 mark does not appear to be different between the progenies of salt stressed and control plants. Thus, it appears that salt stress does not affect the H3K4me2 mark in the progeny of salt stressed Arabidopsis plants, and that this mark has no role in plant adaptation to salt stress.
Figure 5: IGB browser view of the enrichment level of the H3K4me2 mark on the 1,000,000 bp length of chromosome 1 in a library sample of the F1 progeny of salt stressed plants (red peaks) and control plants (green peaks) (A). A representative 100,000 bp close-up of the region is also shown (B). The height of the peaks represents the number of the short reads that were recovered in each region. Along with the peaks, there is an indication of the genes and their direction at the bottom of the picture.
3.3.2 Genomic regions with differential enrichment level of the H3K9ac mark

On the basis of DESeq analysis, a total of 17,931 differential H3K9ac mark peaks were identified on the chromatin of the F1 progeny of salt stressed plants as compared to control plants; however, most of these were not significantly different as indicated by their high padj (> 0.2). This is because the software considered minute differences in the number of short reads among the samples. Two criteria were considered when eliminating the number of insignificant differential peaks in my analysis: i) a padj value of less than 0.2, and ii) only peaks with greater than a 2-fold change between the mean number of short reads in the F1 progeny of salt stress samples compared to the control samples. With these criteria, the number of differential peaks with higher enrichment levels of the H3K9ac mark in the F1 progeny of salt stressed samples compared to the control samples was reduced to 91 (with fold change ranging from 179.919 to 2.004); while the number of peaks with lower enrichment levels of this histone mark was reduced to 318 (fold change ranging from 0.495 to 0.146). These data are available in the online supplementary file. It appears that at the genome wide level, the chromatin of the progeny of salt stressed plants is less enriched in the H3K9ac mark compared to that of the control plants. This may indicate that salt stress induced histone de-acetylation in the progeny of salt stressed plants or that salt stress induced histone de-acetylation that was then inherited by the progeny.

For subsequent experiments, statistically significant differential peaks, ones with more than a 2-fold change and a padj value of less than 0.05 were selected. A total of 45 peaks met both criteria, and each of them was part of an annotated gene. The complete list of these differential peaks as well as their baseMean in the F1 progeny of salt stressed and control samples, fold change, padj value, position, and their corresponding gene is presented in Table 4. As it is obvious from the table, of the 45 candidate genes, only one (AT1G56500) was more enriched for the H3K9ac mark in the F1 progeny of salt stressed plants, while the others showed less enrichment for this histone mark. Only the 45 genes with a statistically significant differential enrichment level of the H3K9ac mark in the progeny of salt stressed plants compared to the progeny of control plants were subjected to further analysis as will be detailed in the following sections.
Table 4: Statistically significant differential H3K9ac mark peaks in the chromatin of the F1 progeny of salt stressed plants compared to that of control plants. The table shows the information relating to the 45 statistically significant differential peaks, including their peak ID, baseMean in the F1 control and the F1 progeny of salt stressed samples, fold change, padj value, exact position including their chromosome (Chr), start and end points, as well as their corresponding gene (see Section 2.6.3-2.6.4).

<table>
<thead>
<tr>
<th>Id</th>
<th>baseMean (F1 control)</th>
<th>baseMean (F1 Salt)</th>
<th>Fold Change</th>
<th>padj</th>
<th>Chr</th>
<th>start</th>
<th>end</th>
<th>feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>peak_3438</td>
<td>59.56573</td>
<td>13.71407</td>
<td>0.230</td>
<td>0.019</td>
<td>1</td>
<td>23953232</td>
<td>23954467</td>
<td>AT1G64500</td>
</tr>
<tr>
<td>peak_169</td>
<td>78.33211</td>
<td>21.73602</td>
<td>0.277</td>
<td>0.019</td>
<td>1</td>
<td>787117</td>
<td>788403</td>
<td>AT1G03220</td>
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<tr>
<td>peak_326</td>
<td>58.51647</td>
<td>19.46184</td>
<td>0.332</td>
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<td>1</td>
<td>1569399</td>
<td>1570151</td>
<td>AT1G05370</td>
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<tr>
<td>peak_1809</td>
<td>70.20733</td>
<td>14.13018</td>
<td>0.201</td>
<td>0.019</td>
<td>1</td>
<td>8959373</td>
<td>8960880</td>
<td>AT1G25510</td>
</tr>
<tr>
<td>peak_3078</td>
<td>10.63554</td>
<td>51.03107</td>
<td>4.798</td>
<td>0.020</td>
<td>1</td>
<td>21166397</td>
<td>21168956</td>
<td>AT1G56500</td>
</tr>
</tbody>
</table>

- **Id**: Unique identifier for each peak.
- **baseMean (F1 control)**: Average baseMean value in the F1 control samples.
- **baseMean (F1 Salt)**: Average baseMean value in the F1 progeny of salt stressed samples.
- **Fold Change**: Ratio of the baseMean in the F1 control to the F1 progeny of salt stressed samples.
- **padj**: Adjusted p-value, indicating statistical significance.
- **Chr**: Chromosome where the peak is located.
- **start**: Start position of the peak.
- **end**: End position of the peak.
- **feature**: Corresponding gene or feature.

The table includes 45 peaks, each with detailed information on their location and expression changes in response to salt stress, providing insights into the molecular mechanisms underlying the stress response in plants.
Table 4. continued. Statistically significant differential H3K9ac mark peaks in the chromatin of the F1 progeny of salt stressed plants compared to that of control plants.

