Genome-wide transcriptional changes and chromatin modifications associated with plant stress memory

Thesis submitted for the degree of Doctor of Philosophy

Emanuela Sani

Institute of Molecular, Cellular and Systems Biology

College of Medical, Veterinary and Life Sciences

University Of Glasgow

January 2013

Abstract

As sessile organisms, plants had to develop various biochemical and physiological mechanisms to respond and adapt to abiotic stress conditions such as salt and drought and thus acquire stress tolerance. A particular interesting mechanism is the so called "priming effect": an application of a mild short stress to plants at an early stage of development appears to enable them to cope better when stressed again at mature stage. However, the molecular effects of salt priming have not been systematically quantified and as a consequence the molecular basis of priming remains unknown.

In this study an experimental procedure was established that allowed to test whether salt priming of young *Arabidopsis thaliana* plants had an effect on plants exposed to more severe salt stress at a later stage of development.

To quantify how primed and non-primed plants responded to the second salt stress, global changes in their transcriptional expression profiles were monitored using Affymetrix GeneChip ATH1 microarray. Results showed that both primed and non-primed plants responded to the salt treatment modulating the same set of known stress responsive genes. However, primed plants differentially regulated a smaller set of genes. Furthermore, the vast majority of the stress responsive genes showed a weaker response in primed than in non-primed plants. These results suggested that primed plants channelled the stress response using only selected genes.

The next question addressed was how primed plants could "remember" the priming treatment after a period of extensive growth. Several studies had indicated that environmental stress induces changes in the chromatin structure thereby modifying the accessibility of the DNA for transcription factors and other regulatory proteins. This suggested a link between epigenetic modification and exposure of plants to stressful conditions, where the chromatin status might act as an epigenetic mark that could be maintained during plant growth and development. To investigate this hypothesis I carried out a comparative analysis of the epigenetic landscapes of primed and non-primed plants combining Chromatin Immuno-Precipitation with Illumina sequencing (ChIP-Seq).

Genome-wide profiles of H3K4me2, H3K4me3, H3K9me2 and H3K27me3 were generated for roots and shoots of plants harvested immediately after the priming treatment. Roots of primed plants showed indeed numerous differences in their epigenetic profiles compared to non-primed roots, in particular at the level of H3K27me3. Therefore, I carried out an additional ChIP-Seq experiment before the application of the second stress to test if the priming induced changes in H3K27me3 were maintained over this period of extensive growth. Results showed that several epigenetic differences caused by priming were still maintained.

Finally, to elucidate the relationship between epigenetic modifications and transcriptional responses the ChIP-Seq profiles were coupled with genome wide transcript profiles obtained by RNA-seq. Results shown that in the non-steady state there was no clear correlation between the differences detected at the transcriptional and at the epigenetic level. The results identified H3K27me3 as a potential mark for salt stress memory and they call for future studies extending both temporal and spatial resolution of epigenetic and transcriptional changes after salt priming.

Table of Contents

List of Figures

List of Tables

Abbreviation list

ABA abscisic acid
AGI arabidopsis genome initiative
ANOVA analysis of variance
ATP Adenosine triphosphate
BABA beta-amino-butyric acid
BLAST basic local alignment search tool
Bp base pair
cDNA complementary DNA
ChIP Chromatin immunoprecipitation
ChIP-Seq chromatin immunoprecipitation sequencing
DAVID database for annotation visualization and Integrated discovery
DNA deoxyribonucleic acid
dsRNA double strand RNA
EDTA ethylene diamine tetra acetic acid
GO gene ontology
FC fold change
FDR false discovery rate
H3 histone 3
HAC histone acetlylase
HDAC histone deacetylase
HDM histone demethylase
HMT histone methyl transferase
HxAC hexanoic acid
IGB integrated genome browser
JA jasmonic acid
K lysine
KEGG Kyoto encyclopedia of genes and genomes
Lys lysine
mRNA messanger RNA
PCA principal component analysis
PCR polymerase chain reaction
PEG polyethylene glycol
Pol polymerase
qPCR quantitative PCR
RdDM RNA directed DNA methylation
RMA robust multichip average
RNA ribonucleic acid
RNA-Seq RNA-sequencing
ROS reactive oxygen species
RT reverse transcription
RT-PCR reverse transcript PCR
SA salicylic acid
siRNA small interfering RNA
ssRNA single strand RNA
TAE Tris base, acetic acid and EDTA
TAIR The arabidopsis information resource
UTR untranslate region
VA vectorial analysis
UV ultra violet

A ma' e Pa'

Acknowledgments

I would firstly like to thank my supervisor Anna Amtmann for her support and encouragement and for giving me the opportunity to work on this project.
I also would like to thank the people at the *Glasgow Polyomics* facility: in particular Pawel Herzyk for his tremendous help in both data analysis and use of bioinformatics tools; Julie and Jin for their help with microarray and sequencing analysis.
Special thanks to Andy for his immense help in both the lab and in my life.
Thanks also to my dear friends Connie, Maria and Annegret for reading and commenting on my thesis, but most importantly, for cheering me up during the (too) long Scottish winters and (too) short summers, thanks girls, you really kept me motivated in the lab every day and on some nights too.
Thanks as well to all the members of the Blatt and Amtmann labs for helping me over the four years including our wee George and his Johnny Cash CD played every Friday and Liz O'Donnel and her great help in growing thousand of plants.
Finally I would like to thank my family who often believed in me more than I did.

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Emanuela Sani

Chapter 1: Introduction

1.1 Plant responses to water stress: mechanisms and strategies employed

Higher plants, owing to their sessile nature, have intricate mechanisms enabling them to respond to environmental changes established over a long period of evolution.

Salinity is one of the major abiotic stresses, which adversely affects the agricultural productivity in many parts of the world. Salinity afflicts 6% of land throughout the world mostly as a natural result of the accumulation of salts over long periods of time in arid and semiarid zones. Significant proportions (20%) of recently cultivated agricultural lands were saline as a consequent of irrigation and clearing. Irrigated land accounts for only 15% of total cultivated land, but productivity is twice that of rain fed land, and furthermore they produce one third of the world's food (Munns and Tester, 2008).

The development of salt tolerant crops is an urgent challenge for plant scientists and requires detailed understanding of plant responses to salinity. Plants respond to high salt with a set of mechanisms that often overlap drought responses. The close relationship between salt and drought responses is due to the dual effect that salinity has on the plants (Fig.1.1). Initially salt increases rapidly the osmotic potential around the roots making the extraction of water difficult from the soil mimicking a drought effect (osmotic stress) and as a result growth is transiently arrested (Zhu, 2002). Secondly, toxic concentrations of ions such as sodium (Na^+) an chloride (Cl^-) are slowly accumulated in the mature leaves (ionic stress) accelerating their senescence and negatively affecting the photosynthetic capacity and ultimately the supply of carbohydrates (Yokoi et al., 2002a; Zhu, 2002).

Plants respond to an increased concentration of NaCl in the root environment within seconds (Knight et al., 1997; Tracy et al., 2008), however, how they sense the salt remains unclear.

Plants gain salinity tolerance using three main strategies: increase protection against osmotic imbalance by preventing the entrance of ions, sequestration of Na^+ in particular in tissue or organs, increase protection against ion toxicity (Na+ and Cl-) by accumulation of soluble organic compounds (Munns and Tester, 2008).

Osmotic stress leads to a rapid inhibition in the rate of expansion of young leaves and to the closing of stomata in mature leaves.

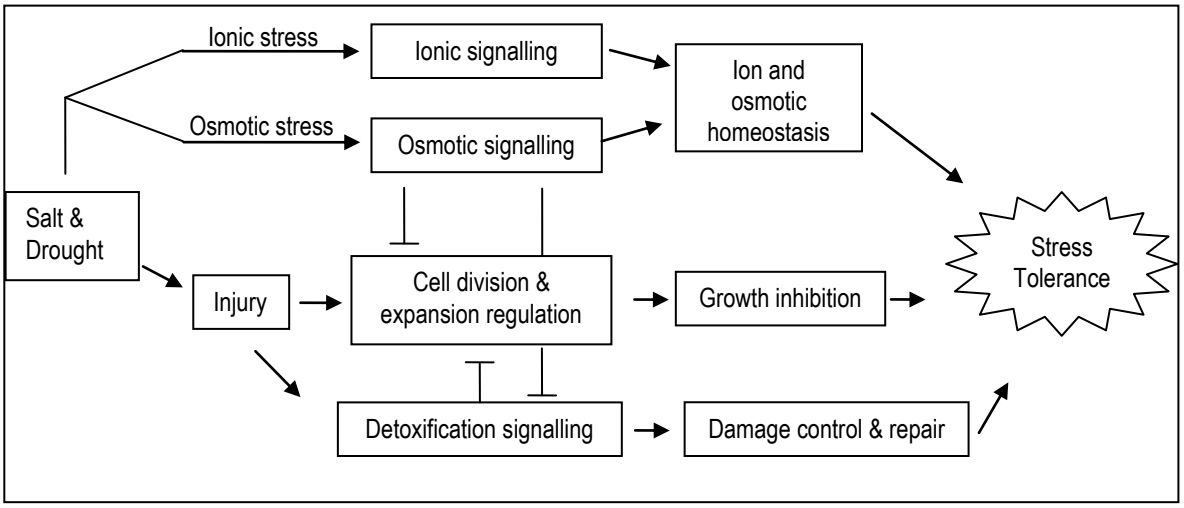


Fig. 1.1 Consequences of salt and drought stress on the signalling pathways.
The signals for ionic and osmotic signalling pathways are ions (excess NaCl) and osmotic changes. The outputs of ionic and osmotic signalling are cellular and plant homeostasis. Direct input signals for detoxification signalling are derived stresses (i.e., injury), and the signalling output is damage control and repair (e.g., activation of dehydration tolerance genes). Interactions between the homeostasis, growth regulation and detoxification pathways are indicated (From Zhu 2002, modified).

Plants cope with ionic stress by preventing the entrance of Na^+ into the root, controlling transport to and allocation of Na^+ within the leaf, and sequestering Na^+ into the vacuole (Apse and Blumwald, 2007). Due to the prevailing negative membrane potential the entrance of Na+ in the roots is a passive mechanism, and conversely the efflux of Na^+ from the cell requires an active process, particularly when external Na^+ concentrations are high. Higher plants lack Na+ pumps energized by ATP hydrolysis (Na^+-ATPases), making the electro-neutral exchange of sodium for protons via Na^+/H^+ antiporters the only mode of transport that has been measured for efflux under physiological conditions (Apse and Blumwald, 2007).

In *Arabidopsis thaliana,* different transporters have been identified as contributing to Na^+ homeostasis at different cellular levels: antiporters at the plasma membrane (e.g. SALT OVER SENSITIVE 1, SOS1)(Shi et al., 2000), vacuolar Na^+/H^+ antiporters (e.g. Na^+/H^+ EXCHANGER 1, NHX1) (Apse et al., 1999; Gaxiola et al., 1999), and the plasma membrane uniporter (e.g. HIGH AFFINITY POTASSIUM TRANSPORTER 1, HKT1) (Uozumi et al., 2000).

The *SOS1* gene encodes a Na^+/H^+ antiporter with 12 transmembrane domains in the N-terminal half and a long hydrophilic C-terminal tail (Shi et al., 2002). *SOS1* is expressed in

epidermal cells at the root tip and in xylem parenchyma cells of roots and shoots; it mediates Na^+ extrusion out of the root and controls long-distance transport. SOS1 is controlled by a pathway that involves a calcineurin-like Ca^{2+} binding protein (SOS3) and a serine/threonine protein kinase (SOS2) that is activated by SOS3 (Liu et al., 2000; Halfter et al., 2000). The concentration of free cytosolic Ca^{2+} increases with the increase in the concentration of Na^+ around the roots (Tracy et al., 2008). Ca^{2+} facilitates the dimerization of SOS3 and the consequent interaction with SOS2 (Liu et al., 2000). Furthermore the SOS3/SOS2 complex is targeted to the plasma membrane via a myristoyl fatty acid chain covalently bound to SOS3 (Ishitani et al., 2000), enabling the phosphorylation and thus the activation of the membrane antiporter, SOS1 (Qiu et al., 2002, 2003; Shi et al., 2002).

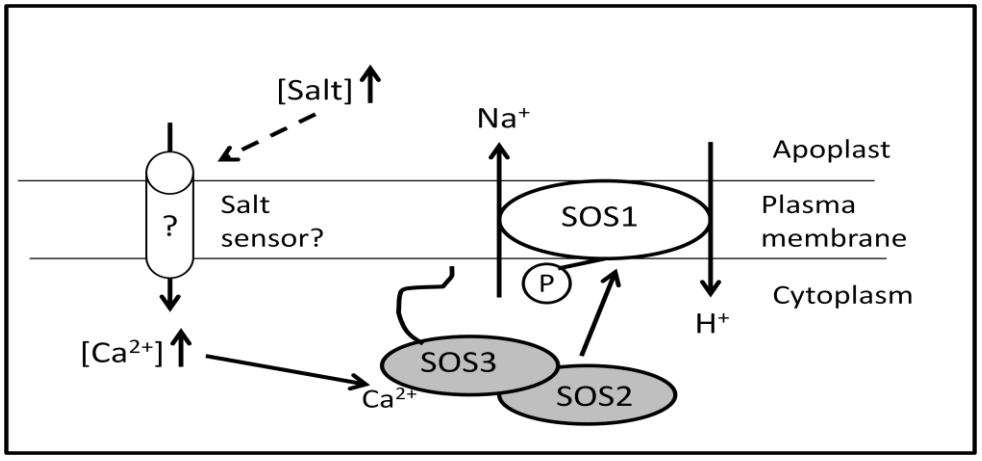


Fig. 1.2 Model for the Salt Overly Sensitive (SOS) regulatory pathway.
Salt induced increases in cytoplasmatic calcium (Ca2+) are detected by SOS3. Ca2+, together with SOS3 activates SOS2, a serine/threonine kinase. Activated SOS2 phosphorylases and stimulates the activity of SOS1, a plasma membrane localized Na+/H+ exchanger leading to regulation of ion homeostasis during salt stress (From Chinnusamy et al., 2004, modified)

Interestingly the mutant *sos3* phenotypes are suppressed by mutations in the *AtHKT1* gene (Rus et al., 2001). HKT1 was initially described in wheat as a high affinity potassium transporter (HKT) acting as a Na^+/K^+ symporter, or as Na^+ selective uniporters depending on the external Na^+ concentration (Schachtman and Schroeder, 1994; Rubio et al., 1995). Other species of plants harbour several HKT proteins combining both functions with different affinities (Munns and Tester, 2008). In *Arabidopsis*, there is only a single member of the HKT family, which acts as a low affinity Na^+ uniporter (Uozumi et al., 2000). AtHKT1 is mostly localized in the xylem parenchyma cells and at least in high-salt conditions unloads Na^+ from xylem vessels into xylem parenchyma cells (Sunarpi et al.,

2005; Shi et al., 2002), giving a significant contribution in the allocation of Na+ from roots to shoots.

The *athkt1* mutant shows an over-accumulation of Na^+ in xylem sap and in the leaves but whether and how HKT1 also impacts on phloem loading remains to be determined (Berthomieu et al., 2003). In addition to SOS1 and HKT1-type transporters non-selective cation channels make a significant contribution to Na^+ influx during salt stress. Volkov and Amtmann (2006) demonstrated that root plasma membrane non selective channels have selectivity for K^+ over Na^+ in *Thellungiella halophila*, a halophyte closely related to *A. thaliana*. Consequently, during the salt exposure, a smaller depolarization occurs in *T. halophila*, and hence the driving force for K^+ uptake is maintained, leading to improved K^+/Na^+ tissue concentrations. Interestingly, this higher K^+/Na^+ selectivity indicates that there are different structural features of root ion channels that might underlie differential ion accumulation in the two species (Volkov and Amtmann, 2006).

In *Arabidopsis*, a good candidate for channels that operate in Na^+ uptake are cyclic nucleotide-gated ion channels (CNGCs), a group of non selective cation channels that are inhibited by calcium and calmodulin (Maathuis, 2001). The *Arabidopsis CNGC* gene family comprises 20 members and several studies indicate that plant CNGCs are involved in the control of growth processes and responses to abiotic and biotic stresses. These channels contribute to cellular cation homeostasis, including calcium and sodium, as well as to stress-related signal transduction. For example CNGC19 and CNGC20, which constitute one of the five CNGC subfamilies, are involved in *Arabidopsis*' tolerance towards salt (Kugler et al., 2009). Upon salinity both genes, *CNGC19* and *CNGC20*, are up-regulated within hours. Mature plants of CNGC10 anti-sense lines were more sensitive to salt stress and contained higher Na^+ concentrations in shoots compared with wild type, indicating that *CNGC10* could assist the plant in the allocation of sodium within the plant (Kugler et al., 2009). Also because of their capacity to mediate calcium influx some CNGCs may potentially act upstream of the SOS pathway (Zhu, 2002).

Another strategy that plants employ under salinity stress is vacuolar compartmentalization of sodium (Apse and Blumwald, 2007). This is a critical mechanism to avoid the toxic effects of Na^+ in the cytosol while providing additional osmotic pressure for water uptake and turgor maintenance. This function has been attributed to tonoplast localized NHX-like antiporters, energized by the ΔpH across the tonoplast that facilitates vacuolar compartmentalization of Na^+ (Rodríguez-Rosales et al., 2009). In *Arabidopsis*, the

family AtNHX contains six members with diverse degrees of response to salt stress. The over expression of *AtNHX1,* for instance, has been shown to improve salt-tolerance in *Arabidopsis* (Apse et al., 1999; Gaxiola et al., 1999). The most constitutively expressed members of the family are *AtNHX1* and *AtNHX2*, found in roots, shoots and seedlings (Apse et al., 2003; Yokoi et al., 2002b; Shi and Zhu, 2002) while *AtNHX3*, *4* and *6* are less expressed in these tissues. In particular, AtNHX1 is localized in all tissues except at tip of the root. Interestingly, high activity has been detected in response to salt stress in root hairs suggesting a role in Na+ accumulation in the enlarged vacuoles of these cells in response to salt stress (Shi and Zhu, 2002). Even though *AtNHX1* and *AtNHX2* are expressed constitutively in shoot and roots, their sensitivity to stress differs depending on tissue. In seedlings, osmotic stress (NaCl, sorbitol) induces the transcription of *AtNHX1* and *AtNHX2* in an abscisic acid (ABA) dependent manner (Shi and Zhu, 2002). However, in mature plants *AtNHX1* expression was shown to be up-regulated in leaves but not in roots by NaCl or ABA (Quintero et al., 2000). In addition, the expression of *AtNHX5* was induced during salt stress by NaCl but not ABA (Apse and Blumwald, 2007).

The constitutive base level of transcript abundance of *AtNHX1*, *2* and *5* was found to be greater in *sos* mutants than wild type indicating that the SOS pathway negatively regulates transcriptional expression of these Na^+ antiporters genes (Yokoi et al., 2002a).

1.2 Effect of salt stress on the transcriptome

The perception of stress and its transmission in plants affects many regulatory elements involved in the synthesis or alteration of different classes of proteins (i.e. ion channels, transporters enzymes or transcriptional factors). Through this signal transduction mechanism plants are able to recognize the stress situation and protect themselves against the adverse conditions. Clearly, understanding of the functions of stress-inducible genes will help to unravel the underlying mechanisms of stress tolerance. However, it should be noted that, especially in the case of plants that are not salt-tolerant, transcriptional responses do not necessarily have a direct benefit or even a consequence at all. Numerous microarray datasets of the response of *Arabidopsis* to NaCl have been made publicly available through databases, such as Gene Investigator and GEO (Hruz et al., 2008; Barrett et al., 2011). One of the early studies carried out with *A. thaliana*,

identified the convergent and divergent pathways between salinity and other abiotic stress responses (Kreps et al., 2002). In this study, a microarray containing 8,100 probe sets was used (Kreps et al., 2002). Expression profiles were obtained separately for roots and leaves isolated from plants exposed for 3 or 27 h to a salt (100mM NaCl), osmotic or cold stress. Results show that a total of 440 genes were differentially expressed only under salt stress. However, the vast majority of genes were showing only a transient change in expression, with only 22 genes showing the same response at both time points, all of which were expressed in the roots. Among differentially expressed genes, the largest category (50%) was related to oxidative stress related enzymes (e.g. glutathione reductase and cytochrome P450) (Kreps et al., 2002). Ma et al., 2006 investigated the global transcriptional response to different stresses using datasets available from AtGenExpress representing a total of 12 studies made with Affymetrix GeneChip- ATH1, which contains probes for approximately 22000 genes. The authors identified 1500 genes that were most strongly regulated by salt. Among them the vast majority (680 genes) were also induced by at least two different biotic stress treatments and, in addition, shared common regulation with genes regulated by abiotic stresses related hormones (elicitor, ABA and MeJa). Plants hormones are chemical messengers that are active at extremely low concentrations and play critical roles in regulating developmental processes and signalling networks involved in plant responses to a wide range of biotic and abiotic stresses (Bari and Jones, 2009).