<table>
<thead>
<tr>
<th>Id</th>
<th>baseMean (F1 control)</th>
<th>baseMean (F1 Salt)</th>
<th>Fold Change</th>
<th>padj</th>
<th>Chr</th>
<th>start</th>
<th>end</th>
<th>feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>peak_7744</td>
<td>54.71733</td>
<td>12.59923</td>
<td>0.230</td>
<td>0.026</td>
<td>3</td>
<td>1241025</td>
<td>1241694</td>
<td>AT3G04590</td>
</tr>
<tr>
<td>peak_8989</td>
<td>101.43727</td>
<td>33.79229</td>
<td>0.333</td>
<td>0.036</td>
<td>3</td>
<td>6978676</td>
<td>6980185</td>
<td>AT3G20015</td>
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<tr>
<td>peak_7986</td>
<td>135.94566</td>
<td>51.3805</td>
<td>0.378</td>
<td>0.036</td>
<td>3</td>
<td>2347443</td>
<td>2348828</td>
<td>AT3G07350</td>
</tr>
<tr>
<td>peak_8089</td>
<td>110.10465</td>
<td>35.72378</td>
<td>0.324</td>
<td>0.040</td>
<td>3</td>
<td>2768796</td>
<td>2771357</td>
<td>AT3G09070</td>
</tr>
<tr>
<td>peak_9152</td>
<td>87.62805</td>
<td>34.23512</td>
<td>0.391</td>
<td>0.043</td>
<td>3</td>
<td>7916228</td>
<td>7918971</td>
<td>TIC</td>
</tr>
<tr>
<td>peak_11984</td>
<td>99.56782</td>
<td>30.24529</td>
<td>0.304</td>
<td>0.019</td>
<td>4</td>
<td>9467521</td>
<td>9469238</td>
<td>PLA-I[beta]2</td>
</tr>
<tr>
<td>peak_12811</td>
<td>58.53718</td>
<td>15.9304</td>
<td>0.272</td>
<td>0.019</td>
<td>4</td>
<td>13982897</td>
<td>13983807</td>
<td>AT4G28180</td>
</tr>
<tr>
<td>peak_11081</td>
<td>143.66647</td>
<td>47.84906</td>
<td>0.333</td>
<td>0.022</td>
<td>4</td>
<td>57223</td>
<td>59327</td>
<td>HAM3</td>
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<tr>
<td>peak_13290</td>
<td>47.35134</td>
<td>16.70256</td>
<td>0.353</td>
<td>0.043</td>
<td>4</td>
<td>16375927</td>
<td>16376852</td>
<td>EDA9</td>
</tr>
<tr>
<td>peak_16332</td>
<td>37.94589</td>
<td>8.82303</td>
<td>0.232</td>
<td>0.019</td>
<td>5</td>
<td>18963889</td>
<td>18965083</td>
<td>AT5G46730</td>
</tr>
<tr>
<td>peak_16015</td>
<td>128.80193</td>
<td>35.75271</td>
<td>0.277</td>
<td>0.020</td>
<td>5</td>
<td>16829669</td>
<td>16831335</td>
<td>ATBG_PPAP</td>
</tr>
<tr>
<td>peak_13841</td>
<td>121.20616</td>
<td>34.00592</td>
<td>0.280</td>
<td>0.028</td>
<td>5</td>
<td>313418</td>
<td>314997</td>
<td>ATSR1</td>
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<tr>
<td>peak_14229</td>
<td>60.82184</td>
<td>21.1797</td>
<td>0.348</td>
<td>0.036</td>
<td>5</td>
<td>2183429</td>
<td>2184806</td>
<td>AT5G07030</td>
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<td>peak_17768</td>
<td>96.18181</td>
<td>34.93606</td>
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<td>26394813</td>
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<td>AT5G65990</td>
</tr>
<tr>
<td>peak_13843</td>
<td>45.24366</td>
<td>7.00724</td>
<td>0.155</td>
<td>0.043</td>
<td>5</td>
<td>324585</td>
<td>325313</td>
<td>ATOFP1</td>
</tr>
<tr>
<td>peak_14525</td>
<td>67.61371</td>
<td>17.1031</td>
<td>0.253</td>
<td>0.043</td>
<td>5</td>
<td>3708147</td>
<td>3709247</td>
<td>AT5G11550</td>
</tr>
<tr>
<td>peak_15086</td>
<td>96.89695</td>
<td>37.33709</td>
<td>0.385</td>
<td>0.043</td>
<td>5</td>
<td>6494007</td>
<td>6495224</td>
<td>AT5G19290</td>
</tr>
<tr>
<td>peak_14982</td>
<td>127.8447</td>
<td>56.15584</td>
<td>0.439</td>
<td>0.043</td>
<td>5</td>
<td>5953601</td>
<td>5955777</td>
<td>AT5G17980</td>
</tr>
</tbody>
</table>
3.4 Gene Ontology Classification of the Candidate Genes

Gene ontology classification of the 45 candidate genes, which were differentially enriched for the H3K9ac mark in the F1 progeny of salt stressed plants relative to F1 control plants, was performed using the online tool at www.arabidopsis.org (Berardini et al., 2004). The gene ontology is based on three aspects of gene products, namely, molecular function, cellular component, and biological process. Figures 6 shows this classification based on molecular function (Figure 6A) and biological process (Figure 6B). On the basis of the molecular function classification, the genes could be grouped into three different categories (with the exception of 19% that had an unknown molecular function), including: enzymatic activity (hydrolase activity, kinase, and others), binding activity (to nucleic acid, nucleotide, protein), and transferase or transport activity.

Furthermore, most of these genes could be classified into a variety of biological processes (with the exception of 14% that had an unknown biological process), including metabolism, development, cell organization and biogenesis, transcription regulatory activity, transport (including energy pathway), and signal transduction. It is important to note that only 16% of these genes were found to be associated with different biotic and abiotic stress responses. Among these, only one gene, AT1G03220, was previously reported to be involved in salt stress response (Gong et al., 2001).
Figure 6: Gene ontology classification of the candidate genes based on the molecular function (A) and biological process (B). The heights of columns represent the percentage of genes in each group.
3.5 Validation of the ChIP-seq Data by ChIP-qPCR

Since the ChIP-seq method is a quantitative method based on the number of short reads which are immuno-precipitated in each sample, the ChIP-seq data was validated by performing qPCR on the ChIP-DNA. To this end, new ChIP assays using an antibody against the H3K9ac mark was performed on the progeny of salt stressed and control plants. Thereafter, the amount of the precipitated DNA in each sample was quantified by qPCR using primers specific for each of the 45 candidate genes. Primers were designed at the summit regions of the differential peaks of the genes (after visualization of the differential peaks in the IGB). The experiments were performed in four biological repeats. The qPCR results for each gene in all samples were normalized against the *ACTIN7* gene, which did not show noticeable changes in the enrichment level of the H3K9ac mark in the progeny of the salt stressed relative to control plants according to the ChIP-seq results. The normalized quantity value was calculated using the $2^{-\Delta\Delta CT}$ method, and then the mean-normalized value of four independent biological replicates was obtained.

Figure 7 shows the chromosomal regions of three representative genes containing the differential H3K9ac mark obtained from the ChIP-seq data in the two library samples (one ac_salt and one ac_ct samples) visualized in the IGB. The figure also displays the normalized relative enrichment level of the H3K9ac mark at these three genes in the progeny of the salt stressed and control samples obtained by ChIP followed by qPCR. For example, in the region containing the AT2G48010 gene, differential H3K9ac peaks are obvious among the F1 progeny of salt stressed and F1 control library samples in the IGB view. This gene has lower enrichment level of this histone mark in the ac_salt library compared to ac_ct library, and consistently this gene has a lower enrichment level of this mark in the F1 progeny of salt stressed as compared to the F1 control plants based on the qPCR data.

The qPCR analysis validated the results of the ChIP-seq method for 33 of the 45 genes; 32 genes had a lower enrichment level of the H3K9ac mark and one gene (AT1G56500) had higher enrichment level of this mark in the progeny of salt stressed plants compared
A) AT2G48010

B) AT3G04570

C) AT4G16820
Figure 7: Validation of the ChIP-seq results using ChIP followed by qPCR. The IGB view of the chromosomal region of three selected genes containing a differential H3K9ac mark in the two library samples, ac_ct (upper green peaks) and ac_salt (lower red peaks) obtained from ChIP-seq data are presented to the left. The height of the peak correlates with the number of times the region was covered by the read. The position of the genes and their transcription directions are also indicated at the bottom of the IGB view. The normalized relative enrichment of the H3K9ac mark at the differential peaks of these genes obtained by ChIP-qPCR is also represented by the bar graphs to the right. The mean-normalized quantity values are an average of four independent biological replicates (each value was the mean of two technical replicates). The small yellow lines in the pictures represent the position of the primers. The error bars represent standard deviation (SD). The asterisks represent a significant difference between the progeny of salt stressed samples to those of the control samples using Student’s t-test (* represents $P < 0.05$).
to that in the progeny of control plants. However, the remaining 12 genes showed the same enrichment or even higher enrichment level (contrary to the ChIP-seq data) in the progeny of salt stressed plants when tested using ChIP-qPCR. According to the qPCR results, in eight of the 45 candidate genes (AT3G01820, AT3G01820, AT2G17880, AT1G07250, AT1G15825, AT3G07350, AT5G65990, AT4G34200), there were no differences in the enrichment level of the H3K9ac mark between the progeny of salt stressed and control plants. Further, according to the qPCR results, in four of the 45 candidate genes (AT2G48030, AT5G42100, AT2G19570, and AT1G09750), the progeny of salt stressed plants showed higher enrichment level of the H3K9ac mark as compared to the control plants; a result that is inconsistent with the ChIP-seq data. Nevertheless, data for 33 candidate genes, or 73% of the total, were consistent between ChIP-seq and ChIP-qPCR. Table 5 shows a summary of the validation results along with the corresponding ChIP-seq data for these 33 genes.

It is important to mention that the six known stress-responsive genes (AT1G03220, salt stress-responsive gene), AT3G04570, AT2G39980, AT1G18080, AT1G05630, and AT3G22380) were also confirmed by qPCR to have a lower enrichment level of the H3K9ac mark in the progeny of salt stressed plants. Generally speaking, all of these 33 genes could play a role in plant salt stress adaptation through trans-generational transmission of the epigenetic modifications, i.e. changes in H3K9ac mark. In order to examine the possibility of these 33 candidate genes being involved in trans-generational salt stress adaptation, and to determine the extent to which this histone modification affects the corresponding genes, experiments were conducted to determine the expression of these genes in the progeny of salt stressed relative to control plants.
Table 5: Validation of ChIP-seq results by The ChIP followed by qPCR for the 33 candidate genes containing a differential H3K9ac mark. Mean-normalized enrichment ratios of the H3K9ac mark at the 33 genes in the progeny of salt stressed to control samples obtained by qPCR along with the corresponding fold change ratios obtained by NGS. The mean-normalized value of each gene is the average of four independent biological replicates (each value was the mean of two technical replicates).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>ChIP-qPCR Results (Ratio: F1 Salt/ F1 Control)</th>
<th>ChIP-seq Results (Fold Change: F1 Salt/ F1 Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G63200</td>
<td>0.680</td>
<td>0.173</td>
</tr>
<tr>
<td>AT1G64500</td>
<td>0.714</td>
<td>0.230</td>
</tr>
<tr>
<td>AT1G03220</td>
<td>0.833</td>
<td>0.277</td>
</tr>
<tr>
<td>AT1G05370</td>
<td>0.730</td>
<td>0.332</td>
</tr>
<tr>
<td>AT1G25510</td>
<td>0.709</td>
<td>0.201</td>
</tr>
<tr>
<td>AT2G48010</td>
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<td>0.273</td>
</tr>
<tr>
<td>AT4G16820</td>
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<td>0.304</td>
</tr>
<tr>
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<td>AT5G46730</td>
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<td>0.232</td>
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<td>AT1G56500</td>
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<td>AT4G00150</td>
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<tr>
<td>AT3G04570</td>
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<td>0.230</td>
</tr>
<tr>
<td>AT2G43540</td>
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</tr>
<tr>
<td>AT3G04590</td>
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</tr>
<tr>
<td>AT5G01820</td>
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</tr>
<tr>
<td>AT1G01900</td>
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</tr>
<tr>
<td>AT2G37580</td>
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</tr>
<tr>
<td>AT3G20015</td>
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<td>AT5G07030</td>
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<td>AT2G45680</td>
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</tr>
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<td>AT2G39980</td>
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<tr>
<td>AT1G18080</td>
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</tr>
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<td>AT2G40230</td>
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<td>AT5G01840</td>
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</tr>
<tr>
<td>AT5G11550</td>
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<td>AT5G17980</td>
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</tr>
<tr>
<td>AT5G21482</td>
<td>0.672</td>
<td>0.365</td>
</tr>
</tbody>
</table>
Expression Analysis of the Genes Showing Changes in the Enrichment Levels of the Histone H3K9ac Mark in the Progeny of Salt Stressed Plants

To determine whether the changes in the enrichment level of the histone H3K9ac mark induced by parental salt stress had an effect on the expression of the corresponding genes, qRT-PCR assays were performed to measure the transcript levels of the 33 candidate genes in the shoots from the progeny of the salt stressed and control plants. The qRT-PCR data were normalized against the PP2A gene, which showed the most consistent expression in the progeny of the salt stressed and control plants when compared to the other three tested reference genes, ACTIN2, UBQ5, and UBQ10. Figure 8 shows the chromosomal region of three selected genes from the ChIP-seq experiment that contained a differential H3K9ac mark between an ac_salt and ac_ct library samples. The figure also shows the normalized relative enrichment level of the H3K9ac mark obtained by ChIP-qPCR, and the normalized relative transcript levels obtained by qRT-PCR for these genes in the progeny of salt stressed and the progeny of the control samples. The graphs clearly indicate that the lower enrichment levels of the H3K9ac mark in the progeny of the salt stressed plants are correlated to the lower expression levels of the corresponding genes.

The qRT-PCR results showed that of the 33 confirmed genes with a different H3K9ac enrichment level, 10 genes did not show any changes in expression level, including AT1G56500 gene with higher enrichment level of H3K9ac in the progeny of salt stressed plants. From the other 23 genes, with lower enrichment level in the progeny of salt stressed plants, 2 genes were up-regulated, while the other 21 genes were down-regulated in the salt stressed progeny. However, results of the student t-test revealed that the decrease in the transcript levels of only 10 of the 21 genes was statistically significant (P value below 0.05).