Only 171 genes were strictly salt-specific and they responded only in roots. Among this set of transcripts nearly 20% of the genes were transcription factors such as *MYB, WRKY* and *AP2* genes. Several families of transcription factors are known to translate stress signals into changes in gene expression. Many ethylene-synthesis and signalling genes were observed, including *ERF1* and *ACS8* (Ma et al., 2006). Another transcript expression study performed with the Affymetrix ATH1 microarray analysed the kinetic of the salt stress by exposing the plants to 150 mM NaCl and harvesting separately roots and shoots at several different time points (0.25, 0.5, 1, 3, 6, 12 and 24h) (Kilian et al., 2007). The results showed that the alterations in gene expression occur rapidly. The first changes are observed within 30 min after the application of stress in both tissues even though only the roots were directly exposed to the stress. The latter observation suggests the immediate production of a systemic signal, which is transferred between organs. As before, it was found that salt stress caused a predominantly transient alterations in gene

activity most of which were over after 24h (Kilian et al., 2007). Other studies investigated the salt response focusing on tissue-specific response to the stress. Roots are the primary site of perception for several types of water-limiting stresses, (i.e salinity and drought) and in many circumstances the stress-sensitivity of the roots limits the productivity of the entire plant (Steppuhn and Raney, 2005). Jiang and Deyholos, (2006) used Array-Ready Oligo Set for Arabidopsis genome Version 1.0 (Qiagen Operon), a microarray representing 23,686 *Arabidopsis* genes, in order to identify root transcripts that changed in relative abundance following 6, 24, or 48 h of hydroponic exposure to 150 mM NaCl. The microarray profiling revealed that dynamic changes in transcript abundance occurred in roots for at least 20% of the genome. Results of functional enrichment analysis were consistent with generally observed stress response genes including hundreds of transcription factors, kinases/phosphatases, hormone-related genes and effectors of homeostasis, and overall emphasized the complexity of this stress response. Also several novel classes of genes were found to be induced by NaCl treatment including transporters (i.e. MATE, LeOPT1-like), signalling molecules (i.e. PERK kinases, MLO-like receptors), carbohydrate active enzymes (i.e. XTH18), transcription factors (i.e. members of *ZIM*, *WRKY*, *NAC*), and others (i.e. 4CL-like, COMT-like, LOB-Class1) (Jiang and Deyholos, 2006). Taken together these data provided a detailed understanding of the pathways that are induced by salinity stress in *A. thaliana,* the kinetics of the transcriptional response and also reveal that only a small number of genes were strictly salt-specific and all are localized in the roots. However, there are no available transcriptomic data describing the effect of salt priming on the transcriptional responses of *Arabidopsis* roots and shoots to NaCl treatment.

1.3 Concept of priming and memory in plants

Breeders and farmers have commonly observed an interesting event in the field: if at the beginning of the season seedlings experience a moderate abiotic stress, caused for example by adverse weather conditions, the plants show an enhanced tolerance if the stress reoccurs. This phenomenon is known as priming or hardening and is a common theme underlying responses to a range of stress factors. In the literature the term “priming” is often used interchangeably with “acclimatization”. In this thesis we use the

term “priming” to describe a short, brief and mild stress treatment that occurs for a limited period of time leading to an increased tolerance when the stress re-occurs. While “acclimatization” is here defined as a gradually increased or repeated stress, that leads to a gradual acquisition of tolerance if the stress becomes more severe.

Priming can be induced through different environmental or chemical stimuli. Primed plants display either faster or stronger activation of the various defence responses if the stress recurs (Bruce et al., 2007).

1.3.1 Effect of priming on biotic stress tolerance

Among the chemical inducers of priming, the elicitor β-aminobutyric acid (BABA) is a potent inducer of resistance against a wide range of pathogens. BABA acts by potentiating pathogen-specific plant resistance mechanisms through activation of defense mechanisms such as callose deposition, hypersensitive response, and the formation of trailing necroses (Zimmerli et al., 2001).

Several studies have shown that pre-treatment with BABA directly up-regulates and potentiates gene expression (e.g. SA-responsive genes) during bacterial infection. Tsai et al. (2011) showed that BABA inhibits the *Arabidopsis* response to the bacterial effector coronatine (COR). COR is known to promote bacterial virulence by mimicking jasmonic acid (JA), which in turn antagonizes salicylic acid (SA) signalling. BABA specifically represses the JA response induced by COR without affecting other plant JA responses (Tsai et al., 2011).

Priming BABA treatment of tomato seeds induced defence against the fungal pathogen, *Oidium neolycopersici*. Primed seeds showed a long lasting increase in resistance to up to 8 weeks associated with enhanced defence without influencing the growth and thus making it suitable for commercial exploitation (Worrall et al., 2012).

Van Hulten et al. (2006) demonstrated that low doses of BABA caused only minor reductions in relative growth rate and had no effect on seed production. However, high doses of BABA strongly affected both fitness parameters (Van Hulten et al., 2006). This implies that the benefits of priming prevail over its costs when disease occurs. Consequently, plants in the primed state are efficiently protected against stresses without major adverse effects on commercially and ecologically important traits, such as growth and seed set.

Another compound that acts as an inducer of plant defences by means of a priming mechanism is hexanoic acid (HxAc). Root treatment with hexanoic acid (HxAc) protected tomato plants against *Botrytis cinerea* by enhancing callose accumulation in an ABA dependent manner (Vicedo et al., 2009). By contrast in *Arabidopsis,* plants primed with HxAc showed a transient enhanced expression of JA- dependent defence, but the mechanism of induced resistance by HxAc seems to be independent of callose deposition or ABA after *B. cinerea* infection (Kravchuk et al., 2011). These results indicate that this particular chemical priming activated mechanisms specific to the plant considered.

Furthermore, plants can secrete Volatile Organic Compounds (VOCs) and use them as a message to warn other plants of the risk of herbivore attack in the environment, and consequently preparing them to modulate their defensive strategy (Heil and Kost, 2006). Experiments conducted in the field demonstrated that plants that had been exposed to VOCs thereafter responded to subsequent leaf damage with an increased secretion of extra-floral nectar (Heil and Kost, 2006). Whereas BABA induced resistance associated with SA-dependent defence against biotrophic pathogens, VOC induced resistance seems to function through priming by jasmonate (JA)-dependent defence against necrotrophic pathogens and insects (Ton et al., 2009)

1.3.2 Effect of priming on abiotic stress responses

Several studies have also shown that applying a number of different abiotic priming treatments can reduce the damage caused if the same stress reoccurs compared to non-primed. Even though priming can be applied at different stages of development, the vast majority of the studies investigated seed priming due to the easier application of the treatment and thus better suitability for economical exploitation. Seed pre-treatments are common practice in agriculture: typically antimicrobial or fungicidal are applied to enhance crop protection prior to planting. However, it is important to mention that it is also common agricultural knowledge that some plants, especially trees, need an appropriate seed pre-treatment (i.e. cold exposure, soaking in water, acid treatment) for breaking dormancy and synchronizing germination. These effects could mask other effects that are obtained with similar pre-treatment technology. For this reason the following literature report will distinguish between priming applied to seeds and priming applied at later stages of plants development. It is important to point out that all carried

out studies to date have described the effect of abiotic priming without fully investigating the molecular basis of this phenomenon and thus the underlying mechanism still remains unknown.

Priming of seeds

In a study by Cayuela et al. (1996) salt priming was applied by soaking the seeds of tomato in a 6M NaCl solution and afterwards exposing the plants to a prolonged salt stress from 11 to 60 days after sowing. Results showed that primed seeds germinated earlier with a shorter shoot and root dry weight reduction than non-primed seeds. Moreover, seed priming led to a significant reduction in Na^+ and Cl^- accumulation in roots. Organic acids accumulated in roots of primed plants while leaves showed a significant increase in sugars and organic acids. The authors ascribed the faster growth of tomato plants from primed seeds to be the result of a higher capacity for osmotic adjustment (Cayuela et al., 1996).

Other reasons for the better performance of primed seeds may be lower electrical conductivity (EC) of the seed, higher sugars content along with increased α- amylase activity (Nawaz et al., 2011). In this study priming was applied by exposing tomato seeds to different concentrations of NaCl and KNO_3 for 24 h. The seedling emergence observed for seeds primed with 25 mM KNO_3 was two times faster than for non-primed seeds along with an increased percentage of seedling emergence and seedling growth; however, NaCl priming resulted in poor seedling emergence and growth.

Seed priming of lettuce alleviated the thermo-inhibition that most cultivated varieties of lettuce (*Lactuca sativa*) show during germination (i.e., above 25–30 °C) (Schwember and Bradford, 2010). Priming treatment consisted in controlled hydration followed by drying and led to an increased germination rate at high temperature. In order to further investigate this phenomenon the authors carried out a quantitative trait locus (QTL) analysis of seed germination after priming using a recombinant inbred line (RIL) population derived from a cross between *L. sativa cv. Salinas* and *L. serriola* accession UC96US23. The results showed that priming significantly increased the maximum germination temperature of the RIL population. Also the authors identified a single major QTL responsible for 47% of the phenotypic variation. The QTL collocated with Htg6.1, a

major QTL from UC96US23 associated with high temperature germination capacity (Schwember and Bradford, 2010).
Interestingly, within the QTL, was found the gene LsNCED4 that encodes a key enzyme (9-cis-epoxy-carotinoid di-oxygenase) in the ABA biosynthetic pathway.

Priming applied at later stages of plant development

Several studies suggest that abiotic stress tolerance can be also improved by priming seedlings. For example drought and salt stress tolerance of *Arabidopsis* plants increased following treatment with the amino acid β-aminobutyric acid. BABA pre-treated plants showed increased tolerance probably due to enhanced ABA accumulation that led to accelerated stress gene expression and stomatal closure (Jakab et al., 2005).
In sugarcane, primed plants exhibited less salt and dehydration-induced leaf senescence (Patade et al., 2009). Field experiments showed when salt priming was applied during germination growth performance in terms of shoot length, shoot and root fresh weight was improved in two-month old sugarcane plants subjected to 15 days of iso-osmotic NaCl or polyethylene glycol (PEG) stress (Patade et al., 2009).
Priming applied to tomato seedlings (5 leaves stage) of a salt sensitive variety grown for 15 days in 35 mM NaCl resulted in an increased fruit yield by 29%. Moreover, the primed plants accumulated about 20 mM less Na^+ and 40 mM less Cl^- in their adult leaves than non-primed plants after 75 days of 70 mM NaCl treatment. Interestingly, seedlings primed at 2-leaves stage did not show any long-term adaptive response. These results suggest that there is a developmental window where priming has an effect on the adaptive response (Cayuela et al., 2001). Different stages of development show significant differences in morphology, membrane permeability and expression of specific genes therefore the perception of the stress has a different impact on the plants at different stages of development.
Finally, there are several reports suggesting that salt stress stimulates cold hardiness in potatoes and spinach, water stress induces chilling resistance in rice and heat stress increases tolerance to several abiotic stresses (Cuartero et al., 2006). Also water stress in general induces salt tolerance: tomato plants pre-treated with drought stress were able to grow better than non pre-treated plants after 21 days of salt stress. Furthermore pre-treatment with PEG had a positive effect on salt tolerance tomato seedling (Cuartero et

al., 2006). This intriguing phenomenon known as cross-tolerance could be potentially used as strategy to protect crops against a wide spectrum of abiotic stresses (Mittler, 2006).

1.3.3 Does priming establish "stress memory"?

It is has been suggested that priming generates some sort of "stress imprint" lasting for minutes, days, weeks or even generations (Conrath et al., 2006), and that the ability to recall and to use this information ('memory') enhances the chance of survival of the plant (Gális et al., 2009). However, the exact molecular nature of stress memory in plants is still under debate. Priming could induce the accumulation of signalling compounds in active configuration, or transcription factors or other regulatory proteins that enhance defence gene transcription after stress recognition. Modification of structural elements such as membrane lipids and cell wall components has also been proposed (Gális et al., 2009). However, one could argue that due to the relatively fast turnover of proteins and lipids, and due to dilution through growth, these changes should not be able to persist over expanded periods between the priming treatment and the second stress exposure. An alternative possibility is that the beneficial priming effect occurs as a result of changes in DNA or chromatin structure. Numerous studies in the past decade have pointed out the importance of the structural proprieties of DNA and chromatin for transcriptional regulation of gene expression (Conrath, 2011; Conrath et al., 2006). Changes in DNA and chromatin structure can be maintained during mitosis and even meiosis and they therefore could provide an explanation for the so-called 'epigenetic memory', where heritable traits occur without changing the DNA sequence.

The expression of the genes is enhanced or repressed through the interaction of transcription factors and other regulatory proteins with short, non-coding DNA sequences, such as promoters, introns and 5' or 3' UTRs. The accessibility of these particular sequences for regulatory interactions can be modulated by the changes in the conformation of the chromatin fibre including chemical modifications of the DNA strand itself as well as the association of the DNA with histone proteins. Very recently an increasing collection of data suggests that histone modifications could hold the information of a primed state of transcription (Conrath, 2011). For example the promoter of the defence gene *PR1* is associated with high levels of H3 acetylation and H3K4

methylation (H3K4me) in the permanently primed *sni1* mutant with constitutively enhanced immunity without the gene being activated (Mosher et al., 2006). Also several *WRKY* transcription factors known to be involved in priming abiotic response were found to be associated with a range of histone modifications. In particular, after priming with benzothiadiazole the promoter of the transcription co-activator gene *WRKY29* was associated with H3K4me3 and H3K4me2, as well as acetylation of H3K9, H4K5, H4K8, and H4K12 (Jaskiewicz et al., 2011). Interestingly the gene is not activated when the plants do not encounter a further stress stimulus. Similar observations were made for *WRKY6* and *WRKY53* when H3K4me3, H3K4me2, and activation of transcription were assayed (Jaskiewicz et al., 2011).

These results suggest that certain *WRKY* promoters are subjected to chromatin modifications that facilitate transcription activation of *WRKY* genes by subsequent stress.

Treatment with acibenzolar S-methyl (a synthetic version of the plant hormone salicylic acid) or pathogen infection of distal leaves induced chromatin modifications on defence gene promoters that were normally found on active genes, although the genes remained inactive. This led to an amplified gene response upon exposure to stress (Jaskiewicz et al., 2011).

Due to the relatively stable nature of biochemical modifications of DNA and histones, they are very suitable candidates for stress imprints with the function of retaining stress memory over longer time periods (Chinnusamy and Zhu, 2009). Unlike the biosynthesis of proteins and metabolites, which requires a constant energy input during plant growth, chromatin modifications, once established, are self-propagating. A role of epigenetic modifications for plant stress memory is therefore an attractive hypothesis that merits further investigation.

1.4 Chromatin: a dynamic and complex structure

In eukaryotes, genomic DNA is tightly packed into a complex structure of DNA and proteins known as chromatin. The fundamental unit of chromatin is the nucleosome (Fig.1.2), which is formed by wrapping ~147 bp of DNA around a histone octamer (two copies each of histones H2A, H2B, H3 and H4). The chromatin complex is a truly dynamic structure that can be subjected to several processes that, acting in concert, can change its

structure (Roudier et al., 2009). Every single component (nucleosome, histone, DNA) can be modified at different levels and thus create a cell-specific or stimulus-specific accessible genome (the 'epi'genome). Chromatin structure influences the accessibility of factors and cofactors to the DNA and therefore gene regulation (Roudier et al., 2009).

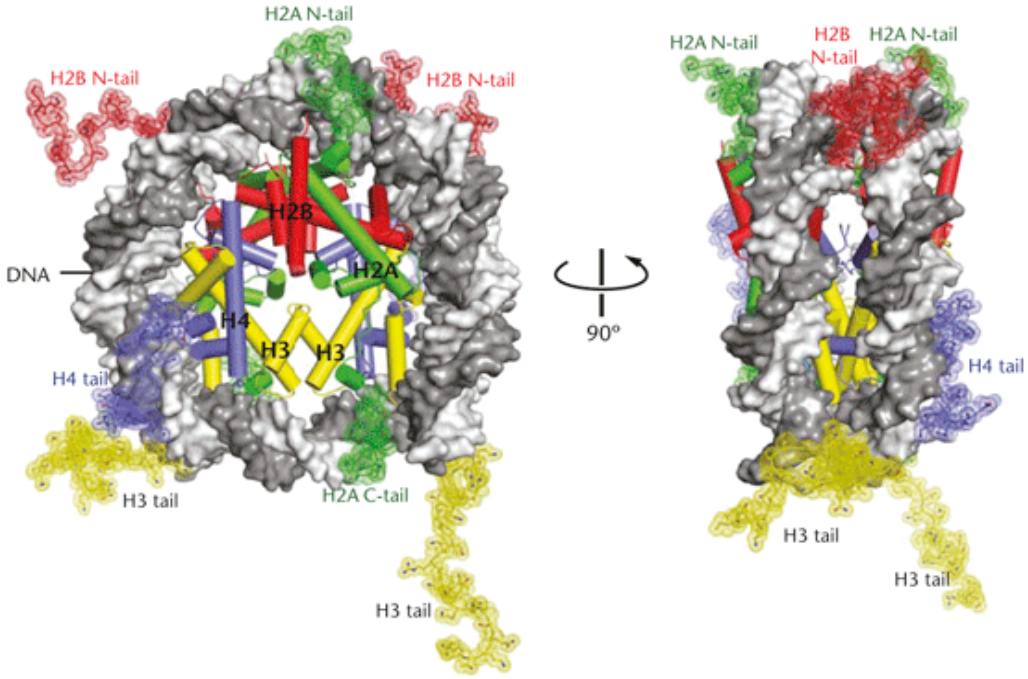


Fig. 1.3 Nucleosome core with DNA and histone sites of post-translational modifications.
The nucleosome is formed by two copies of each histone (H2A, H2B, H3 and H4) and 147 bp of DNA that wraps the histone octamer forming a left-handed super helix. The histone globular regions are shown in schematic with helices illustrated as cylinders, and the DNA shown in grey. The histone tails protrude from the nucleosome core allowing modifications to occur (picture taken from Lallous et al., 2011).

Chromatin ('epigenetic') modifications play an important role in genome organization and stability as well as the control of gene expression without altering the nucleotide sequence (Gendrel and Colot, 2005). Nucleosomes are able to change in position and composition through placement of histone variants by chromatin remodelling ATPases. The histone proteins within the octamer present a tail of amino acids in their N-terminal protruding from the nucleosome core. These amino acid residues can be covalently modified by particular enzymes and ultimately affect the histone-DNA interaction (Gendrel and Colot, 2005). Some histone modifications, such as acetylation of histones H3 and H4 and tri-methylation of H3 lysine 4 (H3K4me3) are known as euchromatic marks because they are often associated with high transcription rate, whereas other

modifications, such as methylation of H3K9 and H3K27, are known as heterochromatic marks and related to gene repression (Jenuwein and Allis, 2001; Li et al., 2007a).

At the DNA level, particular cytosine residues can be methylated, which interfere with binding of transcriptional factors and other proteins (Chinnusamy and Zhu, 2009). In plants, cytosine residues in a DNA molecule can be methylated in three different sequence contexts: CG, CHG "Symmetric methylation" and CHH "asymmetric methylation" (H=A, T or C). In *Arabidopsis*, multiple DNA methyl-transferases are involved in the establishment and maintenance of methylation process: CHG methylation is established by CHROMOMETHYLASE 3 (CMT3), while maintenance of CG methylation is maintained by METHYLTRANSFERASE1 (MET1). CHH methylation is maintained by continual de novo methylation by DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2 (DRM2) guided by small RNAs in a process termed RNA-directed DNA methylation (RdDM) (Simon and Meyers, 2011). In the current model for the RdDM pathway (Simon and Meyers, 2011; Wierzbicki et al., 2012) the plant specific DNA-dependent-RNA polymerases IV (Pol IV) transcribe transposons and other genomic regions copying single-stranded RNA (ssRNA) into double-stranded RNA (dsRNA). Pol IV acts in a complex with chromatin remodelling factors and the RNA-dependent RNA (RDR2) that copies single-stranded RNA (ssRNA) into double-stranded RNA (dsRNA). The dsRNA molecules are then cleaved into 24 nt RNAs by the double-stranded RNA endonuclease DICER- LIKE3 (DCL3) and then loaded into a complex containing Argonaute 4 (AGO4).