Since H3K9ac is a positive mark for gene transcription, it is expected that a low enrichment level of this mark in the progeny of salt stressed plants would result in low levels of gene expression. My results are consistent with this fact, considering that only 2 genes (AT1G64500 and AT2G39980) showed higher levels of gene expression in the progeny of salt stressed plants, despite having lower enrichment level of H3K9ac mark.
A) AT1G03220

B) AT4G28180

C) AT1G05370
Figure 8: ChIP-seq, ChIP-qPCR, and qRT-PCR data for three selected regions/genes. The IGB view of the chromosomal region of three selected genes containing differential H3K9ac enrichment in the two library samples, ac_ct (upper green peaks) and ac_salt (lower red peaks) obtained from ChIP-seq data are presented to the left. The height of the peak correlates with the number of times the region was covered by the read. The position of the genes and their transcription directions are also indicated at the bottom of the IGB view. The normalized relative enrichment level of corresponding gene regions obtained by ChIP-qPCR, as well as the normalized relative transcript level of these genes obtained by qRT-PCR in the progeny of salt stressed and control plants are also represented by the bar graphs to the right. The mean normalized quantity values are an average of four independent biological replicates (each value was the mean of two technical replicates). The small yellow lines in the pictures represent the position of the primers. The error bars represent SD. The asterisks represent a significant difference between the progeny of salt stressed samples to those of the control samples using Student’s t-test (* represents \( P < 0.05 \) and ** represents \( P < 0.01 \)).
This could be due to the fact that other epigenetic modifications, besides H3K9ac, are involved.

Subsequently, genes with lower enrichment levels of H3K9ac mark that also had significantly lower expression in the progeny of the salt stressed plants compared to control plants were analyzed in more detail. First, the positions of the differential peaks in the corresponding genes were analyzed to determine whether the position of the H3K9ac mark could be correlated with changes at the transcriptional level of the corresponding genes. For this purpose, the differentially enriched peaks were visualized in the IGB and their positions were identified. This analysis showed that most of the genes with significantly lower transcription levels contained a decreased enrichment level of H3K9ac at the 5’ end and/or gene body as opposed to the 3’ end. Out of the 10 down-regulated genes, six (AT1G03220, AT2G48010, AT4G28180, AT2G37580, AT2G40230, AT5G17980) showed the enrichment levels of H3K9ac decreasing throughout the whole gene body; the first two are visualized in Figure 7 A and B. Further, three genes (AT2G45750, AT1G05630, AT5G42100) had a significant decrease in the enrichment level of this histone mark at the 5’ end, and only one, AT1G05370, showed a significant decrease of this mark at the 3’ end (Figure 7C). It is interesting to note that differences in the enrichment levels of the H3K9ac mark at the 3’ end do not seem to correlate with changes in gene expression; only one gene (AT1G05370) with reduced enrichment level of this mark at the 3’ end correlated with reduced gene expression in my study.

Next, I compared my data on the expression changes of the 10 significantly affected genes in the progeny of salt stressed plants with the expression changes of these genes shortly after exposure to salt stress (Kilian et al., 2007; Table 6). Killian et al. (2007) carried out a study on the genome expression changes in roots and shoots of Arabidopsis seedlings that were exposed to different abiotic stresses at different exposure times; the data from this study is available on the AtGeneExpress Visualization Tools (AVT) website (http://jsp.weigelworld.org/expviz/expviz.jsp).
Table 6: Transcription changes of the 10 genes with a differential H3K9ac mark and a statistically significant lower transcript level in the progeny of the salt stressed plants. Column 2 shows stress-related domains of proteins encoded by corresponding genes. Columns 3 and 4 show the mean-normalized transcription ratio in the F1 progeny of Arabidopsis salt stressed to control shoots (average of four biological repeats, our data), and the mean-normalized transcription ratio in Arabidopsis salt stressed to control shoots (Killian et al., 2007, 24 hours after exposure to 150 mM NaCl). The asterisks represent a significant difference between the transcription of the genes in the progeny of salt stressed plants to those of the control plants using Student’s t-test (* represents P < 0.05 and ** represents P < 0.01).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Stress-related domain</th>
<th>Transcription ratios (salt stress/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F1 Progeny of salt stressed plants (my data)</td>
</tr>
<tr>
<td>AT1G03220</td>
<td>peptidase aspartic catalytic</td>
<td>0.671**</td>
</tr>
<tr>
<td>AT1G05370</td>
<td>phosphatidylinositol transfer protein-like</td>
<td>0.813*</td>
</tr>
<tr>
<td>AT2G48010</td>
<td>kinase</td>
<td>0.83**</td>
</tr>
<tr>
<td>AT4G28180</td>
<td>myristoylation</td>
<td>0.794*</td>
</tr>
<tr>
<td>AT2G37580</td>
<td>C3HC4-type RING zinc finger</td>
<td>0.813*</td>
</tr>
<tr>
<td>AT2G40230</td>
<td>chloramphenicol acetyl-transferase-like and transferase</td>
<td>0.770*</td>
</tr>
<tr>
<td>AT2G45750</td>
<td>methionine methyltransferase</td>
<td>0.794**</td>
</tr>
<tr>
<td>AT5G17980</td>
<td>C2 calcium/lipid-binding</td>
<td>0.820*</td>
</tr>
<tr>
<td>AT1G05630</td>
<td>inositol polyphosphate-related phosphatase</td>
<td>0.83**</td>
</tr>
<tr>
<td>AT5G42100</td>
<td>glycoside hydrolase</td>
<td>0.741*</td>
</tr>
</tbody>
</table>
From Table 6, it is obvious that the expression changes observed for the majority of these genes 24 hours after salt exposure (AVT data) correspond to the changes I observed in the transcript levels of the F1 progeny of salt-stressed plants. This indicates that the salt stress induced down-regulation of these genes in the Arabidopsis shoots shortly after exposure to stress, and this reduction in the transcript levels of these genes was maintained and then transferred to their progeny, which is related to the lower enrichment levels of the H3K9ac mark. However, there is an exception in two genes, namely AT1G05630, AT5G42100, wherein the trend in the expression changes in plants exposed to salt stress and progeny of the stressed plants are not consistent. These two genes showed higher expression levels shortly after exposure to salt compared to the control samples, even though these genes showed lower expression and lower enrichment level of the H3K9ac mark in the progeny of salt stressed samples.

The 10 genes that showed lower enrichment levels of the H3K9ac and significantly lower gene expression in the progeny of salt stressed plants as compared to the progeny of controls could possibly participate in adaptation of plants to salt stress through changes in epigenetic regulation, namely H3K9ac mark, induced by parental salt stress.
4 DISCUSSION

4.1 Overview

Epigenetic changes at different levels, including DNA methylation, histone modifications, and small RNAs are involved in the regulation of chromatin structure and gene expression in plants (Chen et al., 2010b). A significant body of evidence shows that the levels of DNA methylation, histone modifications, or both alter gene expression levels at specific loci in response to abiotic stress, which indicates the effective role of epigenetic changes in plant abiotic stress responses (Choi and Sano, 2007; Sokol et al., 2007; Chen and Wu, 2010; Bilichak et al., 2012). Furthermore, some studies have shown a strong coordination or “cross-talk” among these epigenetic factors, especially between DNA methylation and histone modification, in response to abiotic stresses (Bilichak et al., 2012; Song et al., 2012). However, most of these studies analyzed the epigenetic changes shortly after exposure to abiotic stresses, and only a few studies have investigated the stability of these changes in the progeny of stressed plants (Boyko et al., 2010; Bilichak et al., 2012). Therefore, I was interested in investigating the role of these epigenetic changes in the transmission of stress memory to the next generation and ultimately in plant stress adaptation. Boyko et al. (2010) showed that the progeny of Arabidopsis salt, UV-C, cold, heat, and flood-stressed plants displayed a higher HRF, as well as a higher global genome methylation level compared to the progeny of control plants. Therefore, it was hypothesized that stress memory could be transferred to the progeny of stressed plants epigenetically and that epigenetic stress memory might play a role in plant stress adaptation (Boyko and Kovalchuk, 2011). Recently, changes in histone acetylation and methylation of certain hyper-methylated loci were shown in the progeny of salt stressed plants compared to the progeny of control plants (Bilichak et al., 2012). To date, there have been no other studies on the heritable impact of stress on histone modifications to assess the role of histone modifications in the establishment of epigenetic stress memory in plants, and further, to identify loci with differential histone marks, which may be involved in plant stress adaptation.
In this research, I assessed the global changes in histone modifications of two marks, namely H3K9ac and H3K4me2, in the progeny of Arabidopsis salt stressed plants relative to control plants. The loci with differential histone modifications were then assessed for transcriptional changes. This research is the first study to explore genome-wide changes in histone modifications that have been induced by parental salt stress.

4.2 Global Changes in Histone Modifications Induced by Stress

Studies using ChIP have shown that changes in histone modifications occur at specific loci in response to abiotic stresses (Kim et al., 2008; Kwon et al., 2009; Bilichak et al., 2012; Song et al., 2012). However, only a few reports have investigated the stress-induced dynamic changes in histone modifications in the whole genome (Charron et al., 2009; van Dijk et al., 2010). For example, Charron et al. (2009) studied the effect of light on genome-wide changes in histone methylation marks (H3K9ac/me3, H3K27ac/me3) in Arabidopsis using the ChIP-chip technique. They revealed that although a large number of genes had similar enrichment levels of specific histone marks between dark and dark to light conditions, many genes showed differential enrichment levels in response to each condition. For example, 17% of the genes in the genome were targeted differently by the H3K9ac mark between the two conditions. This implied differential changes in histone modifications in response to the different light conditions. They also suggested that histone acetylation, in particular H3K9ac, was an important regulator of light-regulated genes and chromatin changes. In addition, van Dijk et al. (2010) studied the dynamic changes in several histone methylation marks (H3K4me1/2/3) in response to dehydration stress in the entire genome of Arabidopsis using ChIP-seq methods. They found that the enrichment level of H3K4me1 and H3K4me2 marks changed only slightly in response to dehydration stress, while the enrichment level of H3K4me3 changed drastically. Further, they showed that these histone marks predominantly targeted genes more than inter-genic and TEs regions. In their experiment, the number of genes targeted by the H3K4me2 mark increased by only 0.9% in response to dehydration stress conditions compared to normal conditions. Finally, they concluded that different histone modifications have distinct roles in the response of affected genes to stress. These genome wide histone
modification studies, further confirmed the important role of histone modifications in the response of plants to abiotic stresses, and provided a more comprehensive picture of dynamic changes in histone modification during plant stress response. However, there have been no studies on global changes in histone modifications in the progeny of stressed plants. This would be useful to determine the stability and transmission of histone changes to the next generation.

In this research, after a suitable ChIP protocol was established, the enrichment level of histone acetylation and methylation marks (H3K9ac and H3K4me2) throughout the Arabidopsis genome was compared between the progeny of Arabidopsis salt stressed and control plants using the ChIP-seq technique. The results showed that while the global enrichment level of the H3K4me2 mark did not change significantly, the enrichment level of the H3K9ac mark, at certain regions of the genome, changed significantly between the two sets of plants. Therefore, I concluded that various histone modifications contribute in different ways to the transmission of epigenetic stress memory and to plant stress adaptation. Some of them, such as H3K9ac, may be of significance, while others, such as H3K4me2, may not be of importance.

4.2.1 Histone H3K4me2 mark

Previous studies suggested that the H3K4me2 mark was associated with transcriptionally active genes (Santos-Rosa et al., 2002). Despite these findings, there has been no evidence to support that stress-induced changes in gene expression are correlated with changes in H3K4me2 levels. For instance, although the enrichment level of this histone methylation mark decreased at the submergence-inducible genes (ADH1 and PDC1) in response to flooding stress in rice, the increased expression levels of these genes were correlated with increased enrichment levels of other transcriptionally active histone marks, such as H3K4me3 and H3K9/14ac (Tsuji et al., 2006). In addition, the enrichment level of this mark changed only moderately in the entire Arabidopsis genome in response to dehydration stress (van Dijk et al., 2010). My findings also showed that there was no significant change in the enrichment level of this mark in the progeny of salt stressed
plants compared to control plants. Taken together, these data seem to suggest that the H3K4me2 mark plays no significant role in either plant stress response or in the transmission of epigenetic stress memory to successive generations.