In addition, another independent pathway containing the DNA-dependent-RNA polymerases V generates other non-coding RNA. Pol V acts in a complex termed DDR composed of DRD1 (DEFECTIVE IN RNA DIRECTED DNA METHYLATION a chromatin remodelling factor), DMS3 (DEFECTIVE IN MERISTEM SILENCING 3, a protein related to cohesions/condensins) and RDM1 (RNA-DIRECTED DNA METHYLATION1 ssDNA-binding protein with a preference for methylated DNA). The Pol V non-coding RNAs serve as "scaffolds" to which AGO4–siRNA complexes bind through RNA–RNA base-pairing. In addition AGO4 can also interact with the C-terminal domain of the Pol V largest subunit, presumably stabilizing the complex through protein–protein interactions.

Subsequently, recruitment of DRM2 and other chromatin-modifying machinery is observed at the Pol V transcribed loci and then cytosines are methylated *de novo*.

Although the exact mechanism is still not clear, the recruitment of DRM2 to AGO4-siRNA-PolV transcript complexes is possibly mediated by RDM1, which has been shown to interact with both AGO4 and DRM2 (Wierzbicki et al., 2012).

1.5 Histone acetylation in plants

Histone acetylation is a dynamic and reversible mark estabilished by the deposition of an Acetyl-CoA group to lysine residues within histones (Servet et al., 2010). When the mark is in place there is a neutralization of the positive charge of the histone tails which in turn decreases their affinity to DNA and thereby forming a more relaxed structure. This facilitates the association of transcription factors and other proteins involved in replication, transcription etc to the DNA (Servet et al.2010). Histone acetylation is usually associated with transcriptional activation whereas lack of acetylation forms a tighter association between DNA and chromatin leading to transcriptional repression (Berger, 2007).

Histone acetylation in *Arabidopsis* occurs at the N-terminal lysine residues of histone H3 (K9, K14, K18, K23, and K27) and H4 (K5, K8, K12, K16, and K20) although acetylated H3K27 is not detected by mass spectrometry (Zhang et al., 2007a; Earley et al., 2007).

The acetylated histone residues can be recognized and bound by chromatin remodelling factors through a 'bromodomain' which therefore is considered as a reader of histone acetylation markers (Zeng and Zhou, 2002). Histone acetyltransferases (HATs) (of which there are numerous in plants) are the enzymes that add the Acetyl-CoA group to the histone lysine residues, which in turn can be removed by histone de-acetylases (HDACs). In *Arabidopsis* there have been 12 *HAT* and 18 *HDAC* genes identified, as part of multigene families (Pandey et al., 2002).

Several studies showed that histone acetylation has an important influence on numerous developmental and biological processes in plants (Chen and Tian, 2007, Jasencakova et al., 2001, Chua et al., 2003, 2001)). In the cell cycle, mitosis is associated with changes in the acetylation status of histones that are correlated with dynamic changes in chromosome structure and function (Jasencakova et al., 2001). Furthermore acetylation of histones has been shown to play an important role in light-responsive gene activation in plants, for example it was first demonstrated in tobacco that the transcriptional

activation of the light-induced pea plastocyanin gene is associated with hyperacetylation of histones H3 and H4 (Chua et al., 2003, 2001). Flowering time is also influenced by changes in histone acetylation. The repressor of flowering *Flower locus C* (*FLC*) is repressed by vernalization and this is proportionally correlated with the reduced levels of histone acetylation at the *FLC* locus (Sheldon et al., 2006). Finally the *Arabidopsis* histone de-acetylase HDA6, has been found to interact with COI1, an F-box protein, which is required for JA-mediated plant defence responses (Devoto et al., 2002). Overall histone acetylation plays a major role during plant development and growth and also in response to a number of environmental stimuli.

1.6 Histone methylation in plants

Histone methylation is generally recognized as a dynamic epigenetic mark often recruiting other protein complexes to regulate diverse developmental processes (Gendrel and Colot, 2005). It is also involved in silencing repetitive sequences in order to maintain genome stability (Gendrel and Colot, 2005). The histone methylation status is dynamic: methylation marks can be established on lysine or arginine residues by histone lysine methyltransferases (HKMTs) and removed by histone demethylases (HDMs) (Liu et al., 2010). HKMTs can add up to three methyl groups to the residues (mono- (me1), di- (me2) and tri- (me3)). Unbiased mass spectrometry in *Arabidopsis* has identified four major sites on histone H3 of lysine methylation; Lys4 (K4), Lys9 (K9), Lys27 (K27), Lys36 (K36). Two other residues with minor levels of methylation were also detected Lys18 (K18) and Lys23 (K23) (Johnson et al., 2004) (Fig.1.3).

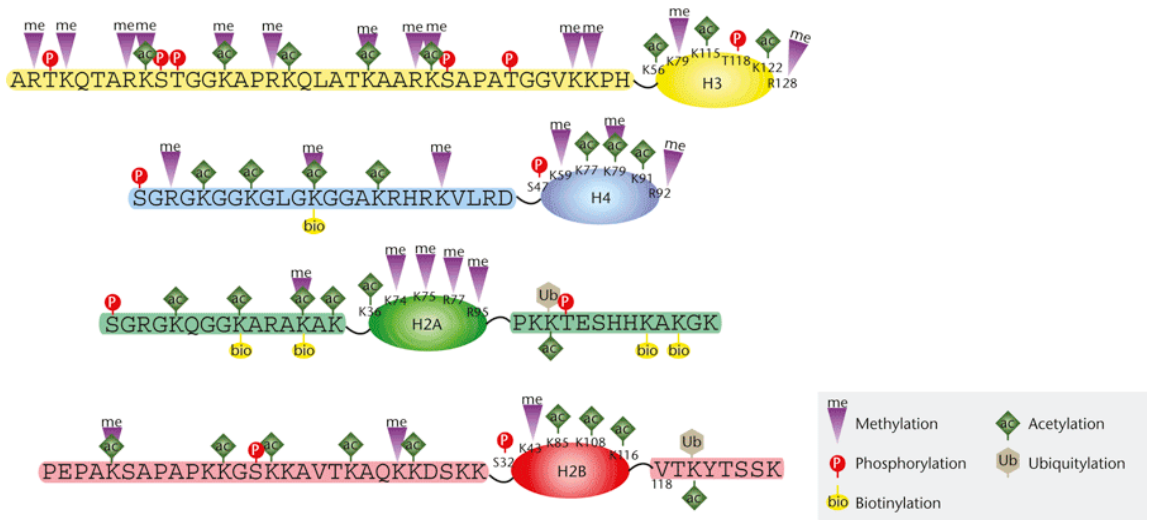


Fig. 1.4 Representation of histone post-translational modifications.
The histone cores are represented as ellipsoids and the amino acid sequences of the tails are shown. These modifications may not occur in every organism or simultaneously in the same histone tail. Different combinations of modifications allow the generation of specific patterns ('epigenetic code'). (Picture taken from Lallous et al., 2011).

Approximately two-thirds of the *Arabidopsis* genome is associated with methylated histones (Chinnusamy and Zhu, 2009). Histone methylation plays an important role in transcriptional regulation. Depending on the type of amino acid residue and on the degree of methylation, this type of modification can be correlated with both silent and transcriptionally active chromatin domains (Fig.1.4). For example H3K4me3 is associated with active genes and their promoters (Zhang et al., 2009). H3K9me2 is mainly associated with the hetero-chromatic zone of the chromosome and is enriched in transposons and repetitive sequences, and is therefore required for transcriptional silencing (Berger, 2007).

H3K27me3 and H3K36me are primarily involved in the repression of developmental genes and are required for normal plant development, but their exact role in transcriptional regulation remains unclear (Zhang et al., 2007b; Xu et al., 2008b).

H3K36 is mostly associated with actively transcribed genes while H3K27me3 causes silencing of genes in euchromatic regions (Berger, 2007).

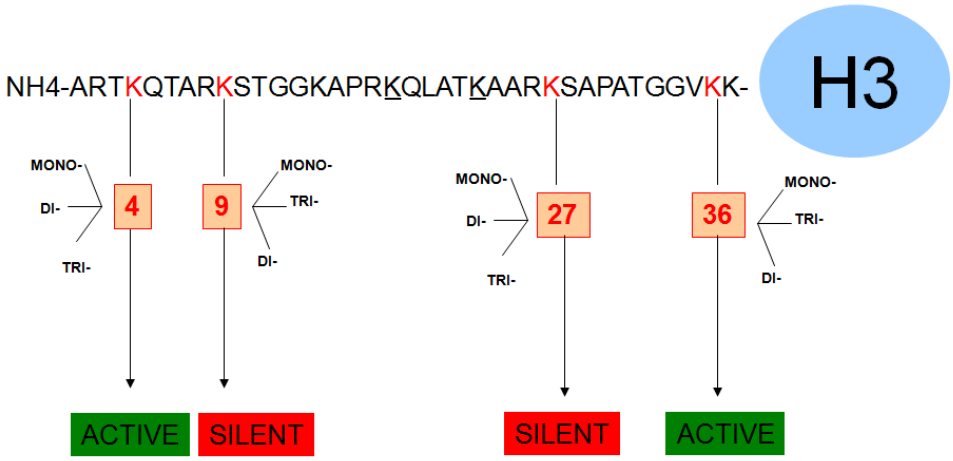


Fig. 1.5 Methylation occurs at specific lysines along the histone H3 tails in *Arabidopsis*. In red are the four major sites of lysine methylation Lys4 (K4), Lys9 (K9), Lys27 (K27), Lys36 (K36), and underlined the other two with minor levels of methylation detected in Lys18 (K18) and Lys23 (K23).

1.6.1 Histone H3 Lysine 4

Molecular features and distribution

Approximately two-thirds of genes are associated with H3K4 methylation. 6.45%, 6.0% and 12.1% of the sequenced nuclear genome are associated with mono-, di- or tri-methylated H3K4 respectively (Zhang et al., 2009). Methylated H3K4 is highly enriched in euchromatic regions and is absent from peri-centromeric heterochromatin regions. H3K4 methylation occurs almost exclusively within genes and their promoters; only a small fraction of intergenic repetitive sequences or transposons are associated with this mark. Zhang et al., 2009, showed that H3K4me3, H3K4me2 and H3K4me1 are distributed with a 5'-to-3' gradient along genes. H3K4me3 and H3K4me2 are enriched in the promoters and within the 5' end of transcribed regions with H3K4me3 occurring slightly upstream of H3K4me2. H3K4me1 is enriched in the transcribed regions with an apparent 3' bias. These results suggest that H3K4me2 and H3K4me3 may be involved in both transcription initiation and the early stages of transcript elongation. In contrast, it has been suggested

that H3K4me1 may be primarily involved in the elongation step during the transcription of longer genes. Comparative analysis from microarray datasets found that genes associated with different combinations of H3K4me, me2 and me3 are expressed at different levels and with different tissue specificity, suggesting that the three types of H3K4me may have different effects on chromatin structure and transcription (Zhang et al., 2009). In particular, H3K4me3 appears to be generally associated with actively transcribed genes, but there is no evidence for a direct role of H3K4me1 and H3K4me2 in transcriptional activation. H3K4me1 and H3K4me2 do not appear to have an additive effect to H3K4me3 on transcription levels and in the absence of H3K4me3 they are not preferentially associated with actively transcribed genes. Furthermore, H3K4me2 (but not H3K4me1 or H3K4me3) often overlaps with H3K27me3 (Zhang et al., 2009).

Impact of H3K4me on the plant life cycle

To date several studies have been carried out using *Arabidopsis* mutant lines for different methyl-transferase enzymes in order to unravel the impact of H3K4 methylation on plant life. Four of the seven class III HMTase genes (*ATX1*/*SDG27, ATX2*/*SDG30, ATXR7*/*SDG25* and *SDG2*) act on the H3K4 (Alvarez-Venegas et al., 2003; Saleh et al., 2008; Tamada et al., 2009; Guo et al., 2010). These enzymes showed differential abilities of methylation: ATX1 mediates H3K4 tri-methylation, ATX2 mediates only di-methylation (Pien et al., 2008; Saleh et al., 2008) whereas SDG2 is capable of catalyzing all three types of methylation *in vitro*. The loss of SDG2 activity in the *sdg2* mutant leads, to a strong decrease of H3K4me3 at numerous loci *in vivo* (Guo et al., 2010). The analysis of the knockout mutants generally shows that a defect in the methylation of H3K4 leads to severe abnormalities in development, with a particularly strong influence on flowering time, flower development and fertility. For example *atx1* mutants were reported to display an altered leaf morphogenesis and an early flowering phenotype, via the epigenetic regulation of the floral repressor FLOWERING LOCUS C (FLC), a MADS box transcription factor, which controls the transition from vegetative development to reproductive phase (Alvarez-Venegas et al., 2003; Saleh et al., 2008). Interestingly, some of these mutants also show particular phenotypes that might suggest other roles of the HTMase. For example, homozygous *sdg2* seedlings present significantly shorter root and

the plants remain dwarfed. In addition, flower organs are normal in morphology but the plants are male sterile due to pollen defects (Guo et al., 2010).

1.6.2 Histone H3 Lysine 9

Molecular features and distribution

Histone H3K9 methylation occurs as mono (H3K9me1), di- (H3K9me2), and tri-methylation (H3K9me3) and mass spectrometry in *Arabidopsis* revealed that the degree of this modification is variable (Johnson et al., 2004). The main status is mono-methylation, in fact 20% of the genome was found to be associated with mono, followed by 10% of di-methylation, whereas only a small percentage of tri-methylation was detected (100 fold lower than mono) (Johnson et al., 2004).

Genome wide ChIP assays in *Arabidopsis* coupled with high-resolution microarray analysis (ChIP on ChIP) revealed that H3K9me2 covered a total of 27 Mb of the sequenced genome (22.5%). It was found that di-methylation of H3K9 is highly enriched in peri-centromeric regions and appears over long uninterrupted regions (3.4-350 kb), while euchromatic arms showed isolated patches of smaller regions (0.6 kb) (Zhou et al., 2010; Bernatavichute et al., 2008). Around the centromere H3K9me2 is more frequently associated with transposons/pseudogenes (77% of all elements) than in the euchromatic arms (23%). H3K9me2 is rarely found in genes. Genes lacking H3K9me2 were found to have a higher average expression level than genes harbouring this modification, suggesting that H3K9me2 correlates with gene suppression (Zhou et al., 2010; Bernatavichute et al., 2008). H3K9me2 is localised either in promoters (46.4%) or in gene bodies (48.8%), rarely in both (4.8%). Consistent with a function of H3K9me2 in repressing transposon activity, further analysis showed that the majority of siRNA clusters present in peri-centromeric regions are highly associated with H3K9me2 (90% or higher overlap) (Bernatavichute et al., 2008).

H3K9 methylation is carried out by proteins of the SU(VAR)3-9 subgroup of methyltransferases, which consists of 14 proteins in *Arabidopsis*: the SU(VAR) 3-9 HOMOLOGs SUVH1-SUVH9, and the more distantly related SU(VAR) 3-9 RELATED proteins SUVR1-5 (Veiseth et al., 2011). Unlike SUVH proteins, which contain a YDG/SRA domain, the SUVR1, SUVR2 and SUVR4 proteins contain an N-terminal WIYLD domain of

unknown function, suggesting different modes of action of these enzymes (Veiseth et al., 2011). Interestingly, the mechanism of action of SUVH on K9 is tightly correlated to methylation occurring at the DNA level. For example, SUVH4 and SUVH6 bind the methylated CHG through their YDG/SRA domains suggesting a role of DNA methylation in recruiting H3K9 methyltransferases. In particular, SUVH4 binds the chromodomain of the Chromomethyltransferase 3 (CMT3) enzyme that mediates CHG DNA methylation through its SRA domain (Veiseth et al., 2011). Once recruited to the target regions, the SET domains of these histone H3K9 methyltransferases methylate adjacent histones, resulting in mutually reinforcing H3K9 methylation and CG DNA methylation in heterochromatin formation (Liu et al., 2010). SET domains and YDG/SRA domains of SUVH2 and SUVH9 were also shown to be required for RNA-directed DNA methylation (RdDM). The YDG/SRA domain of SUVH2 preferentially binds to methylated CG sites, while that of SUVH9 mainly binds to methylated CHH sites (Liu et al., 2010). Thus the SUVH proteins in *Arabidopsis* link the epigenetic gene-silencing marks H3K9me2 and DNA-methylation to control constitutive heterochromatin formation and work in parallel as transcriptional repressors of transposons or inverted repeat sequences (Pontvianne et al., 2010).

More recently, the more distantly related SU(VAR) 3-9 RELATED has also been investigated: while either SUVR1 or SUVR2 do not have detectable methyltransferase activity, SUVR4 can add methyl groups to the mono-methylated H3K9 peptide as substrate (Veiseth et al., 2011). SUVR4 is preferentially localized in non-condensed nuclear bodies suggesting it acts on euchromatin regions and may function as a repressor of rDNA gene clusters (Veiseth et al., 2011).

Impact of H3K9me on the plant life cycle

SUVH4, also known as KRYPTONITE (KYP), was the first plant histone H3K9 methyltransferase identified. Although histone H3K9 methylation is essential for gene silencing, *kyp* lack of function mutants show no obvious phenotypes apart from the reactivation of loci that were transcriptionally silenced by DNA methylation, suggesting its role in DNA methylation–mediated gene silencing (Liu et al., 2010; Pontvianne et al., 2010). Also the triple mutant *suvh4/suvh5/suvh6* and the loss-of- function of *suvh2* show no visible phenotype (Liu et al., 2010; Pontvianne et al., 2010). Only the combined

mutation of *suvh2/suvh9/suvh4* show pleiotropic developmental defects, including curled leaves and short stature, which are also seen in the *drm1/drm2/cmt3* triple mutant. The cause of these phenotypes is the activation of the F-box gene SUPPRESSOR of *drm1 drm2 cmt3* (SDC), which in wild type is silenced by a combination of the RNA-directed DNA methylation (RdDM) pathway and CMT3 (Liu et al., 2010; Pontvianne et al., 2010).

Interestingly, over-expression of SUVH2 induces development changes, such as delayed leaf senescence, by a general chromatin re-organization resulting in increased repressive histone marks including H3K9me2, H3K27me3 and H4K20me2 (Pontvianne et al., 2010).

1.6.3 Histone H3 Lysine 27

Molecular features and distribution

Histone H3 presents two isoforms (H3.1 and H3.2) that vary in the extent of methylation at K27. Although the two variants cannot be separated by HPLC, they present enough difference in the sequences to be separately detected by mass spectrometry (Johnson et al. 2004). H3.1 has a mixture of peptide isoforms dominated by those methylated at K27: 60% K27me1, 16% K27me2 and 5% K27me3. In contrast, the peptide isoform observed for H3.2 shows lower levels of methylation at K27: 36% K27me1, 6% K27me2 and no detectable K27me3 (Johnson et al. 2004). Genome-wide ChIP assays coupled with high-resolution microarray analysis (ChIP on ChIP) identified a total of 8,979 H3K27me3 regions covering 6.9 Mb and representing 5.7% of the sequenced nuclear genome (Zhang et al., 2007b). H3K27me3 was found to be highly enriched in the euchromatic arms, resembling the distribution of genes (Zhang et al., 2007b). This is in contrast to the heterochromatic distribution of several other silencing marks such as DNA methylation, small interfering RNAs (siRNAs) or H3K9me2. H3K27m3 is associated with a large number of genes in *Arabidopsis*: 18.6% of expressed genes with known functions, 15.6% of expressed genes with unknown functions and 7.2% of pseudogenes harbour this mark. Indeed, 70.8% of the H3K27me3 regions were found in promoters (around 200-bp regions upstream of transcription start sites) or in the transcribed regions of genes (Zhang et al., 2007b).

Cluster analysis of genes based on their expression patterns show that most of H3K27me3 target genes are expressed in a very tissue-specific manner, suggesting that H3K27me3

may facilitate tissue-specific repression of these genes (Zhang et al. 2007). Taken together, these results suggest that H3K27me3 is a component of a gene silencing system in *Arabidopsis* that is involved in the regulation of numerous genes and many developmental processes.