4.2.2 Histone H3K9ac mark

Histone acetylation marks, such as H3K9ac, are usually associated with transcriptionally active genes (Zhou et al., 2010). There has been evidence showing that the enrichment level of this histone mark is usually altered at specific genes in response to abiotic stress. For example, studies by Kim et al. (2008) showed an increase in the enrichment level of the H3K9ac mark at several Arabidopsis drought stress-responsive genes in response to dehydration stress. Also, studies performed by Chen et al. (2010a) showed that the enrichment levels of some transcriptionally active histone marks, such as H3K9ac, were increased at ABA- and abiotic stress-response genes in plants exposed to ABA and salt stress. Further, mutations in the two histone de-acetylase genes, HDA6 and HDA19, lead to ABA and salt-stress hypersensitivity in Arabidopsis (Chen et al., 2010a; Chen and Wu, 2010). Also, Arabidopsis seedlings overexpressing the AtHD2C gene, encoding a plant-specific histone de-acetylase, were tolerant to ABA, salt, and drought stress, while up-regulating several ABA-responsive genes (Sridha and Wu, 2006). Furthermore, Zhu et al. (2008) revealed the involvement of HOS15, a histone H4 de-acetylase, in the repression of genes through histone de-acetylation in response to cold stress conditions in Arabidopsis. My genome wide analysis of this histone mark revealed that in total there were 409 genomic regions with differential enrichment levels of the H3K9ac mark in the genomes of the progeny of salt stressed plants compared to the progeny of control plants (regions with more than 2-fold change and padj value < 0.2). Of these 409 regions, 91 showed higher enrichment levels compared to F1 control plants, while 318 regions showed lower enrichment than in F1 control plants. Therefore, the H3K9ac mark could also play a role, not only in plant stress acclimation, but also in the transmission of stress memory to the next generation and in plant stress adaptation.
My global genome analysis comparing progeny of salt stressed and control plants showed that the number of loci with decreased levels of the H3K9ac mark is greater than the loci with increased levels of this acetylation mark in the progeny of salt stressed plants. Since the H3K9ac mark indicates transcriptionally active genes, it could be argued that the progeny of salt stressed plants have more inactive chromatin compared to control plants. Boyko, et al. (2010) also reported that the progeny of salt stressed plants contains higher global DNA methylation levels (10-12%) than the progeny of control plants, although some loci were hypo-methylated. Considering the fact that methylation negatively correlates with gene expression levels, Boyko, et al. (2010) also concluded that the progeny of salt stressed plants exhibited lower global genome activity than the progeny of control plants. Further, it was clear that the progeny of salt stressed plants displayed substantial changes in gene expression levels as compared to the progeny of control plants (Boyko et al., 2010). In total, 678 genes in the progeny of salt stressed plants showed more than 2-fold change in gene expression (P < 0.05). Of these, 181 showed up-regulation, while 506 showed down-regulation (Boyko et al., 2010). Therefore, my results provide more evidence to support the hypothesis proposed by Boyko et al. (Boyko and Kovalchuk, 2011), which states that plants show lower chromatin activation to adapt to stress conditions.

To date, there have been limited data on histone modifications in the progeny of stressed plants. To my knowledge, the only report is by Bilichak et al. (2012) that analyzed the enrichment levels of histone acetylation and methylation (H3K9ac and H3K9me2) at 11 selected hyper-methylated and one selected hypo-methylated loci in the progeny of salt stressed plants. They showed that the enrichment levels of the H3K9ac mark decreased at 10 of the 11 hyper-methylated genes, while it increased at the hypo-methylated gene in the progeny of salt stressed plants compared to the progeny of control plants. Most of these selected genes regulate the chromatin structure, or are involved in transcriptional and posttranscriptional regulation of gene expression. This study provides a glimpse into the global changes in histone modifications induced by parental salt stress, and shows that changes occur at a limited number of genes. My ChIP-seq data confirm the trend observed by Bilichak et al (2012) in the enrichment changes of the H3K9ac mark at five of the 12 genes (SUVH2, SUVH6, DRB2, MOS6, and UVH3) that the authors identified.
For the remaining genes, five showed no changes (SUVH5, SUVH8, WRKY22, MSH6, and UBP26), whereas the other two (ROS1 and APUM3) had higher levels of the H3K9ac mark according to my ChIP-seq data. This discrepancy could be explained by differences in the two experimental conditions such as different plant samples used (shoots in my study versus leaves in Bilichak’s study).

The annotation data using SeqMonk software showed that all of the 409 regions, obtained from my ChIP-seq data, containing the differential H3K9ac mark were designated to specific genes. This result was consistent with that obtained from previous studies. For example, Charron et al. (2009) found that the histone acetylation marks (H3K9ac and H3K27ac) usually target genes, while the histone methylation marks (H3K9me3 and H3K27me3) target genes as well as inter-genic and TE regions. Therefore, my result was a confirmation that this histone mark predominantly targets genes more than inter-genic regions and TEs.

4.3 Regions with Significantly Different Enrichment Levels of the H3K9ac Mark in the Progeny of Salt Stressed Plants

I found that the progeny of salt stressed plants have 409 genomic regions with a differential enrichment level of the H3K9ac mark (more than 2-fold change and padj < 0.2) compared to the progeny of the control plants. However, with a padj value < 0.05, only 45 of the 409 regions had significantly different enrichment levels of this histone mark. Of the 45 regions, only one exhibited hyper-acetylation while the others were all hypo-acetylated in the progeny of salt stressed plants. Based on the gene ontology classification terms that reflect biological processes, a higher percentage of these 45 genes are involved in metabolic process, developmental process, response to stresses, as well as cell organization and biogenesis, respectively. There were also genes which are involved in transport activity (including energy pathway), and signal transduction. Twelve percent of the genes were also involved in transcription activity.

Data obtained for the 45 regions that had significantly different enrichment levels of the H3K9ac mark based on ChIP-seq were validated using ChIP followed by qPCR (ChIP-
qPCR). The qPCR results validated results for 73% (33 of the 45) of the differentially enriched regions. Considering the technical and computational limitations of both techniques, the two sets of data appear mostly in agreement. Studies by van Dijk et al. (2010) also found a quantitative agreement with a correlation coefficient of 0.7 between ChIP-seq and ChIP-qPCR in their studies on global changes in histone methylations in response to dehydration stress. Therefore, the ChIP-seq technique could be considered a reliable method to analyze genome wide changes in histone modifications.

4.4 Correlation between Differential H3K9ac Mark and Gene Expression

Several reports in the literature have pointed to a positive correlation between the enrichment levels of the H3K9ac mark and gene expression (Benhamed et al., 2006; Lang-Mladek et al., 2010; Zhou et al., 2010; Bilichak et al., 2012). However, other reports have shown some exceptions wherein gene expression levels were not related to changes in the enrichment levels of H3K9ac mark (Zhou et al., 2010; Bilichak et al., 2012; Song et al., 2012). These discrepancies could be explained by the still poorly understood complex interactions among various epigenetic factors (Zhou et al., 2010; Song et al., 2012). My results showed that of the 33 genes that were confirmed to have a significantly different enrichment level of H3K9ac in the progeny of salt stressed plants, 10 had a significantly lower gene expression level compared to the progeny of control plants. The other 23 genes did not show any significant differences in their transcriptional levels between the two sets of plants, even though they had a significantly different enrichment level of the H3K9ac mark. Therefore, although my results are generally consistent with the finding that the H3K9ac mark is positively associated with gene expression, interactions of this histone mark with other epigenetic factors likely determine the final status of gene activity (Zhou et al., 2010).