Impact of H3K27me on the plant life cycle

The correct distribution of H3K27 is essential for correct plant development as it provides a cellular memory to maintain the repressed transcriptional states of targets genes during cell division (Zhang et al., 2007b).

The eukaryotic enzymes that methylate H3K27 *in vivo* are all homologous with the *Drosophila melanogaster* SET-domain protein Enhancer of Zeste E(Z). E(Z) acts as part of the Polycomb Repressive Complex 2 (PRC2) and requires the WD-40 protein Extra Sex Combs for activity in *Drosophila*. *Arabidopsis* contains three E(Z) homologs: MEDEA (MEA), CURLY LEAF (CLF) and SWINGER (SWN). Both CLF and SWN are expressed during postembryonic development whereas MEA expression is limited to the female gametophyte and embryo development (Pontvianne et al., 2010).

Mutations in the maternal allele of *MEA* cause embryo abortion and endosperm over-proliferation. In addition, *MEA* has also been found to be involved in the regulation of its own imprinted expression: before fecundation *MEA* is required to repress the expression of the maternal copy of *MEA* while later in seed development the paternally inherited *MEA* allele is kept silent by the maternal copy *MEA* (Baroux et al., 2006). Mutations in *CLF* result in early flowering and pleiotropic phenotypes, including curled leaves and partial homeotic transformation of the sepals and petals to carpels and stamens (Ng et al., 2007). No obvious phenotype is observed in *swn* mutants but it strongly enhances the phenotypes of *clf* and *mea* mutants in *clf swn* and *mea swn* double mutants indicating redundant functions (Ng et al., 2007). Despite their similarity to E(Z) and despite the fact of the mutants show lower level of H3K27me2 and H3K27me3 in immunostaining experiments, the plant proteins remain biochemically uncharacterized; possibly due to the requirement for the whole PRC2 complex for methyltransferase activity to occur (Liu et al., 2010). In addition, the level of H3K27me1 was not affected in *clf swn* double mutants which suggests that H3K27 methylation at centromers is catalyzed by other proteins that are not homologous to E(Z). Recently, Jacob et al. 2009

showed that the divergent SET-domain proteins TRITHORAX RELATED PROTEIN 5 (ATXR5) and ATXR6 are able to catalyze the mono-methylation of H3K27 in *Arabidopsis* (Jacob et al., 2009). Moreover the *atxr5atxr6* double mutant shows partial disruption of constitutive heterochromatin, reduced H3K27me1 around the centromer, and reactivation of silenced genes such as Ta3 and other heterochromatin markers, indicating that both H3K27me1 and H3K9me2 act at common loci (Jacob et al. 2009).

1.7 Dynamic alterations of histone modification profiles in plants subjected to environmental changes

Several studies have indicated a link between epigenetic modifications and exposure of plants to stressful environmental conditions, which could create a stress responsive mechanism that controls gene expression at the chromatin level (Luo et al., 2012).

In tobacco and *Arabidopsis* cells, a change in nucleosome position occurs in response to high salinity and cold stress, accompanied by H3 phospho-acetylation and histone H4 acetylation, and followed by enhanced expression of stress-specific genes (Sokol et al., 2007). In rice seedlings, water submergence induced histone H3K4 tri-methylation and H3 acetylation of alcohol dehydrogenase1 (ADH1) and pyruvate decarboxylase1 (PDC1). As a result the expression level of these two genes was enhanced, but was restored to the basal level when the stress is relieved by re-aeration (Tsuji et al., 2006).

Furthermore, it was shown that K4 tri-methylation and K9 acetylation of the histone H3 was altered under drought stress resulting in gene activation of the affected drought stress-responsive genes (Kim et al., 2008). Both abscisic acid (ABA) and salt stress can induce histone H3K9-K14 acetylation and H3K4 tri-methylation and decrease H3K9 di-methylation of some known ABA and abiotic stress-responsive genes (Chen et al., 2010). , These findings suggest that some functionally related gene groups are regulated co-ordinately through histone modifications in response to abiotic stress in plant cells. Dynamic changes in genome-wide histone H3K4 methylation patterns in response to dehydration stress in *Arabidopsis* were also observed using a ChIP-Seq approach (Van Dijk et al., 2010).

Mutations of histone modifying enzymes also cause altered abiotic stress responses and can influence plant stress tolerance. For example, lack of function of the *Arabidopsis* Trithorax-like Factor (ATX1), which tri-methylates histone H3 at lysine 4 leads to a

phenotype including larger stomatal apertures, increased water loss and hyper-sensitivity to dehydration stress (Ding et al., 2011).

Member of the histone deacetylase (HDAC) family have been found to regulate biotic and abiotic stresses responses. For example, in *Arabidopsis*, knockout/knock-down of histone de-acetylases HDA6 and/or HDA19 makes the plants hypersensitive to ABA and salt stress (Chen and Wu, 2010; Chen et al., 2010). Knockout of HD2C, a member of the plant-specific HD2 histone de-acetylase family that interacts with HDA6, also confers higher sensitivity to ABA and NaCl and altered expression of ABA-dependent genes. Mutation of a putative member of a histone de-acetylase complex, HOS15, caused higher levels of acetylated histones and altered freezing tolerance in *Arabidopsis* (Zhu et al., 2008). In addition to their role in stress responses, histone de-acetylases, as with other histone modification enzymes (see previous sections), are also involved in plant development (Yu et al., 2011; Tian and Chen, 2001; Tanaka et al., 2008). Over-expression of HDA19 led to strong developmental aberrations in *Arabidopsis* (Zhou et al., 2005), and over-expression of histone de-acetylases in maize and rice led to a range of morphological phenotypes (Rossi et al., 2007; Jang et al., 2003). These findings highlight the dilemma of how histone modification act as a process with a fundamental role in determining cell line identity can contribute to dynamic stress signalling without interfering with basal developmental patterns. One possibility is that the targeting of genes is very specific. Another possibility is that changes induced by environmental stimuli are very small compared to the basal patterns determining cell-specific expression.

Tab. 1.1 Chromatin modification and remodelling proteins involved in plant abiotic stress response.

Gene	Histone modification	Stress	Development	Reference
AtHD2C	De-acetylation /catalytic	Salinity/ABA	Not known	Luo et al., 2012
HDA6	De-acetylation /catalytic	Salinity/Cold	Flowering	Chen and Wu, 2010
HDA19	De-acetylation /catalytic	Salinity/ABA	Embryo/Flowering	Chen et al., 2010
HOS15	De-acetylation/ histone-binding	Freezing	Not known	Zhu et al., 2008
ATX1	Methylation /catalytic	Drought	Flowering	Ding et al., 2011

1.8 Aims and methodology of the thesis

The aim of this thesis was to address the question whether and how a transient mild salt priming treatment at an early developmental stage influences the morphological, transcriptional and epigenetic response of plants to a second treatment occurring later in development. To answer this question I used several technical approaches. I first subjected *A. thaliana* plants to a range of potentially effective priming treatments and evaluated differences in stress tolerance as well as transcriptional responses of known salt stress-marker genes between primed and non-primed plants. After developing an optimised priming protocol, I determined genome-wide transcriptional profiles at various stages of the protocol (24h after priming and 10 days after priming) by microarray analysis and RNA-sequencing.

Secondly, I analysed epigenetic profiles of four histone modifications (H3K4me2-me3, H3K27me3 and H3K9me2) 24h after priming and 10 days after priming. These marks are stably transmitted through mitosis but their potential role in long-term somatic memory of abiotic stress in plants was unknown. In addition, H3K27me3 and H3K9me2 were known to be enriched in transcriptionally repressed genes and transposons while H3K4me3 and H3K4me2 were known to be enriched in transcriptionally active genes; in addition a previous study showed that H3K4me2 and H3K4me3 were associated with dehydration-responsive genes, (Kim et al., 2008; Pfluger and Wagner, 2007). For this purpose, DNA samples obtained both 24h after the priming treatment and 10 days after the second treatment by Chromatin Immuno-Precipitation (ChIP) with antibodies against H3K4me2, H3K4me3, H3K9me2, H3K27me3 were subjected to next generation sequencing (Illumina, ChIP-Seq). The large datasets obtained were analysed with a range of statistical methods to separate random noise from priming/stress induced changes. Further data mining and quantitative analyses were subsequently carried out to better understand the biological and molecular events associated with salt priming.

Chapter 2: Materials and methods

2.1 Materials

Except where otherwise stated all chemicals were purchased from Sigma (Pool, UK) or Fisher Scientific (Southampton UK). Custom made primers were purchased from Invitrogen and are listed in Appendix I. Sources of all equipment are given in the text. List of solutions, antibodies and primers referred to in the text are given in the following sections.

2.2 Plant materials and growth conditions

Growth of plants on agar plates:

For growth on agar plates *Arabidopsis thaliana* (ecotype Columbia 0) seeds were first surfaced sterilized. The seeds were placed in a 1.5 ml centrifuge tube with 1 ml of absolute ethanol for 1 minute, and mixed by inversion. The ethanol was then replaced with 1 ml of mild bleach solution (2.5% sodium hypochlorite solution and 0.01% Tween20), left to incubate for 5 minutes and occasionally mixed by inversion. Seeds were washed five times in sterile distilled water and incubated in the dark at 4°C for two days for stratification. To prepare the solid medium for plates, concentrated stocks of each reagent listed in Table 2.1 were mixed and diluted to 1x concentration with distilled water. Sucrose was added to a concentration of 3% and pH was adjusted to 5.6 with 0.1 M NaOH, and lastly 1 % agar was added before autoclaving at 121 °C for 20 minutes. The medium was dispensed into square (120 x 70 mm, 70 ml per plate) tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) using a titration pipette. Ten seeds were individually sown on sterile nylon mesh using a pipette on the surface of the solidified medium in a horizontal line approximately 2 cm from the top of the plate. Plates were sealed with Parafilm and placed vertically in a controlled growth chamber (10 h light/14 h dark photoperiod, white fluorescent tubes L36W/30 with light intensity 120 µmol $m^{-2}s^{-1}$, temperature 21 °C).

Growth of plants in hydroponic culture:

To prepare the liquid nutrient medium, concentrated stocks of each reagent listed in Table 2.1 were mixed and diluted to 1x concentration with distilled water. Three weeks old seedlings (4 true leaves) were carefully peeled from Petri dishes and transferred to a dark-taped 800-mL hydroponic container (dimension: 17 x 11 x 5.5 cm) containing the nutrient medium. The containers were placed in a controlled growth room under a propagator (10 h light/14 h dark photoperiod, light intensity approx 120 µmol $m^{-2}s^{-1}$, 21 °C) and the plants were generously sprayed with double distilled water to avoid desiccation. Plants were sprayed with water every two days and after five days the propagator was removed.

Tab. 2.1 Growth medium used for plates and hydroponic culture.

Control Minimal Medium, (pH= 5.6)	
Reagent:	Final concentration 1x
KNO_3	1.25 mM
$MgSO_4$ $7H_2O$	0.5 mM
KH_2PO_4	0.625 mM
$Ca(NO_3)_2$ $4H_2O$	0.5 mM
FeNaEDTA	42.5 mM
Micronutrients:	1x
$CuSO_4$ 0.25mM, $ZnSO_4$ 0.68mM, $MnSO_4$ 2mM, H_3BO_3 45.3mM	
NaCl	2mM

2.3 Determination of tissue ion content

The sodium content in plant tissue was determined as described by Wang et al., 2006. Four plants (primed and non-primed) were harvested individually at 0, 2, 4, 8 and 24 h after salt treatment. Roots and shoots were separated and dried to determine dry weights before ion content analysis. Dried tissues were incubated overnight in 2 M HCl (1:100 w:v), diluted 50 times with double-distilled H_2O, and analysed by flame photometry using an 410 flame photometer (Sherwood-Scientific Ltd, UK).

2.4 Plant treatments and sampling

Plants were subjected to a transient mild salt treatment ("priming treatment) early in their life and to a second treatment ("salt treatment") after an extensive period of growth (Fig.2.1) at the same time of the day.

Priming treatment:

Priming treatment was applied to three weeks old plants grown on solid media in Petri dishes: plates were opened under a sterile hood and treated with priming solution (sterile liquid medium with addition of 2 mM, 50mM or 100mM NaCl). 5 ml of priming solution were dispensed on the roots using a titration pipette; the plates were then sealed with Parafilm and placed again in the controlled growth room. After 24 hours the nylon mesh with the seedlings was carefully transferred to a fresh new plate containing sterile agar medium without additional salt. Plants were then left to recover for three days before transferring to hydroponics

Salt treatment:

Ten days after the priming treatment plants growing in hydroponic medium received a second salt treatment by addition of granular NaCl to the medium to a final concentration of 80 mM. No salt was added to control plants but they were subjected to the same mechanical treatment (opening of lid and stirring).

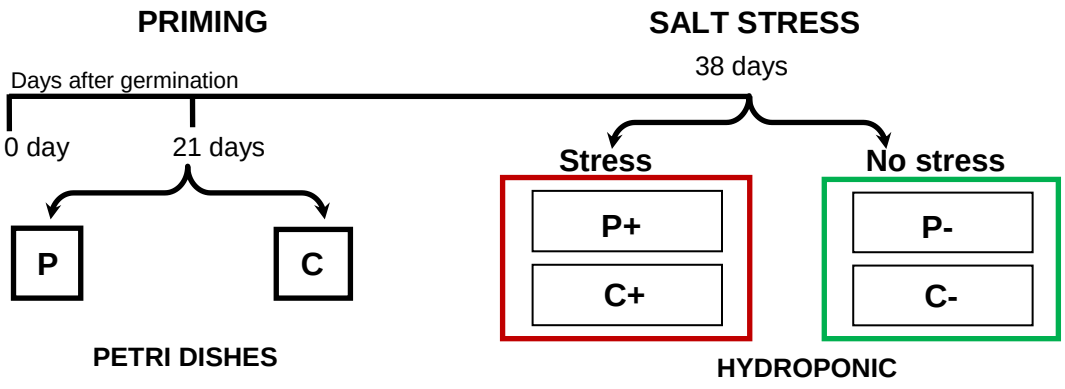


Fig. 2.1 Plant treatments and sample harvesting schematically summarized.
Plants were treated (red box) or not (green box) with salt. On the left: 24h priming treatment on Petri dishes. P: plants primed, C: plants not primed. On the right: salt treatment in hydroponics. P+: plants primed and salt treated, C+: plants not primed and salt treated. P-: plants primed but not salt treated, C-: plants not primed and not salt treated.

Sample harvesting:

Plants were harvested at given time after the treatments, separated into shoots and roots samples and frozen for RNA or DNA extraction or immediately fixed in formaldehyde for isolation of chromatin as described in the following sections.

RNA-sequencing samples

Three week old plants (4 leaves stage) were primed (P) or not (C) with 50mM NaCl for 24h, samples were then separated into shoots and roots and total RNA was extracted. 30 plants have been used for each condition and three independent experiments were made and pooled together.

Microarray samples

Three week old plants (4 leaves stage) were primed (P) or not (C) with 50 mM NaCl for 24h and 10 days later exposed to 80 mM salt treatment (+) or not (-); samples were then separated into shoots and roots and RNA was extracted. 15 plants have been used for each condition and three independent experiments were carried out.

2.5 RNA extraction and cDNA synthesis

RNA extraction:

Total RNA was extracted from A. *thaliana* tissue using Trizol solution (0.8 M Guanidinium Thiocyanate, 0.4 M Ammonium thiocyanate, 0.1 M NaAc pH 5, 5% Glycerol, 38% Phenol saturated in water pH 4.5). Frozen tissue was ground to powder using Mixer Mill MM 300 (Qiagen, Valencia, CA, USA) repeating three cycles of one minute at 25 Hz. The material was kept frozen in liquid nitrogen between the cycles. 1.4 ml of Trizol solution was then added to the frozen powder in a 2 ml centrifuge tube and the mixture was vortexed for one minute and left standing at room temperature for 5 minutes.

Following centrifugation at 10000 x g for 2 minutes at 4 °C, 1.4 ml of supernatant was transferred to a new 2 ml centrifuge tube containing 300 µl of Chloroform-Isoamylic (24 Vol Chloroform, 1 Vol Alcohol Isoamylic). The solution was vortexed for one minute and incubated at room temperature for 2 minutes, then centrifuged at 12000 x g at 4 °C for 10 minutes producing two phases. 750 µl of the upper phase was transferred to a new 2 ml centrifuge tube containing 200 µl of Chloroform-Isoamylic. Following another centrifugation at 12000 x g at 4 °C for ten minutes, 750 µl of the upper phase was added

into a new 1.5 ml tube containing 750 µl of isopropanol. The solution was mixed by inversion and the RNA was left to precipitate at -20 °C for 30 minutes. The tube was then centrifuged at 12000 x g for 20 minutes at 4 °C to produce a pellet. The pellet was washed with 1 ml 70% ethanol centrifuged at 4 °C for 5 minute 12000 x g and washed again with 1 ml 70% ethanol and centrifuged at 4 °C for 5 minutes 12000 x g. The supernatant was discarded and the pellet was dried in a speed vacuum centrifuge (Eppendorf) for 10 minutes before being re-suspended in 30 µl RNase-free water.

Total RNA used for Microarray analysis and RNA-sequencing was extracted and isolated with RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

RNA quantity was determined spectrophotometrically using an Eppendorf spectrophotometer. Absorbance readings were normalized to RNase-free water. RNA quality was checked by agarose gel electrophoresis. All the materials (gel tank, combs, and electrophoresis apparatus) were pre-treated with NaOH 0.1 M for 30 minutes. 2 µl of 6x loading buffer (0.25 % orange G, 40 % sucrose) was added to 5 µl of RNA and loaded on a 1.5 % agarose, 1% TAE gel, Ethidium bromide. Bands were separated at 100 V for 25 minutes and then visualized under UV light using a Gel Doc scanner (Bio-rad).

To avoid contamination with genomic DNA, total RNA was treated with DNA-free Kit (Ambion) according to the manufacturer’s instructions. Efficiency of the DNase treatment was tested on agarose gel electrophoresis and then RT-PCR with primers specifically either designed spanning an intron region or either sides of an intron region.

RNA for microarray and RNA sequencing were tested for quality using Agilent® 2100 Bioanalyzer™ (Fig.2.2).

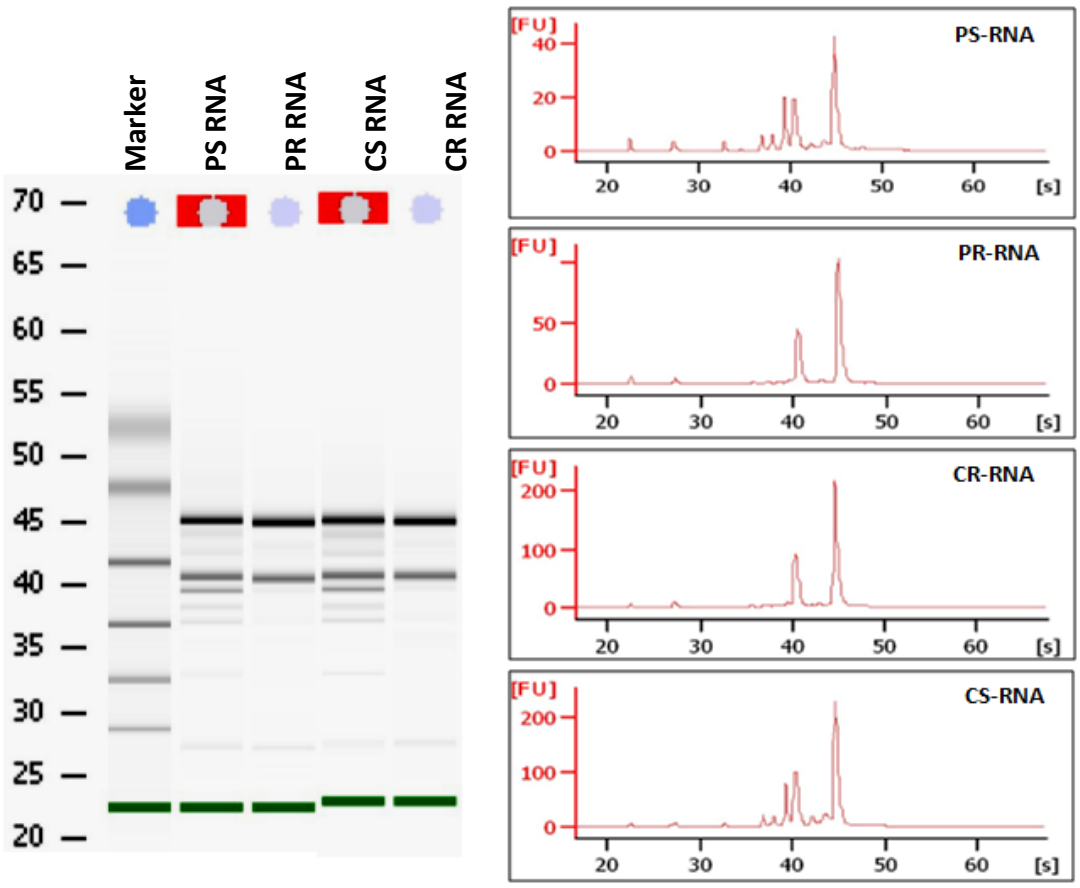


Fig. 2.2 RNA quality check performed for RNA-Seq and Microarray samples. Bioanalyzer profiles for RNA-seq: capillary electropheresis profile (left) and spectrophotometer profile (right). (C: control, P: priming, S: shoots, R: roots)

cDNA synthesis:

cDNA for PCR was synthesized using QuantiTect® Reverse Transcription kit (Qiagen, Valencia, CA, USA). 1 µg of total RNA was used to carry out the first-strand synthesis of cDNA according to the manufacturer's instructions. The RNA was added to 2 µl of gDNA Wipeout Buffer 7 x and RNase-free water up to a final volume of 14 µl. After incubation for 2 minutes at 42 °C the tubes were put on ice and the following mix was added: 1 µl Quantiscript Reverse Transcriptase, 4 µl Quantiscript RT Buffer (5x), 1 µl RT Primer. The tubes were incubated for 15 min at 42°C to allow reverse transcription and finally incubated for 3 min at 95 °C to inactivate reverse transcriptase.

2.6 Polymerase chain reaction (PCR) and microarray hybridisation

2.6.1 Standard PCR

Standard PCR mixes (final volume 25 µl) were set up as shown in Tab. 2.2. Template was genomic DNA or cDNA. Stated cycling conditions were as follows: 1 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and 7 min at 72 °C. Primer annealing temperature and cycle numbers were adjusted as appropriate for each reaction. PCR products were added to 5 µl of 6x loading buffer (0.25 % orange G, 40 % sucrose) and resolved by agarose gel electrophoresis loaded on a 1.5 % agarose, 1 % TAE gel, containing Ethidium bromide. Bands were separated at 100 V for 15 minutes and then visualized under UV light using a Gel Doc scanner (Bio-rad). If necessary, bands were extracted using a QIAquick gel extraction kit (Quiagen) according to the manufacture instructions. DNA was eluted from QIAquick columns using 30 µl of the supplied elution buffer. DNA concentration was determined spectrophotometrically.

Tab. 2.2 Standard reaction mix for PCR.

Component	Concentration	Volume (µl)
5x buffer	1 x	5
dNTP mix (10mM each of dATP, dCTP, dGTP, dTTP)	0.2 mM each	0.5
Forward primer (10 µM)	0.1 µM	0.5
Reverse primer (10 µM)	0.1 µM	0.5
Taq Polymerase (5units/ µl)		0.15
Distilled water	-	variable
Template	variable	

2.6.2 Quantitative PCR

Quantitative PCR was performed in 96-well plates using Mx3000 Stratagene real-time PCR system, and the Brilliant III SYBR Green qPCR kit (Stratagene). SYBR green fluorescence was measured every PCR cycle at the end of the annealing step. As SYBR green fluorescence is greatly increased in the presence of double stranded DNA, product accumulation can be monitored in real time. For each reaction, the Ct (defined as the number of cycles required to reach the threshold fluorescence or the point at which the

fluorescence can be accurately related to initial template quantity) was calculated automatically using Stratagene MX software. Cycling conditions were as follows: 3 min at 95 °C, 40 cycles of 10 s at 95 °C, 20 s at 60 °C, followed by a 60–95 °C dissociation protocol to verify the amplification of a single PCR product. Primers were designed to amplify 100–250 bp products and the primer sequences are given in Appendix I. Target concentration was quantified by comparing its amplification to that of standards of known concentration. Standards consisted of gel-purified PCR products identical to the intended amplicon. They were produced by performing standard PCR on a cDNA sample using the same primers as used for qPCR. PCR products were gel purified and DNA concentration determined spectrophotometrically. Purified PCR products were first adjusted to 10 pg/µl and then further diluted by ten-fold serial dilutions to produce six different standards (ranging from 10 pg/µl to 10^{-4} pg/µl). A standard curve was produced by plotting the Ct values obtained from qPCR for each standard dilution against the logaritm of the initial template quantity. Initial target quantity in cDNA samples was determined by comparing Ct values to this standard curve. Each reaction was performed in duplicate.

As a control for variation in RNA quantification, reverse transcription efficiency and template preparation, the template quantify each genes was expressed relative to the reference gene. Several conventionally used reference genes were evaluated for their stability under our experimental conditions using geNorm (Vandesompele et al., 2002). Based on the results, the gene RpII (At4g26410) was selected for use as internal reference for transcripts.

For determination of enrichment of target sequences in ChIP samples Input samples were adjusted considering that 10% was immune-precipitated and the follow formula was used:

$[(IP_{target}/(10*Input_{target})]/ [(IP_{ref}/(10*Input_{ref})]$

Enrichment for H3K27 tri-methylation was determined using AT5G56920 as a reference gene, while enrichment for H3K4 di- and tri- methylation and was determined using AT1G24560 as a reference with primers designed into different position of the gene.

2.7 Transcriptome profiling by RNA-sequencing and microarray

For RNA sequencing 10 µg of quality checked total RNA was subjected to Illumina sequencing by Polyomics Facility-University of Glasgow, using protocol for provided by Illumina.

10 µg of quality checked total RNA were used for microarray analysis. The procedures of labelling and hybridizing of the RNA to the Affymetrix® GeneChip™ ATH1 were carried out according to manufacture protocols by the Polyomics Facility- University of Glasgow.

2.8 Isolation and immune-precipitation of chromatin (ChIP)

The protocol used for Chromatin Immuno-Precipitation (ChIP) in this study is a modified version of the protocol described by Bowler et al., 2004. An overview is shown in Fig.2.4.

2.8.1 Tissue fixation and nuclei isolation

Approximately 4 g of plant tissue was harvested, separate into shoots and roots samples and fixed in 30 ml of 1% (w/v) formaldehyde for 15 min under a vacuum. Cross-linking was stopped by adding 125 mM glycine and the vacuum was re-applied for another 5 min. The tissue was rinsed twice with distilled water, blotted dry and flash frozen in liquid nitrogen. The tissues were ground in liquid nitrogen to obtain a fine powder and the powder was re-suspended in 30 ml of buffer containing 0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM PMSF and one protease inhibitor mix tablet (Complete Mini, Roche). The homogenate was then filtered through two layers of Miracloth and centrifuged for 20 min at 4,000 g. The pellets were re-suspended in a 1 ml of buffer containing 0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitor, followed by a 10-min centrifugation at 12,000 g. The pellet was resuspended in 1 ml of buffer containing 1.7 M sucrose, 10 mM Tris-HCl pH 8, 0.15% (v/v) Triton X-100, 2 mM $MgCl_2$, 5 mM β−mercaptoethanol, 0.1 mM PMSF and protease inhibitor and was deposited on a layer of 1ml of the same buffer followed by centrifugation for 1 hour at 16,000 g. The pellet

was resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% (w/v) SDS and protease inhibitor).

2.8.2 Sonication

In order to break the chromatin into fragments of approximately 500 base pairs, the re-suspended pellets were fragmented for six 15 sec pulses using a sonicator (Sanyo-Soniprep 150) with an output of 10 - 15% and centrifuged for 10 min at 16,000 g. To assure the correct fragmentation 1 µl of sheared chromatin was added to 1 µl of 6x loading buffer (0.25 % orange G, 40 % sucrose) and loaded on a 1.5 % agarose, 1 % TAE gel, Ethidium bromide. Bands were separated at 100 V for 15 minutes and then visualized under UV light using a Gel Doc scanner (Bio-rad) (Fig.2.3).

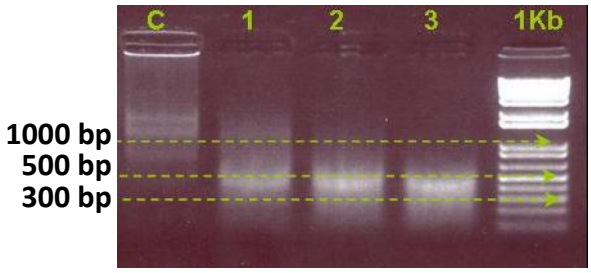


Fig. 2.3 Separation of chromatin on agarose gel.
C: chromatin not sonicated, 1: chromatin sonicated for 4 pulses of 15-sec, 2: chromatin sonicated for five pulses of 15-sec, 3: chromatin sonicated for six pulses for 15-sec (optimal fragmentation length), 1kb: molecular marker.

2.8.3 Immuno-precipitation

100 µl of sheared chromatin was diluted in 900 µl ChIP dilution buffer (1.1 % (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl).The chromatin-enriched solution was pre-cleared with 25 µl of protein A Dynabeads (Invitrogen, Carlsbad, CA), at 4 ºC constant rotation for 1 hour. Protein A beads were collected using a magnet and the pre-cleared chromatin was incubated overnight at 4 °C with antibodies for dimethylated H3K4 (6 µL, Diagenode pAb-035-050 Liège-Belgium), trimethylated H3K4 (1 µL, Diagenode pAb-003-050, Liège-Belgium), dimethylated H3K9 (2 µL, 17-681, Millipore, Billerica, MA), trimethylated H3K27 (2 µL, Diagenode, pAb-069-050, Liège-Belgium). A sample without antibody addition was also used as a mock control. Next the antibody/chromatin complex

was precipitated by incubating the mix with 50 µl of protein A Dynabeads for 2 h: this step allowed the binding of the antibody/chromatin complex to the magnetic beads for separation using a magnet. The immunoprecipitated chromatin and the associated proteins were washed at 4 ºC constant rotating with 1 ml of each of the following buffers: low salt buffer (150 mM NaCl, 0.1 % SDS, 1 % TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1), high salt wash buffer (500 mM NaCl, 0.1 % SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1), LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), TE wash buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA). The beads were washed, with some modifications depending on which antibody was used: for anti- H3K4me2- and H3K4me3 beads were washed twice for 10 minutes incubation at 4 ºC, for H3K9me2 once for 5 minutes, for H3K27 twice for 5 minutes.

2.8.4 Reverse cross-linking and DNA purification

The chromatin was eluted and reverse cross-linked in one step by overnight incubation at 65 ºC in presence of 500 µl elution buffer (1% (w/v) SDS, 0.1 M$NaHCO_3$, 0.2 M NaCl). The eluted antibody/chromatin complex was separated from the beads using a magnet.
Residual proteins were removed by Proteinase K (20 µg ml^{-1}) treatment and DNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 1 µl of Glicogen. The purified DNA pellets were resuspended in 50 µl of Tris-EDTA pH 8 and subsequently purified using a Qiagene Mini Elute column following the manufactures instructions.

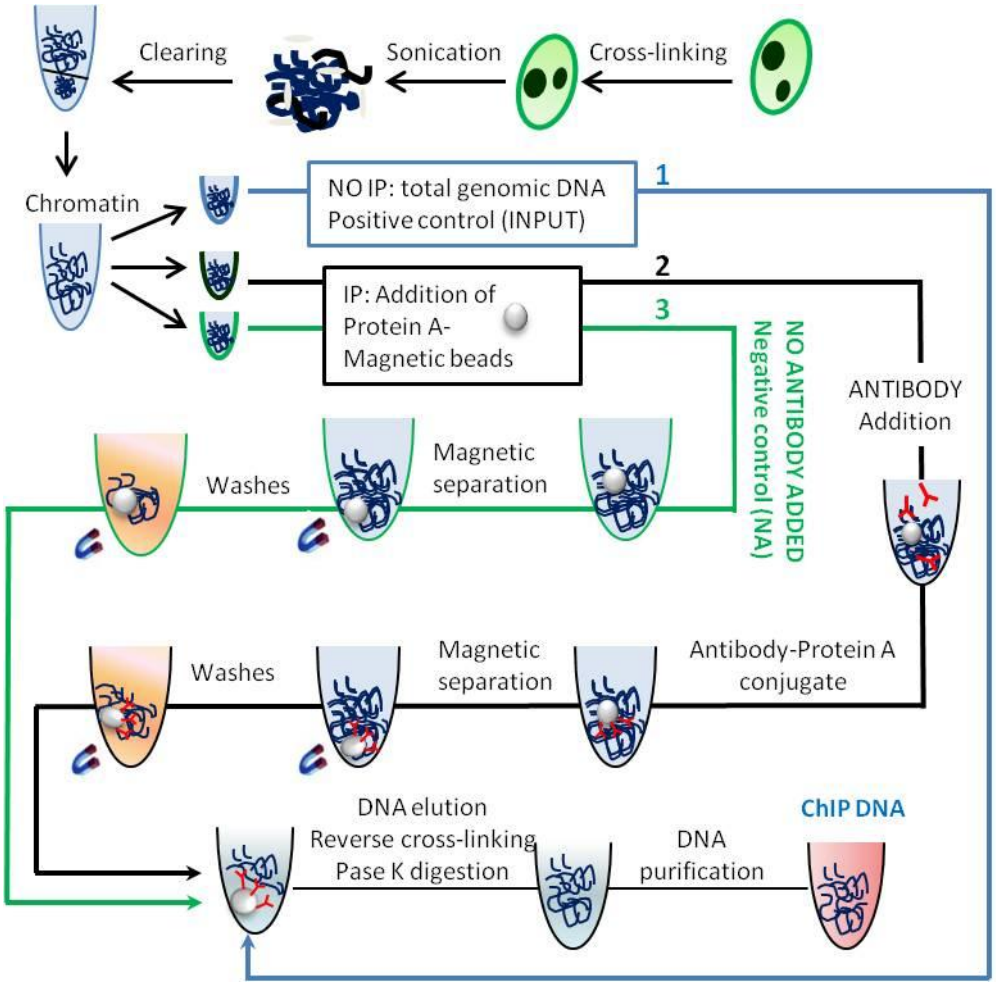


Fig. 2.4 ChIP protocol overview.
After cross-linking of chromatin and DNA by formaldehyde fixation (in fresh plant material), nuclei are isolated and the chromatin is extracted and fragmented to the right size by sonication. An input sample (1) is collected immediately after the sonication and will represent the positive control. The rest of the samples are split in two reactions: a sample (2) is immune-precipitated with the specific antibody (i.e. H3K4me2) and one sample (3) is used as a negative control without addition of antibody. DNA is isolated and purified from the proteins by reverse cross-linking, proteinase (Pase K) treatment and phenol/chloroform extraction.

2.9 Whole genome linear amplification

For qPCR reactions, ChIP DNA samples and Inputs were linearly amplified using GenomePlex Complete Whole Genome Amplification (WGA2) (Sigma-Aaldrich). The reaction was carried out following the manufacture's instructions with the only exception being that no DNA fragmentation was carried over because the samples had previously been sonicated. The kit utilizes an amplification technology based on random fragmentation of genomic DNA and conversion of the resulting small fragments to PCR-amplifiable library molecules flanked by universal priming sites. The library is then PCR amplified using universal oligonucleotide primers and a limited number of cycles.

Library was prepared by adding 1 µL of Library Stabilization Solution and 2 µL of 1x Library Preparation Buffer to 20 µL of each sample. The mix was placed in a thermal cycler at 95 °C for 2 minutes. Samples were cooled on ice, consolidated by centrifugation, and returned to ice where 1 µL of Library Preparation Enzyme was added. Samples were placed in a thermal cycler and incubated as follows: 16 °C for 20 mins, 24 °C for 20 mins, 37 °C for 20 mins, 75 °C for 5 mins, 4 °C hold. Linear Amplification was prepared by adding the following reagents to the 15 µL reactions from the previous step: 7.5 µL of 10X Amplification Master Mix, 47.5 µL of water, 5 µL of WGA DNA Polymerase. Samples were vortexed thoroughly, centrifuged briefly, then placed in a thermal cycler and incubated for Initial Denaturation at 95 °C for 3 minutes. Then 14 cycles were performed as follows: Denature at 94 °C for 15 seconds, Anneal/Extend at 65 °C for 5 minutes. The amplified samples were used for PCR (section 2.6).

Chapter 3: Physiological analysis of priming

3.1 Introduction

3.1.1 Background

If at the beginning of the season plant seedlings experienced a moderate abiotic stress, the mature plants often show an enhanced tolerance when the stress condition reoccurs. This phenomenon, known as priming, has often been reported by farmers, breeders and gardeners. However, the reports are mostly anecdotal and the effect lacks quantification and still awaits confirmation through rigorous scientific testing. As described in the general Introduction (Chapter 1.3) several studies have shown that prior application of either a biotic stress or a pathogen elicitor enables plants to respond quicker or stronger to a subsequent pathogen infection (Tsai et al., 2011; Van Hulten et al., 2006; Worrall et al., 2012; Ton et al., 2009; Conrath et al., 2006, 2001; Conrath, 2011; Kravchuk et al., 2011; Heil and Kost, 2006). Other studies have found that pre-treatment of plants with an abiotic stress reduced the damage caused if the same stress reoccurred (Cayuela et al., 1996; Jakab et al., 2005; Schwember and Bradford, 2010; Sedghi et al., 2010; Nawaz et al., 2011; Patade et al., 2009; Farooq et al., 2008). However, in all of these studies the priming effect was only investigated on seeds and the molecular basis of this phenomenon was not explored and thus the underlying mechanisms remain still unknown. In this first part of the project the effect of an abiotic priming treatment on the response of *A. thaliana* plants to a later abiotic stress was investigated systematically. The aim was to optimise the experimental design for subsequent exploration of the molecular processes underlying abiotic priming of plants.

3.1.2 Aims of the chapter

In this chapter I investigated the effects of different abiotic priming treatments on the physiology of the model organism *Arabidopsis thaliana*. In particular, I focused on the effect of salt priming applied at different key-stages (seeds, one day after germination, 2 true leaves, 4 true leaves) of development of *Arabidopsis*. I will describe how these observations influenced the choice of the final experimental design that I used in all further experiments presented in this thesis.

3.1.3 Systematic optimization of an experimental protocol to test the effect of priming on stress responses of *A. thaliana*

The optimization of a priming protocol requires consideration of many different factors (Fig. 3.1). Clearly not all of these can be tested experimentally in all combinations. The optimization of the protocol was therefore based on a combination of existing knowledge, informed guess and experimental testing.

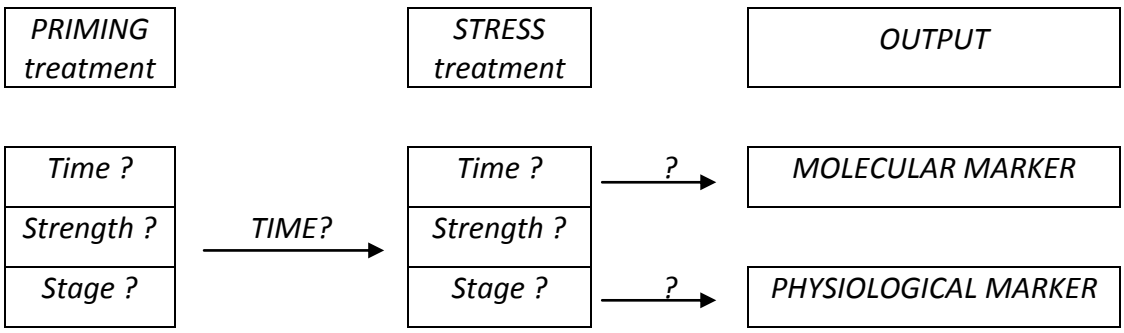


Fig. 3.1 Schematic illustration of the factors that need to be considered in the experimental design.

Factors that are expected to determine the outcome of a priming experiment are the following: biological system (plant species, developmental stage), growth system (plates, hydroponics, soil), type of abiotic stress to be investigated (salt, osmotic, drought), strength and duration of the priming treatment, length of period between priming and (second) stress treatment, strength and duration of (second) stress treatment. In addition, decisions had to be taken on how to measure the outcome of the experiment (physiological and molecular marker). In this section I present the individual factors

together with a reasoning of choices made to narrow down the number and the range of conditions that were tested.