My results further showed that most of the genes with significantly altered expression levels in the progeny of salt stressed plants exhibited the presence of the differential peak more frequently at the 5' end of the gene and/or in the gene body than at the 3' end of the gene. Studies conducted by Bilichak et al. (2012) also showed a positive correlation
between gene expression levels and enrichment levels of the H3K9ac mark at both the promoter and gene body regions at eight hyper-methylated loci in the progeny of salt stressed plants. Similar enrichment patterns of the H3K9ac mark were also observed in the coding region of several drought stress responsive loci in Arabidopsis (Kim et al., 2008). Zhou et al. (2010) also revealed a strong correlation between global gene expression levels and enrichment levels of the H3K9ac mark at both the promoter and body regions of genes. Charron et al. (2009) further stated that the enrichment levels of this acetylation mark at the 5' end of the genes correlate well with gene expression levels in the entire genome of Arabidopsis plants.

4.5 Genes with Significantly Different Enrichment Levels of H3K9ac Mark and Expression in the Progeny of Salt Stressed Plants

It was previously documented that the progeny of salt stressed plants were more tolerant to salt stress than those of the control plants, and this increase in tolerance to salt stress was attributed to heritable epigenetic changes, such as altered levels of genome methylation (Boyko et al., 2010), as well as histone modifications (according to my data). My results identified 10 genes that contained reduced enrichment level of the H3K9ac mark as well as a lower level of gene expression in the progeny of salt stressed plants. Therefore, I hypothesize that at least some of these 10 genes may be involved in the establishment of stress memory and plant salt stress adaptation through histone modifications. Below is a brief introduction to these genes along with their involvement in biological pathways, which further illustrate their possible roles in plant stress adaptation.

AT1G03220 encodes a protein, which belongs to the eukaryotic aspartyl protease family protein, containing a peptidase aspartic catalytic domain. This protein family is known to be involved in several physiological processes, including protein processing, senescence, and stress response in plants (Mazorra-Manzano et al., 2010). Gong et al. (2001) also showed that the expression of this gene increased at 4 h after salt treatment.
AT2G37580 encodes a protein, which belongs to the RING/U-box superfamily protein, containing zinc finger, C3HC4 RING-type, and RING/FYVE/PHD-type domains; these domains have a putative transferring activity. Many of these proteins are known to be necessary in controlling various plant processes, including response to abiotic stress in plants (Jung et al., 2012). For example, zinc finger transcription factors, such as ALFIN1, interact with promoters of osmotic-regulated genes (Gong et al., 2001). In addition, it was proposed that Arabidopsis AtING and Alfin-like proteins, containing PHD-type domains, are nuclear proteins that bind to active H3K4me3/2 marks, and therefore, are involved in chromatin regulation (Lee et al., 2009). Recently, a new C3HC4-type RING zinc finger protein (BrRZFP1) was identified in *Brassica rapa* (Jung et al., 2012). This study showed that cold, salt, and ABA treatments in *B. rapa* induced transcription of this gene. They concluded that the BrRZFP1 transcription factor plays an important role in regulating stress response and tolerance in plants (Jung et al., 2012).

AT5G17980 encodes a protein from the C2 calcium/lipid-binding plant phosphoribosyltransferase family, which contains a C2 calcium/lipid-binding, phosphoribosyltransferase C-terminal and C2 calcium-dependent membrane targeting domains. Ca<sup>2+</sup> is an important secondary messenger in plant signal transduction pathways that regulate stress-induced gene expression (de Silva et al., 2011). For example, another member of this gene family, *AtCLB*, negatively regulates responses to abiotic stresses in Arabidopsis. The loss of *AtCLB* gene function confers enhanced drought and salt tolerance (de Silva et al., 2011). Also, the *BAP1* gene, which has similar domains to the AT5G17980 gene, binds to phospholipids in a calcium-dependent manner and is associated with membranes. *BAP1* has been shown to negatively regulate defense responses and cell death in Arabidopsis. For example, the loss of BAP1 function resulted in increased biotic stress resistance (Yang et al., 2006). Furthermore, it was suggested that BAP1 interacts with another calcium-dependent lipid-binding protein, BON1 (which has similar conserved C2 domains), to negatively control defense responses in plants (Yang et al., 2006).

AT1G05630 (*INOSITOL-POLYPHOSPHATE 5-PHOSPHATASE 13, 5PTASE13*) encodes a protein with phosphatase activity towards an inositol 1, 4, 5-trisphosphate
(InsP3). Inositol signaling pathways in plants are activated in response to different developmental and stress signals, such as ABA, salt, gravity, and pathogens (Ananieva et al., 2008). InsP3 is a secondary messenger that activates intracellular Ca\(^{2+}\) release from different sources within the cell (Tang et al., 2007). Inositol polyphosphate 5-phosphatases, such as 5PTASE13, terminate the signaling event initiated by InsP3 via the removal of the 5-phosphate (Astle et al., 2007). Ananieva et al. (2008) showed that the WD40 repeat region of 5PTASE13 interacts with AKIN10 (a sucrose nonfermenting-1-related kinase). AKIN10 acts as a sensor of energy and stress in plants (Baena-Gonzalez et al., 2007). Using 5ptase13 defective mutants, Ananieva et al. (2008) showed that 5PTASE13 acts as a regulator of AKIN10 activity in response to different nutrient availability conditions. They also showed that a loss of function of 5PTASE13 resulted in ABA and sugar insensitivity. They suggested that 5PTASE13 and AKIN10 form a complex to play a regulatory role associated with inositol, sugar, and stress signaling (Ananieva et al., 2008).

AT1G05370 encodes a protein belonging to the Sec14p-like phosphatidylinositol transfer family protein; this protein contains phosphatidylinositol transfer protein-like and cellular retinaldehyde-binding/triple function domains. AtSfh1p, which has a similar domain to that of the protein encoded by AT1G05370, is a phosphatidylinositol transfer protein (PITP) that regulates a specific stage in root hair development (Vincent et al., 2005). PITPs control signal coordination between lipid metabolism and membrane trafficking. Vincent et al. (2005) proposed that AtSfh1p regulates plasma membrane trafficking, Ca\(^{2+}\) signaling, and cytoskeleton functions in the growing root hair apex. Further, COW1 protein, another PITP protein with a similar domain, was shown to be also essential for proper root hair growth (Bohme et al., 2004).