- Strength of treatments

The selection of the priming/stress treatment strength was made taking into consideration the salt sensitive nature of *A. thaliana*, which prevents the plants from completing their life cycle if continuously exposed to 100 mM NaCl (Munns and Tester, 2008). This indicates that a harsh priming treatment could severely damage the plant, leaving a choice between applying a solution with a high concentration of salt for a short period of time, or a solution with a lower concentration for a longer time. It was decided to test a concentration range of 20-100 mM NaCl.

- Length of treatments

From the literature it is known that the response to salt stress in plants is rapid. The vast majority of transcriptional changes are detectable after only 30 minutes and are completed after 24 hours (Kilian et al., 2007). Therefore, considering this time frame it is possible to conclude that a 24 hour treatment is a good informed guess. It should be long enough to induce a strong response to the salt but not long enough to change the morphology of the plants.

- Time between treatments

Another essential parameter to be considered is the time that elapses between the priming and the stress treatment. The purpose of the experiment was to leave the plants growing for an extensive period of time, during which the plants have gone through numerous cell divisions but without switching from vegetative growth to reproductive phase. In fact after this switch, the cell differentiation profile changes completely from leaves to flower and consequently all the patterns of gene expression change as well. An informed guess of 10 days was made. This period of growth should be long enough to

ensure that transcripts and other molecular entities transiently induced by the priming treatment are substantially diluted and/or turned over.

- Choice of species

A. thaliana is a widely used model plant. A large body of information already exists on its transcriptional responses to stress and also some epigenetic studies are avaible .

- Stage of development

It is also important to establish at which developmental stage plants are most responsive to the priming/stress treatment, bearing in mind the practical amount of material that further analysis required. It is likely that priming has a different effect depending on the stage of development when it is applied. Thus, priming of seeds could have a different effect from priming seedlings or mature plants due to development specific gene expression or structural properties (Catusse et al., 2008). Here, four different key stages of development (seed, one day after germination-G0, two real leaves-2L and four real leaves-4L), were considered for the priming treatment.

- Evaluation method to test the effectiveness of the treatment

The final outcome of the experiment was difficult to establish because the priming treatment did not result in a clearly visible phenotype. Hence, a molecular marker was needed. Two candidate responsive genes (rd29A and P5C1S) were selected on the basis of previously published transcriptional responses to high salinity stress (Yoshiba et al., 1999; Yamaguchi-Shinozaki and Shinozaki, 1993). However, the choice of a few individual molecular markers without knowing if they are targets of priming treatment is problematic. For this reason a wider approach using microarrays was also taken.

3.2 Results

3.2.1 Experimental design

Figure 3.2 details the experimental protocol designed on the basis of the reasoning given in the previous section.

Four different developmental stages of *A. thaliana* (seed, one day after germination-G0, two real leaves-2L and four real leaves-4L), were subjected to the priming treatments, one moderately harmful (50 mM) and one rather critical (100 mM). The salt priming solution was left in direct contact with the roots (or seeds) for 24 hours. Non-primed control plants were treated with 0 mM NaCl. After 24 hours both primed and non-primed plants were transferred to new plates without the salt solution. The plants were then grown until they reached the 4L stage (three weeks), and then individually transferred to hydroponic culture. The second salt stress was provided 10 days after priming treatment occurred, by adding granular salt directly to the hydroponic media to reach the final concentration of 80 mM.

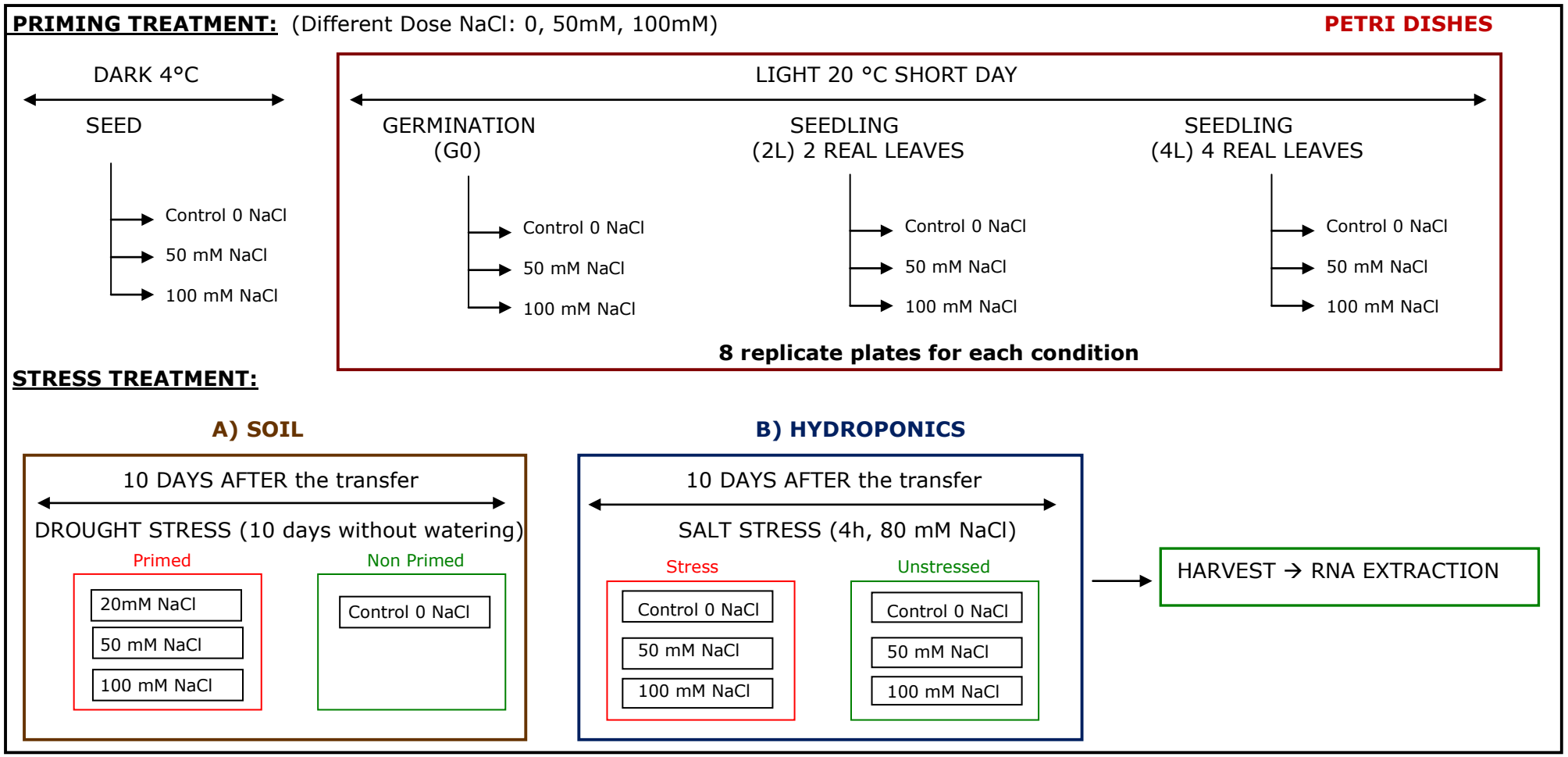


Fig. 3.2 Optimisation of the salt priming protocol.

Three concentrations of salt (0 mM, 50 mM and 100 mM) were tested as priming treatments at four different stages of development (seed, G0, 2L, 4L) of *A. thaliana* seedlings sown on petri dishes. Plants were left to recover and grown for another 10 days before being subjected to different experimental condition: A) Plants were transferred to soil and left to dry for 10 days before visual inspection for phenotypical differences. B) Plants were transferred to hydroponics, where an 80 mM salt stress was applied for 4 hours. Samples were harvested and the RNA was isolated for further analysis.

3.2.2 Effect of priming before second stress

To investigate a somatic memory that is not simply due to differences in growth or development caused by the priming treatment, it was important that the priming treatment had no effect on the plant morphology. In accordance with this Fig. 3.3 shows that there was no delay in growth or development of plants that had been primed with 50 mM NaCl.

Two days (upper panel Fig.3.3) or ten days (lower panel Fig.3.3) after the transfer from plates to the hydroponics culture, there were no visible differences in phenotype between primed plants and non-primed plants. Thus, at the time when the second stress was applied (lower panel Fig.3.3) the primed plants were morphologically indistinguishable from non-primed plants. The same was true for plants primed at other developmental stages with the same or lower concentrations of NaCl (data not shown). However, plants primed with 100 mM NaCl sometimes showed slower growth (see for example Fig.3.4)

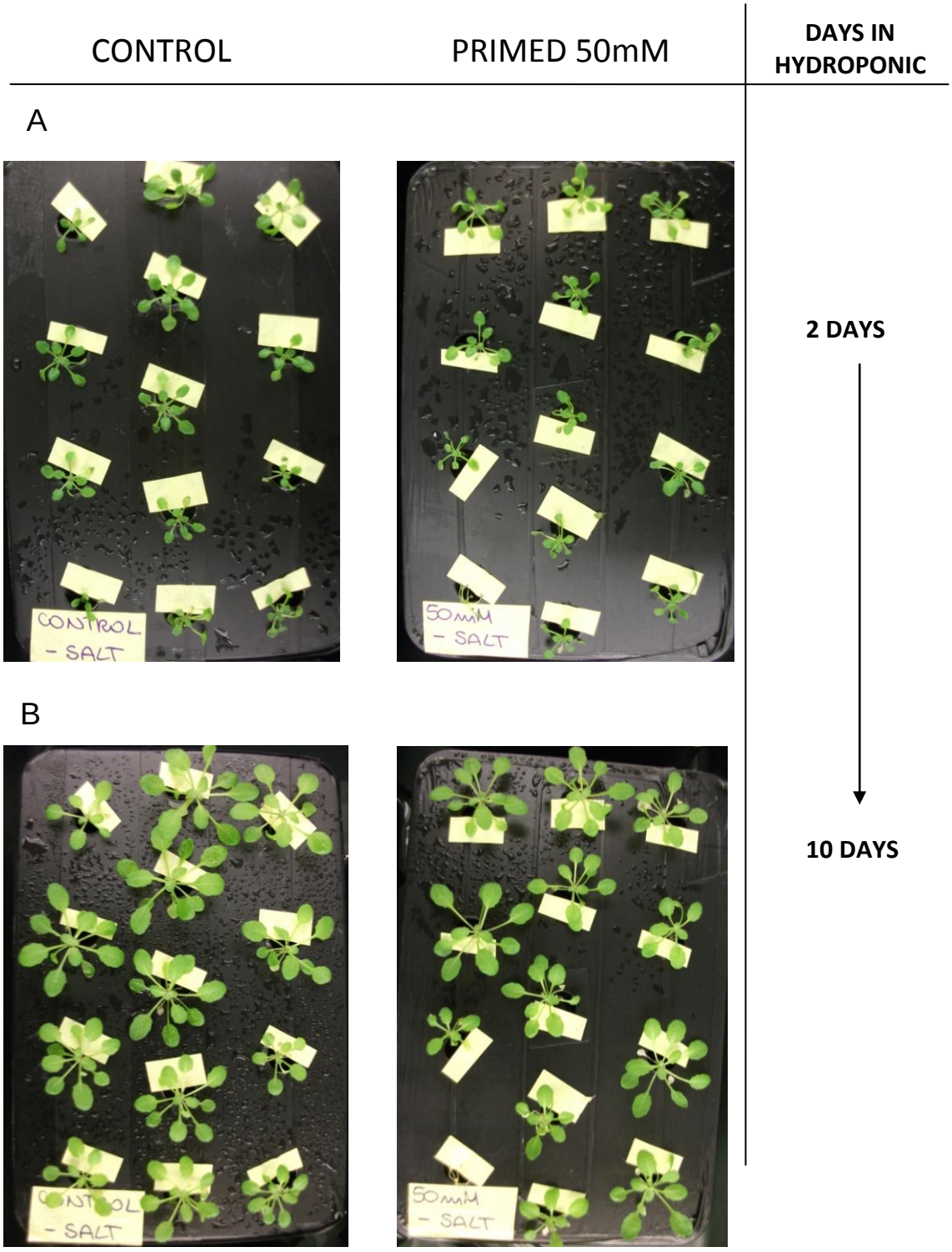


Fig. 3.3 Effect of salt priming on plant growth.
In A are shown plants that had been primed at 4L stage with 50 mM salt for 24h or not (control), two days after transfer from plates to hydroponics. In B are shown plants that had been primed at 4L stage with 50 mM salt for 24h or not (control), ten days after transfer from plates to hydroponics, the day when the salt stress was applied.

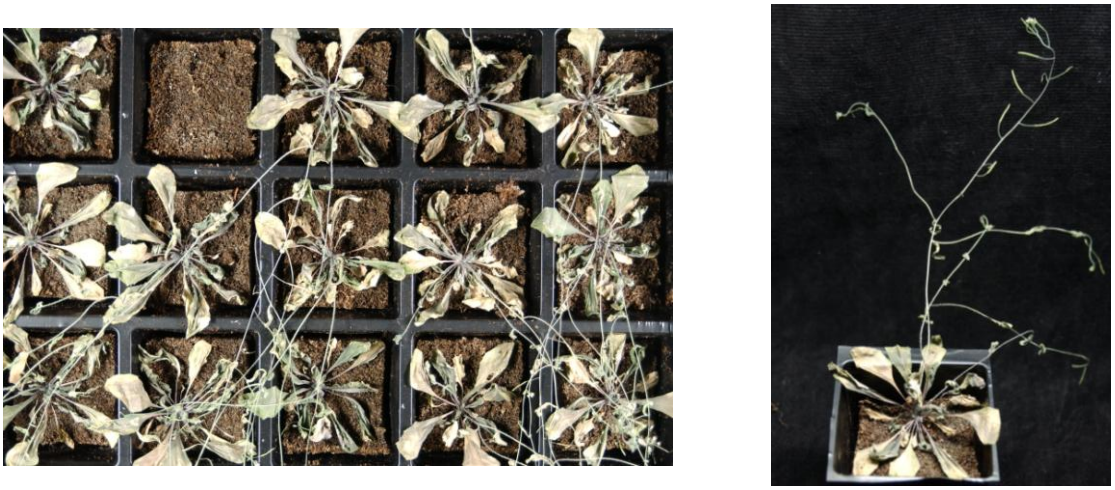
3.2.3 Effect of priming on plant drought tolerance

As discussed in the general Introduction, treatment of plants with salt for a short period of time induces osmotic stress rather than sodium toxicity. It is therefore reasonable to speculate that a 24h salt priming treatment could protect the plant against osmotic stress e.g. drought. Moreover, several studies indicate that, at the beginning of a stress, plants activate non-specific protective responses independently of the nature of the stress applied (Golldack et al., 2011; Mittler, 2006; Kilian et al., 2007).

To investigate this hypothesis in a first experiment *A. thaliana* plants were grown under long-day conditions (16 h light/8 h dark) for three weeks at 22 °C in a growth chamber and were primed on plates for 24 h with different concentrations of salt (0, 50, 100 mM). The day after, 30 plants were transferred to soil in two trays containing 15 plants each, and then watered for 10 days. Plants were then transferred into a greenhouse and left to dry for 10 days. Visual inspection showed clear differences between primed and non-primed plants (Fig.3.4). Primed plants survived the drought stress while non-primed plants were dead. To assess whether the priming effect was dependent on other environmental factors an additional experiment was carried out.

10 days after the salt priming, all plants (including non-primed) were exposed to a heat stress of 38 °C for 6 and 24 hours. Plants were watered once after the heat treatment and then left to dry. The plants coped generally better with the drought stress than the plants in the previous experiments that had not been exposed to a short period of heat stress. Nevertheless, the difference between primed and non-primed plants was still apparent. Fresh weight, dry weight and rosette area determined 10 days after the last watering were always greater in primed plants than non-primed plants, and primed plants looked overall healthier than non-primed plants (Fig. 3.5). However, the length of the heat treatment modified the sensitivity of the plants to the salt concentration applied during priming. The strongest effect was obtained with 50 mM NaCl-priming and at 6h heat treatment.

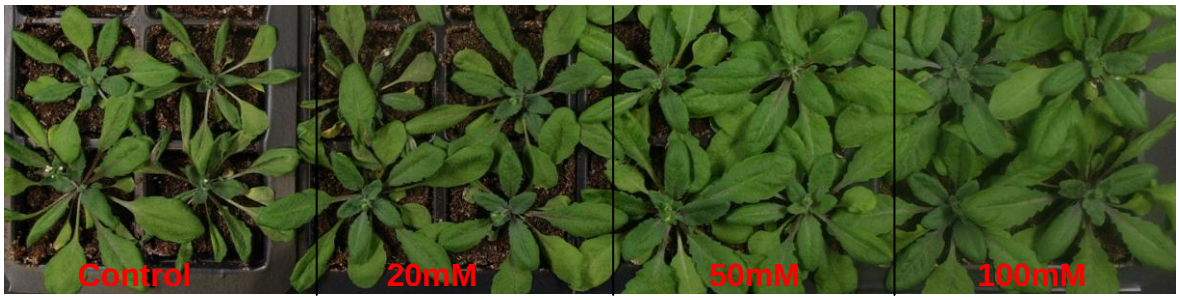
Non-primed



50 mM primed

100 mM primed

Fig. 3.4 Effect of drought stress on primed and non-primed plants.
Control (non-primed) plants (upper panel), 50 mM NaCl-primed plants (central panel) and 100 mM NaCl-primed plants (lower panel) 10 days after the last watering. This experiment was carried out in duplicate and the two replicates showed the same phenotypical result.



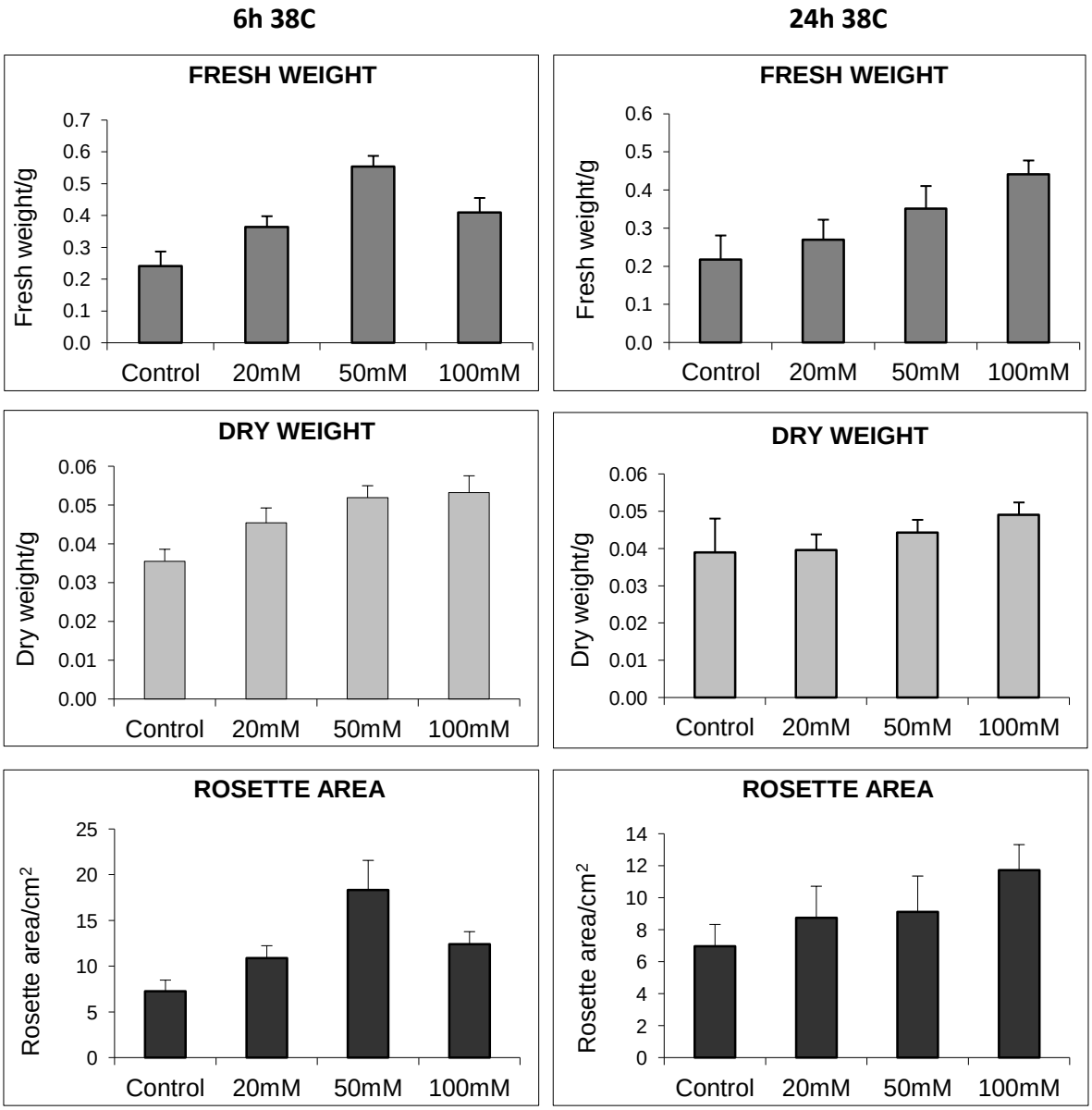


Fig. 3.5 Effect of drought stress on primed and non-primed plants after additional pre-exposure to a short heat treatment.