AT2G48010 (RECEPTOR-LIKE KINASE IN FLOWERS 3, RKF3) encodes a protein with kinase domains and is involved in protein phosphorylation. Although there have been no studies that directly report the role of this gene in salt stress response and/or adaptation, many genes with similar domains, including RPK7 (Hong et al., 1997), SOS2 (Liu et al., 2000), and AtLecRK2 (He et al., 2004), have been reported to be involved in salt stress responses. These reports discussed the possible role of these genes in signal
transduction pathways such as intracellular Na\(^+\) and K\(^+\) homeostasis and ethylene signaling pathway in response to salt stress.

AT5G42100 (β-1, 3-GLUCANASE_PUTATIVE, BG_PPAP) encodes a plasmodesmal associated membrane protein, with a glycoside hydrolase domain. This membrane protein is involved in plasmodesmal callose (β-1, 3-glucan) degradation, cell communication, and regulation of protein localization (Levy et al., 2007). When plants undergo developmental processes or exposure to stresses such as wounding or aluminum toxicity, callose is transiently deposited in the cell walls surrounding the plasmodesmata at both ends of the channel, and thus, the passage of the molecules through the plasmodesmata is blocked (Levy et al., 2007). In Arabidopsis \textit{bg-ppap} mutants, cell-to-cell movement between epidermal cells, as well as the conductivity coefficient of the plasmodesmata is reduced (Levy et al., 2007).

AT2G40230 encodes a protein that belongs to the HXXXD-type acyl-transferase (BAHD) family; these proteins have chloramphenicol acetyl-transferase-like and transferase domains. Some proteins from this family, ASFT and FACT, with similar domains were shown to be involved in the synthesis of components of cell walls, especially in seed and root tissues of Arabidopsis (Gou et al., 2009; Kosma et al., 2012). For example, the formation of suberin, a polyester polymer in the cell wall, is primarily dependent on the activity of the \textit{ALIPHATIC SUBERIN FERULOYL-TRANSFERASE} (\textit{ASFT}) gene (Gou et al., 2009). Suberin, which controls transport of water and nutrients, has an important role in maintaining cell wall integrity to protect plants from environmental stresses (Gou et al., 2009).

AT4G28180 encodes a protein, which has been predicted to have myristoylation activity. N-myristoylation is an irreversible modification that influences the membrane binding properties of critical cytoplasmic proteins from signal transduction cascades (Boisson et al., 2003). The Arabidopsis N-myristoylome family consists of 437 proteins, which account for 1.7% of the complete Arabidopsis proteome (Boisson et al., 2003).

AT2G45750 encodes a protein, which belongs to the S-adenosyl-L-methionine-dependent methyltransferases superfamily, and is predicted to have methyltransferase
activity. Methionine is converted to S-adenosylmethionine (SAM), which is a precursor of metabolites, such as glycinebetaine, polyamines, and ethylene, that accumulate in response to salinity (Ogawa and Mitsuya, 2012) and drought (Quan et al., 2004). A link between salinity tolerance and methionine biosynthesis was established by Ogawa and Mitsuya (2012) who showed that the Arabidopsis methionine methyltranferase mutant, \textit{mmt}, had a reduced germination rate and severely repressed shoot growth compared to the wild type plants in response to salt stress.

The aforementioned analysis of my data suggests that these genes harbor salt stress memory through altered H3K9ac enrichment level, and could be involved in different pathways related to plant stress response and adaption. Although the biological functions of most of the proteins encoded by these genes are not fully elucidated, these proteins contain well-known and important domains for plant stress response. Specifically, proteins with zinc finger domains could act as regulatory determinants that post-transcriptionally activate other effectors. Proteins with Ca\textsuperscript{2+} binding and protein kinase domains could have a role as intermediates in signaling pathways; a number of proteins with these domains have well-known functions in plant response and tolerance to salt stress (Gong et al., 2001). However, further molecular characterization of the 10 candidate genes is clearly needed to definitely illustrate their functions in trans-generational epigenetic memory and adaptation to abiotic stress.

4.6 Conclusions and Prospective for Future Studies

My research work assessed the role of histone modifications in the establishment of trans-generational epigenetic stress memory in the progeny of salt stressed plants. I used ChIP-seq to analyze genome-wide enrichment levels of histone acetylation and methylation marks in the progeny of salt stressed Arabidopsis plants. My genome-wide analysis revealed that, except for some regions that were hyper-acetylated (H3K9ac), the chromatin of progeny of salt stressed plants contained global less enrichment levels of the transcriptionally active H3K9ac mark as compared to that of the progeny of control plants. Furthermore, I showed that there was no difference in the enrichment levels of the
H3K4me2 mark between the progeny of salt stressed and control plants. Therefore, I conclude that different histone marks contribute differently to the establishment of stress memory and possibly stress adaptation; with some, such as H3K9ac, playing important roles while others, such as H3K4me2, being unessential. My results are in agreement with previous findings which showed that subjecting parental lines to salt stress results in higher global genome methylation and lower global genome expression levels in the progeny (Boyko et al., 2010). These findings suggest that a global decrease in chromatin activity or genome expression levels may be a mechanism employed by plants to adapt to stress. Plant stress adaptation appears to be dependent on the involvement of a number of epigenetic factors, including the H3K9ac mark, DNA methylation, and possibly other epigenetic factors (Boyko et al., 2010; our data). My findings also support the hypothesis that stress-induced trans-generational responses in Arabidopsis depend on an altered epigenetic status (Boyko and Kovalchuk, 2011).

Among the loci with significantly reduced enrichment levels of the H3K9ac mark, I found 10 genes in the Arabidopsis genome showing lower levels of expression in the progeny of salt stressed plants. These 10 genes, most of which are novel and/or poorly characterized, may represent a new set of genes involved in plant abiotic stress response and in plant adaptation to salt stress via epigenetic stress memory. Further, characterization of these genes using overexpression and knockout mutants would shed more light on their exact role in plant stress adaptation. Some genes in the progeny of salt stressed plants that harbored hypo-acetylation were expressed at similar levels in the progeny of control plants, which suggests that the regulation of these genes is independent of the levels of histone acetylation, and thus other epigenetic factors or mechanisms could be involved in regulating their expression. In conclusion, these results demonstrate the role of epigenetics, especially histone modifications, in the establishment of trans-generational stress memory and plant stress adaptation. The genome-wide analysis of histone modification in the progeny of salt stressed plants that was performed in this study is the first of its kind and provides a good understanding of the role of histone modifications in the establishment of plant stress memory. However, further ChIP-seq experiments to analyze other histone marks would be needed for a full understanding of the role of histone modification in the establishing salt stress memory.
Furthermore, investigation of histone changes in more plant species exposed to a variety of stresses would identify those marks that are commonly affected in response to stress, and those that are affected in response to specific types of stress. Findings from these studies would open the door to the exploitation of these epigenetic factors in developing stress tolerant crop varieties.
References


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Appendices

Appendix A: List of primers used in this study

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