The upper row of panels shows plants primed with different concentration of salt (0, 100, 50, 20 mM), exposed to 24h of heat stress and finally subjected to drought for 10 days. The graphs show fresh weight, dry weight and rosette area of salt primed (20 mM, 50 mM, 100 mM NaCl) and non-primed (0 mM NaCl) plants, exposed to heat (38 C) for 6 hours (left) or 24 hours (right) and left to dry for 10 days.

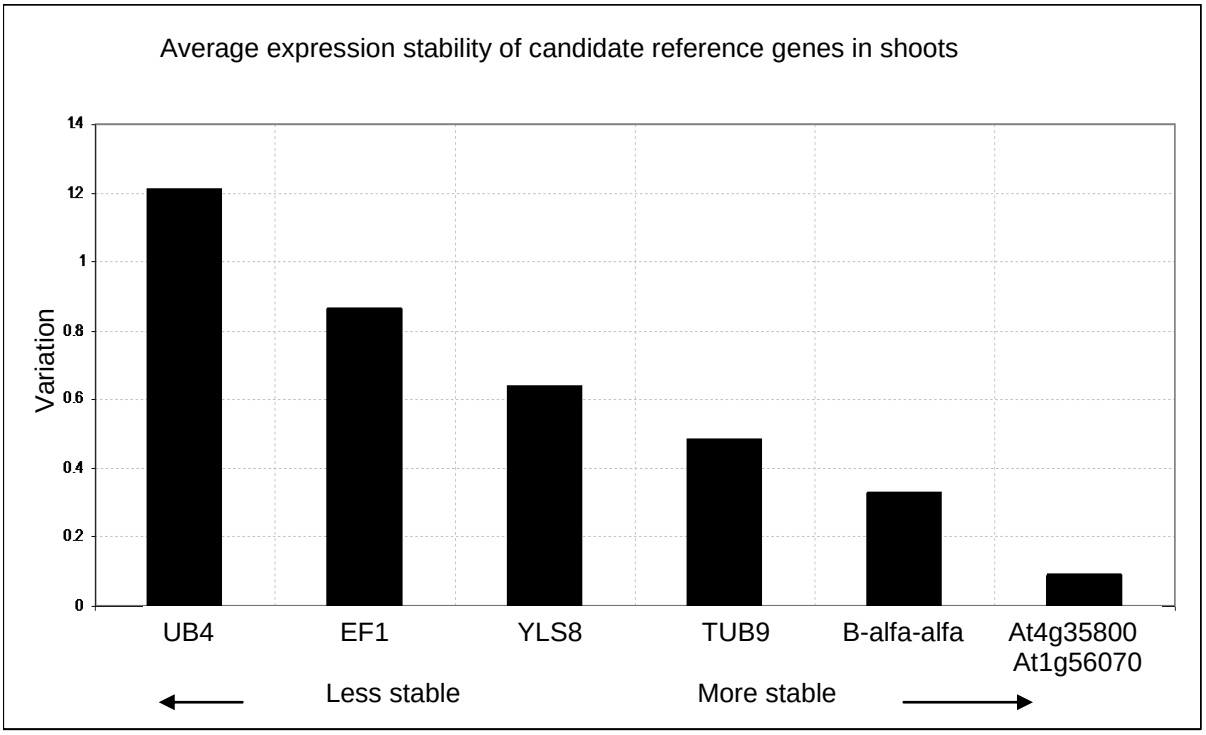
3.2.4 Effect of priming on transcriptional responses to a second salt stress treatment

Optimization of a reference gene for qPCR analysis

Initially, priming-induced differences at the molecular level were investigated by measuring transcript levels of known salt/osmotic stress induced genes by qPCR. This technique permits one to accurately quantify gene expression, measuring the amount of cDNA of a gene of interest. The obtained values are normalized to a "constitutively expressed" reference gene that shows equal levels of expression across conditions. The normalization permits direct comparison of expression of a gene of interest in different samples. However, many studies have reported that the most commonly used reference genes can vary considerably, depending on conditions, tissues or the ecotypes (Vandesompele et al., 2002; Nolan et al., 2006). Therefore, it was of high importance to identify a good reference gene for the experimental conditions and tissues used here. Appropriate reference genes were identified using geNorm software, which determines the stability of expression across a representative set of samples by comparing their Ct values (Vandesompele et al., 2002). geNorm is an algorithm that selects an optimal pair of reference genes out of a larger set of candidate genes. It calculates and compares the so called M-value of all candidate genes, eliminates the gene with highest M-value, and repeats the process until there are only two genes left. An M-value describes the variation of a gene compared to all other candidate genes. The last pair of candidates remaining is recommended as the optimum pair of reference genes.

Five common reference genes (*UBQ4*, *EF1*, *YLS8*, *TUB9*, *ALFA-ALFA*) and two additional genes *At4g35800* and *At1g56070* (selected from publicly available microarray data) were selected as starting set of candidate references genes (Hruz et al., 2008). Ct values for each gene were determined in cDNA sample derived from root and shoot of 4L plants subjected to two priming treatments (0, 50 mM NaCl) and two stress treatments (0, 80 mM NaCl, 4h). geNorm identified *At4g35800* and *At1g56070* as the best reference genes for this particular experiment for both shoots and roots (Fig.3.6).

A)



B)

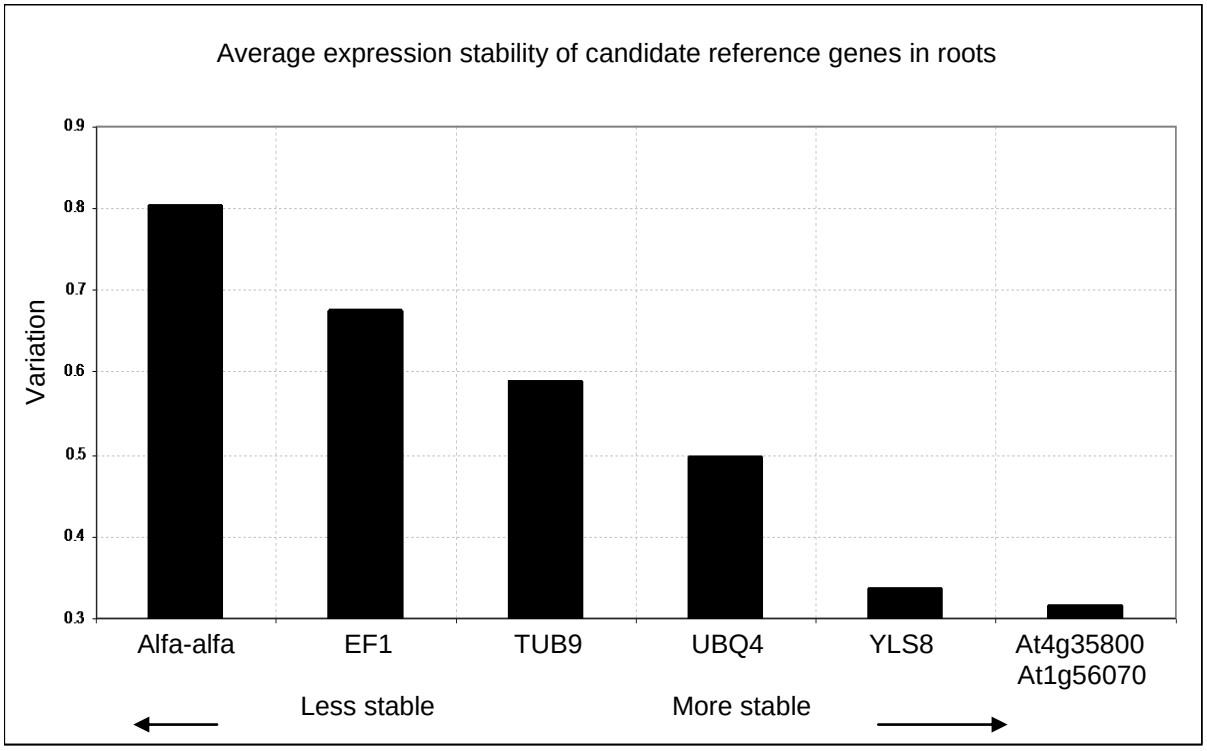


Fig. 3.6 Stability of candidate reference genes in shoots (A) and roots (B).
Variation of Ct values of candidate genes (*UBQ4*, *EF1*, *YLS8*, *TUB9*, alfa-alfa, At4g35800 and At1g56070) in four samples (primed, non-primed, stress and non-stressed) as determined by qPCR and geNorm software. From left to right are plotted the most stable transcripts across all 4L stage shoots samples.

Primed plants respond differently to salt stress depending on the stage of development at which the priming treatment is applied

qPCR analysis was carried out on shoots samples of plants primed at different stages of development to detect changes in the expression level of two known salt stress-responsive genes, *Rd29A* (a transcription factor involved in drought, salt and cold responses) and *P5CS-1* (Delta 1-pyrroline-5-carboxylate synthetase, an enzyme involved in the synthesis of proline, induced by ABA and salt) (Yoshiba et al., 1999; Yamaguchi-Shinozaki and Shinozaki, 1993). Two biological replicates were carried out. The transcript levels of *Rd29A* and *P5CS-1* always increased after the salt treatment independently of the stage of priming or the concentration used. These results clearly attested the efficiency of the treatment (Fig.3.7-3.8). However, the amount of up-regulation ("response") was dependent on the stage of development at which priming was applied and on the concentration of NaCl used for priming. Very similar expression patterns were observed for *Rd29A* (Fig.3.7) and *P5CS-1* (Fig.3.8). Plants primed at the "G0 stage" showed a slightly increased response to salt compared to non-primed plants and this effect was more pronounced in 100 mM NaCl primed plants than in 50 mM primed plants. In contrast, plants primed at seedling stage (2L and 4L) showed a weaker response than non-primed plants. Again this effect was more pronounced in 100 mM NaCl primed plants than in 50 mM primed plants. Plants primed at seed stage showed less clear expression patterns with both base levels and responses of transcripts varying between differentially primed plants. This variability among developmental stages in the transcriptional response to the stress upon priming suggests a different perception of the stress and its signalling network between embryonic tissue (Seed and G0 stage) and adult tissues (2L-4L stage).

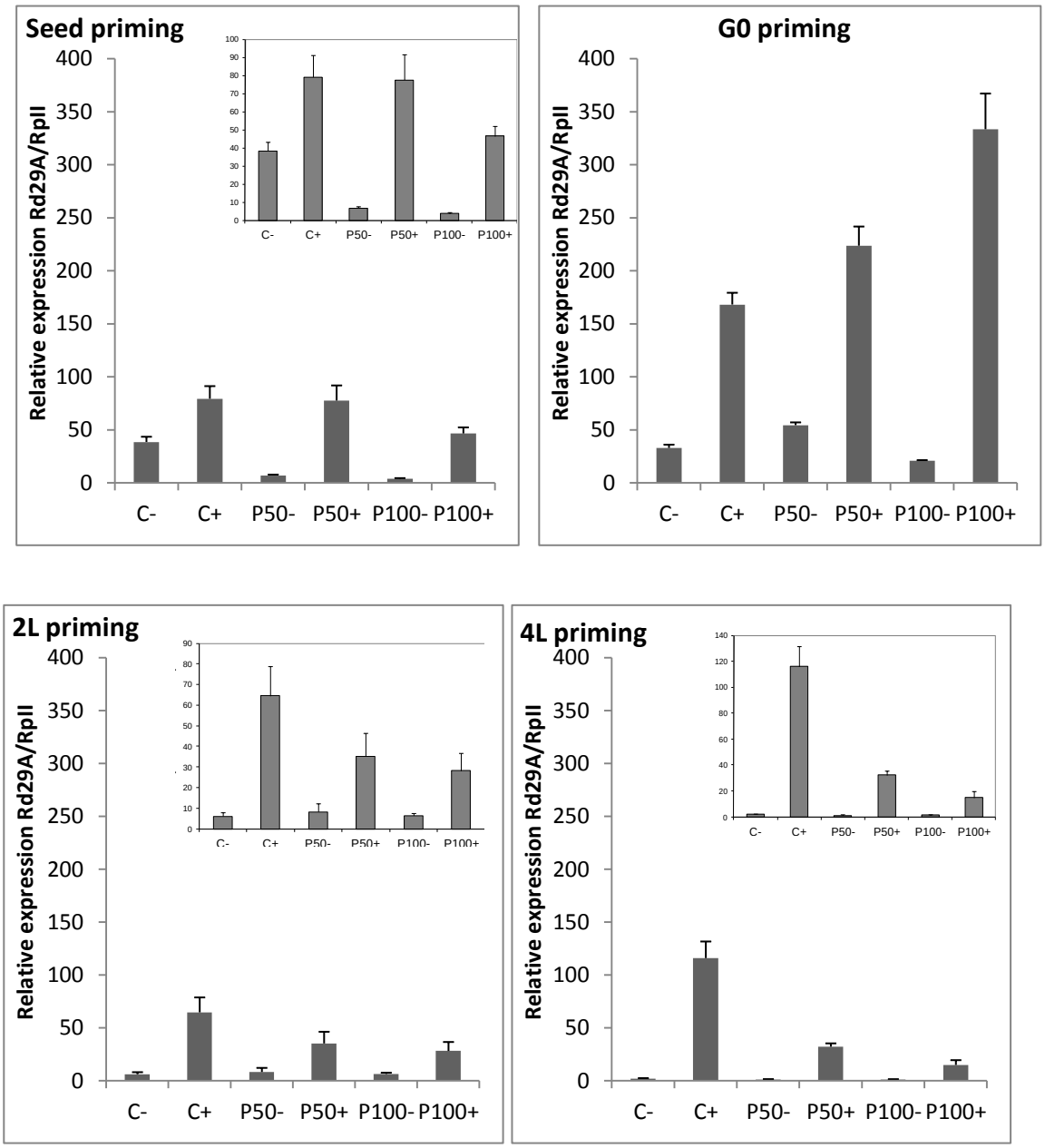


Fig. 3.7 Relative transcript level of *Rd29A* in shoots samples from plants primed at four different stages of development.

RNA was isolated from plants primed with two different concentrations of salt (50 mM, 100 mM) or 0 mM NaCl (C) at the stage of development indicated (seed, G0, 2L and 4L) and later exposed for 4 hours to salt treatment with 80 mM (+) or not (-). Values are averages of 3 technical replicates ± SE, each normalised to the respective value obtained for the reference gene *RpII*.

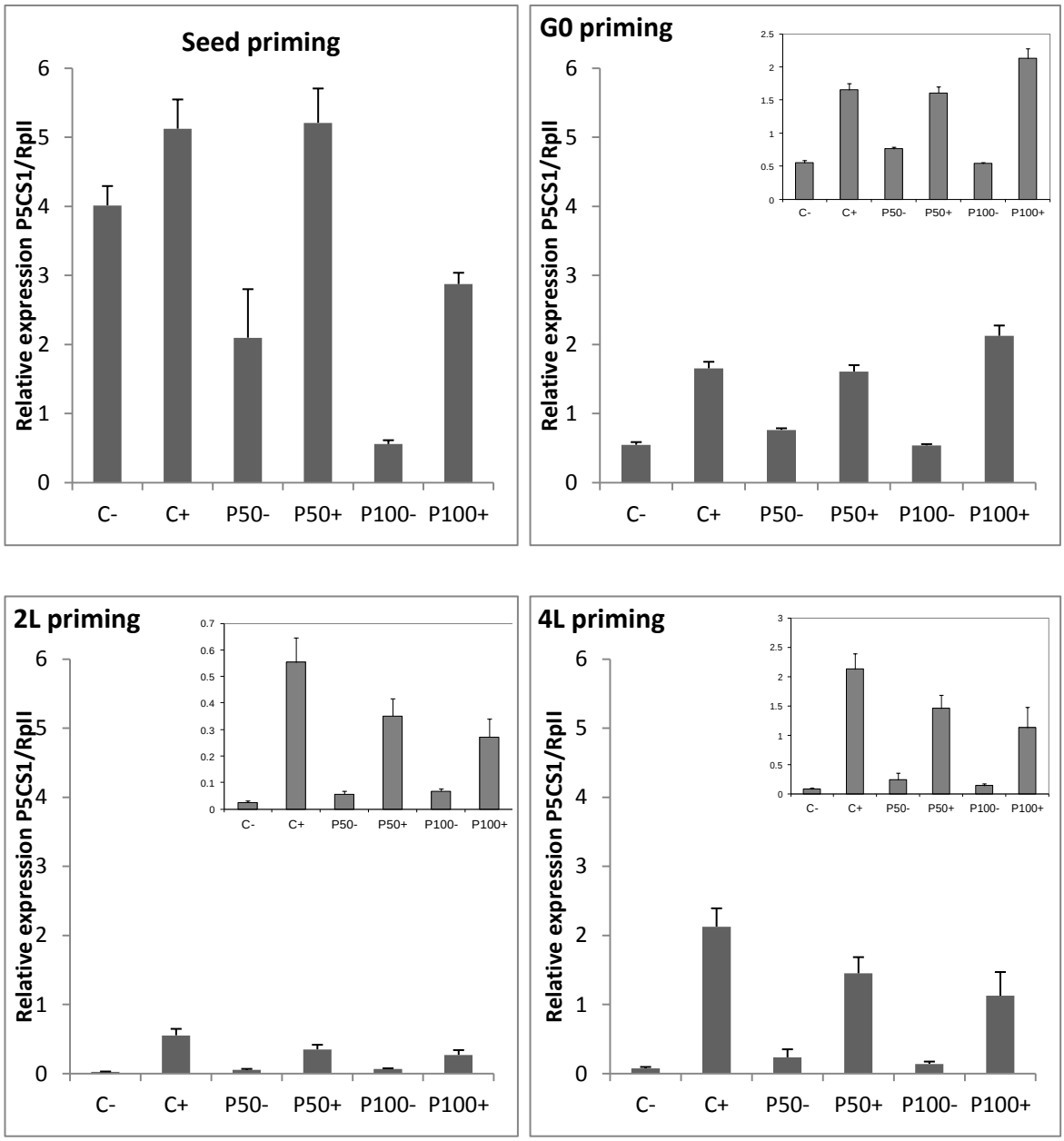


Fig. 3.8 Relative transcript level of Proline Synthase (*P5CS1*) in shoots samples from plants primed at four different stages of development.
RNA was isolated from plants primed with two different concentrations of salt (50 mM, 100 mM) or 0 mM NaCl (C) at the stage of development indicated (seed, G0, 2L and 4L) and later exposed for 4 hours to salt treatment with 80 mM (+) or not (-). Values are averages of 3 technical replicates ± SE, each normalised to the respective value obtained for the reference gene *RpII*.

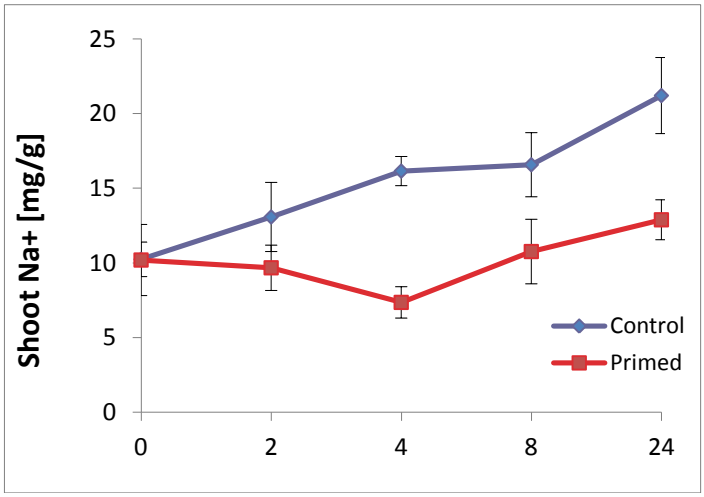
The expression patterns of *P5CS1* and *Rd29A* suggest that when priming was applied to seedlings (2L and 4L) the plants were responding consistently differently to the second salt treatment. The basal level of the transcripts before the stress exposure was very similar in primed and non-primed plants. Thus, the priming treatment affected the transcriptional response to salt without changing the base level of the transcripts. Priming at G0 stage also changed the stress responsiveness of the plants. Also, taking into account that ChIP-Seq analysis requires a large amount of fresh material, it was decided to use plants primed at 4L stage for all subsequent experiments. Priming with 50 mM NaCl was selected as the most efficient concentration of salt for priming with no apparent impact on plants growth and development. The final experimental design is summarized in the chapter Materials and Methods Fig.2.1.

3.2.5 Effect of priming on plant sodium uptake

The results of the previous sections showed that priming modulated early transcriptional responses of genes to the application of 80 mM NaCl. This concentration is known to negatively impact on plant growth if applied for several days due to accumulation of toxic Na^+ concentration in the plant (Wang et al., 2006). But the early response will be dominated by adjustment to the osmotic stress. To investigate whether priming also had an effect on how the plants dealt with the ionic (sodium) stress component, the concentration of Na^+ accumulated in roots and shoots of primed and non-primed plants was measured over a period of 24 hours after salt application. As expected from other studies, non-primed plants showed an initial increase in root Na^+ concentration and a steady increase of Na^+ concentration in the shoots. Primed plants also showed an initial increase in root Na^+, but they accumulated considerably less Na^+ in their shoots over the entire 24 hour time-course (Fig.3.9). Because a similar phenotype had been described for mutants in the root xylem Na^+ transporter HKT1 (see Introduction 1.1), the transcript level of this gene was measured in roots and shoots. qPCR results confirmed root specificity of *HKT1* expression and showed induction of the gene upon salt treatment. It also revealed that *HKT1* transcript levels were expressed in primed roots when the stress occurred (Fig.3.10). However, despite the observed differences in Na^+ uptake no obvious

difference in salt tolerance between primed and non-primed plants was apparent if plants were left in the salt solution for longer periods of time (10 days).

A)



B)

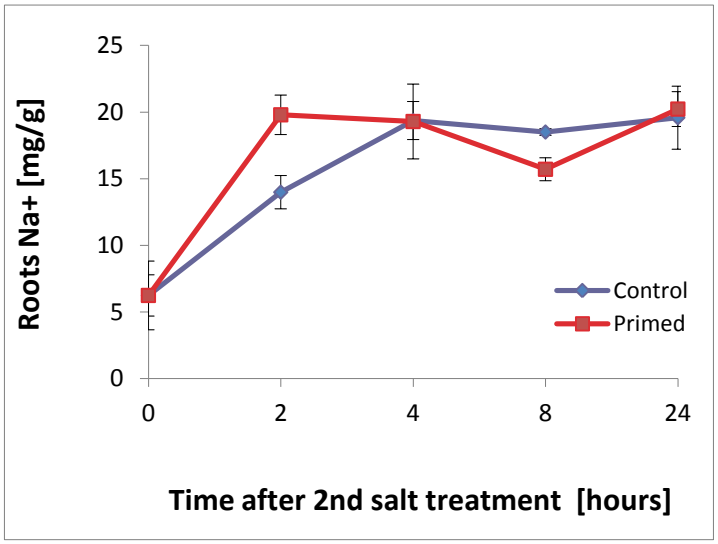
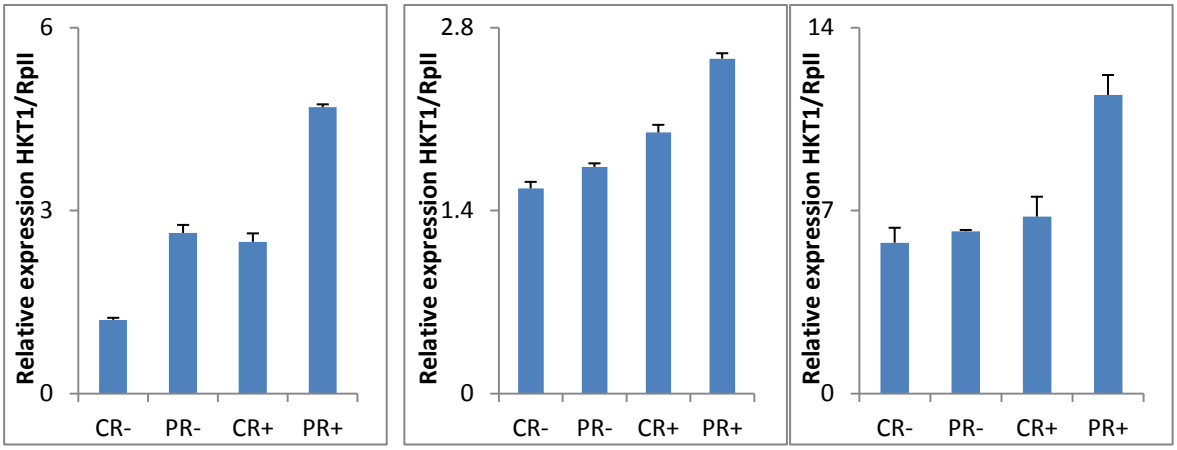


Fig. 3.9 Sodium content in shoots (A) and roots (B) of primed (red) and non-primed (blue) plants at different time points after application of 80 mM NaCl.
Values are means ± SE of four plants.

A) ROOTS



B) SHOOTS

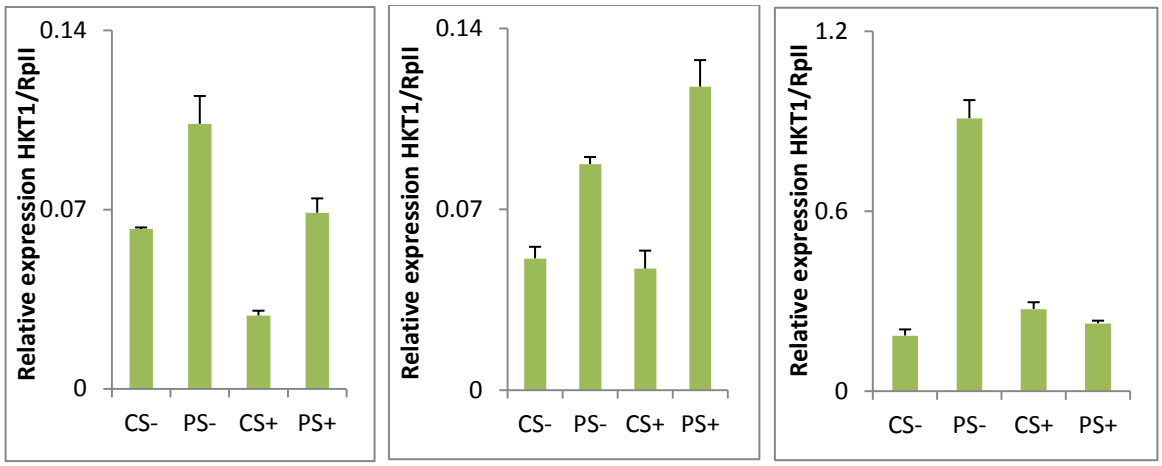


Fig. 3.10 Transcript levels of *HKT1* in primed and non primed plants.
RNA was isolated from shoots (A) and roots (B) of plants primed with 50 mM (P) or 0 mM NaCl (C) at 4L stage of development and later exposed for 4 hours to salt treatment with 80 mM (+) or not (-). Values are averages of 3 technical replicates ± SE, each normalised to the respective value obtained for the reference gene *RpII*. Results obtained in three independent experiments are shown.

3.3 Discussion

3.3.1 Establishment of an effective priming protocol

In this first part of the thesis it was shown that a transient priming treatment of *A. thaliana* plants with 50 mM NaCl at the 4L-seedling stage led to enhancement of drought tolerance of adult plants and modulation of early transcriptional responses to a second salt treatment applied 10 days after the priming treatment. In addition, the expression pattern of the Na^+ transporter HKT1 and Na^+ uptake into shoots upon the second stress treatment were altered in primed plants. However, these effects did not lead to enhanced long-term tolerance to salt. This finding is in accordance with the notion that a 24h priming treatment is not long enough for the plants to accumulate Na^+ to toxic levels and hence to induce protective mechanisms that are needed to deal effectively with ion toxicity, which is the predominant stress factor during long-term salt stress (Munns and Tester, 2008). This work therefore established an effective protocol for osmotic priming of *A. thaliana* plants and provided a first quantitative proof of a long-term somatic "memory" of *A. thaliana* plants for osmotic stress, both at physiological and molecular level. The period of time between the transient priming treatment and the second stress was at least 10 days. During this time the plants increased their biomass by a factor of approx. 10 times (average fresh weight of 4L-seedlings was 0.01 g, while average fresh weight of plants at time of second stress was 0.1 g, respectively). This means that any molecular entities (e.g. transcriptional factors, etc.) would have been considerably diluted in the tissues at the time of the second treatment. The established protocol therefore provides an excellent basis for investigating the involvement of epigenetic processes in somatic stress memory.

3.3.2 Developmental stage is important to overcome of priming

Plants primed at the seedling stage (2 and 4 real leaves) showed lower expression levels of *Rd29A* and *P5CS1*, when exposed to salt stress compared to the non-primed plants. Interestingly priming applied at G0 stage of germination had the opposite effect. G0 priming was applied after the seeds were completely imbibed and put on plates. This phase is recognized as germination *in sensu strictu*, during which important metabolic processes are initiated. At this crucial point a decision was made whether to continue towards radicle emergence and plantlet establishment or to remain in the dormant state, as would be the case during adverse environmental conditions such as salt or drought (Finch-Savage and Leubner-Metzger, 2006; Catusse et al., 2008). Our results indicate that transcriptional responses to the same treatment differ depending on whether the stress occurs before or after this checkpoint, but they do not differ during the later stages of development (2L to 4L). This also suggests a different perception of the stress and its signalling network between the embryonic tissues and the adult tissues might occur.

3.3.3 Effect of priming on drought tolerance is additive to the effects of temperature

Plants often respond to different stresses by activating the same protective mechanism (Kilian et al., 2007; Mao et al., 2011; Kreps et al., 2002). This could be because the simultaneous combination of stresses is frequently imposed on plants, e.g. drought stress is often accompanied by high salinity or high temperature (Mittler, 2006) or because different stresses often cause similar problems particularly in early stages of growth (Wang et al., 2003; Mahajan and Tuteja, 2005; Hussain et al., 2010; Golldack et al., 2011). For example, a high external salt concentration lowers the water potential in the soil and hence presents a challenge for root water uptake that is similar to what is caused upon drought or freezing (Munns and Tester, 2008). Table 3.1 summarises the problems caused and the protective mechanisms induced for a number of environmental conditions. Overlapping responses also occur at the level of transcription. It has been shown that a large number of drought-inducible genes are also induced by salt stress (Kilian et al., 2007). The observation that salt priming had a positive effect on drought tolerance is therefore reasonable and it suggests that drought tolerance is based on protection

against osmotic stress. Furthermore, it was found here that the salt priming effect was additive to the temperature-priming, re-enforcing the hypothesis that overlapping mechanisms can enhance the stress tolerance. In both experiments the plants generally coped better with the drought stress showing an enhancement in all the parameters previously mentioned when they had experienced the salt-priming treatment. In summary, the results indicate a scenario as shown in Fig. 3.11: (1) Priming treatment leads to osmotic protection and drought tolerance (green arrows). (2) A short heat treatment, was found to be also beneficial when plants were under drought stress (red arrows) possibly due to the induction of additional protective mechanisms (e.g. ROS detoxification). (3) Long-term salt tolerance would require additional protection against ion toxicity (blue arrows) which is not provided by the short priming treatment.

3.3.4 Potential role of HKT1 in primed plants

The sodium uptake experiment showed that primed plants accumulated less Na^+ in their shoots. Shoot Na^+ accumulation is known to be controlled by the root xylem Na^+ transporter HKT1 and indeed *HKT1* was found to have higher transcript levels in primed than in non-primed plants. The results are interesting in several aspects. Firstly, they indicate that the priming treatment did induce ionic and not just osmotic responses triggered directly by high external Na^+ rather than high internal Na^+. The notion that external Na^+ can act as a signal in its own right is supported by the fact that it induces a cytoplasmic calcium signal, which in turn regulates Na^+ transporters via the CBL/CIPK (SOS) pathway (Liu et al., 2000; Halfter et al., 2000). Secondly, a lower Na^+ content in shoots of primed plants 4 hours after application of the second stress (Fig. 3.9) could explain why the stress marker genes showed a weaker transcriptional response (Fig. 3.7-3.8); the primed plants were simply less 'stressed'. Thirdly, lower Na^+ uptake should delay the onset of Na^+ toxicity and therefore delays, if not prevents, stress symptoms and plant death. This is in contrast to the finding that primed plants were not more tolerant to salt in the long term. However, the tolerance was assessed here by scoring survival after 10 days. A more detailed scoring of symptoms at a better time resolution needs to be carried out to assess whether salt stress was delayed in primed plants. It is also possible that the beneficial effect of *HKT1* induction is only transient.

Tab. 3.1 Summary of cross protection mechanisms enhancing stress tolerance.

Stress	Problem at tissue/cell level	Protective mechanism	Tolerance
Salt	Na/Cl toxicity	Ion transport e.g. HKT1, and compartmentalization	Salt, drought
	Osmotic imbalance	Increase osmotic potential inside the cells (e.g. compatible osmolites such as proline)	
	Water loss	Stomatal closure	
Drought	Protein denaturation	Chaperones	Salt, drought, freezing
	DNA damage	DNA repair	
	ROS, oxidative stress	Anti-oxidant	
Freezing			Salt, drought, freezing
	Structural instability of proteins/nucleic acids	Chaperones	
	Cellular dehydration	Osmo-protecting	
	ROS, oxidative stress	Anti-oxidant	
	Membrane fluidity	Changes in lipid components	
Heat	ROS, oxidative stress	Anti-oxidant	Salt, drought, heat
	Water loss	Stomatal closure	

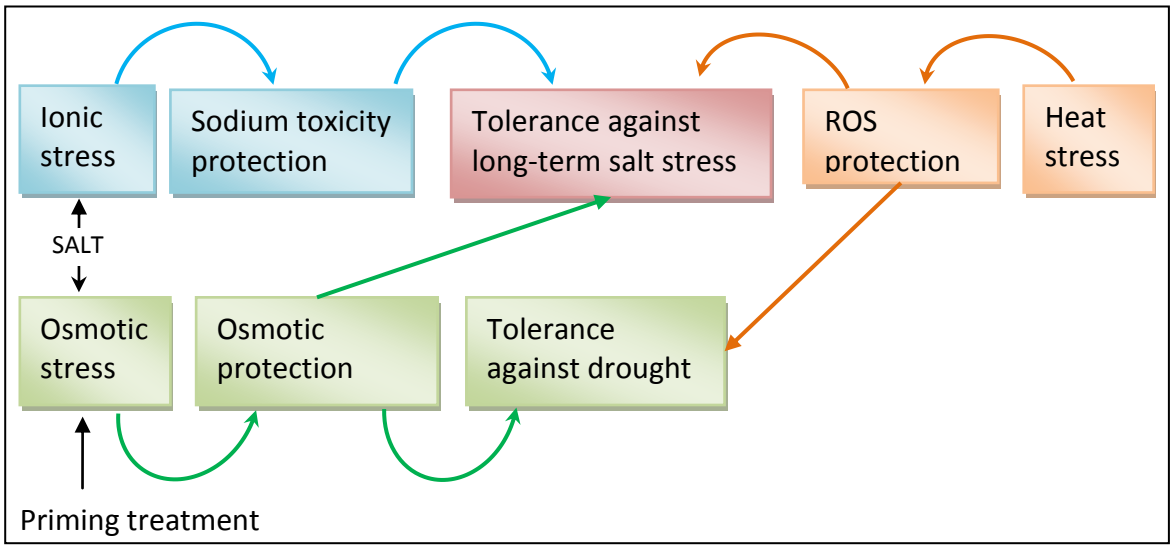


Fig. 3.11 Salt priming and cross protection.

Chapter 4: Quantifying the effect of priming: expression profile

4.1 Introduction

4.1.1 Background

In the previous chapter it was shown that a transient priming treatment of seedlings modified the transcriptional response of two osmotic-stress marker genes to a second salt treatment 10 days later. This result raised the question which other set of genes respond differently in primed plants? In this chapter the effect of priming on the transcriptional responses of primed and non-primed plants was analysed at the level of the entire transcriptome using microarrays.

Numerous microarray analyses, studying the response of *Arabidopsis* to NaCl, have been made publicly available through several databases (See Introduction 1.2). For example, some studies have identified the convergent and divergent pathways between salinity and other abiotic stress responses (Ma et al., 2006; Kreps et al., 2002). While others have investigated the kinetics of salt stress at several different time points (Kilian et al., 2007), or the tissue-specific response to the stress (Jiang and Deyholos, 2006). All together the results from these microarray profiles have revealed that the vast majority of the genes identified are responding to a range of different stresses and only a small number of genes were strictly salt-specific but importantly all were localized in the roots (Ma et al., 2006; Kreps et al., 2002). Also the alterations in gene expression occur rapidly within 30 min after the application of stress (Kilian et al., 2007). The vast majority of the stress responsive genes showed dynamic changes in transcript abundance with different turnovers. Finally, results of functional enrichment analysis generally showed stress response genes including hundreds of transcription factors, kinases/phosphatases, hormone-related genes and effectors of homeostasis, and overall emphasized the complexity of this stress response (Jiang and Deyholos, 2006). Even though these data provided a detailed understanding of the pathways that are induced by salinity stress in

A. thaliana, no available transcriptomic data describes the effect of salt priming on the transcriptional responses of *Arabidopsis* roots and shoots to NaCl treatment.

4.1.2 Aims of the chapter

In this chapter I describe the influence of priming on the genome-wide transcriptional response of *Arabidopsis* plants after exposure to salt treatment. This is the first study to my knowledge that investigated the effect of salt priming using a global expression profiling strategy. *Arabidopsis* Affymetrix GeneChip ATH1 microarray was used, which provided probe sets for approximately 23,000 genes. When a broad approach of this type is taken technical factors, such as salt applications, processing of samples or quality of probe hybridisation might obscure the biological responses. It is therefore very important to identify which factors are influencing the data set, and also to eliminate undesired effects in order to extract reliable results. For these reasons several different statistical approaches were carried out to identify genes and pathways related to salt priming. The differences detected by microarray analysis were further confirmed by Real Time quantitative PCR. The final part of the chapter presents an analysis of functional annotations of the genes differentially responsive to salt stress between primed and non-primed plants. Also, possible roles of priming and molecular targets are discussed.

4.2 Methods for the chapter

Plants growth and treatments, RNA extractions, microarray hybridisation and PCR methods are described in Chapter 2.

4.2.1 Statistical approaches and software used

Affymetrix® Expression Console™ version 1.1 was used in order to analyze the microarray quality of the samples. Partek® Genomics Suite™ version 6.10.1020 was used to normalize the microarray data and to analyse differential gene expression in the samples by Analysis of Variance (ANOVA). Functional annotation gene clusters, were made using the free access software The Database for Annotation, Visualization and Integrated Discovery (DAVID).

Quality control and normalization

Quality control on the microarray data was performed using the software Expression Console™ (version 1.1). The software also performs data normalization using robust multi-array average (RMA) of the signal intensities adjusting the signal values to a common median for subsequent comparison.

Vector Analysis (VA)

The challenge of this study resided in the two-factor nature of the comparison (+/- salt, +/- priming). Vector analysis provides a tool to quantitatively compare transcriptional responses (+/-salt) between two different backgrounds (e.g. wild type/mutant, here primed/not-primed) (Breitling et al., 2005). The basic principle is to represent the response of each gene by a vector in a Cartesian plane. Various sectors of the plane will correspond to various prototypical behaviour of gene: genes that respond the same in both factors, genes that respond in opposite directions or genes that are changed only in one of the factors. Depending how the vector is oriented along the plane it is possible to establish how the gene(s) behave (Fig.4.1). Importantly, this method allows one to

establish the statistical significance of the behaviour of each gene by calculating a sum vector based on all replicates.

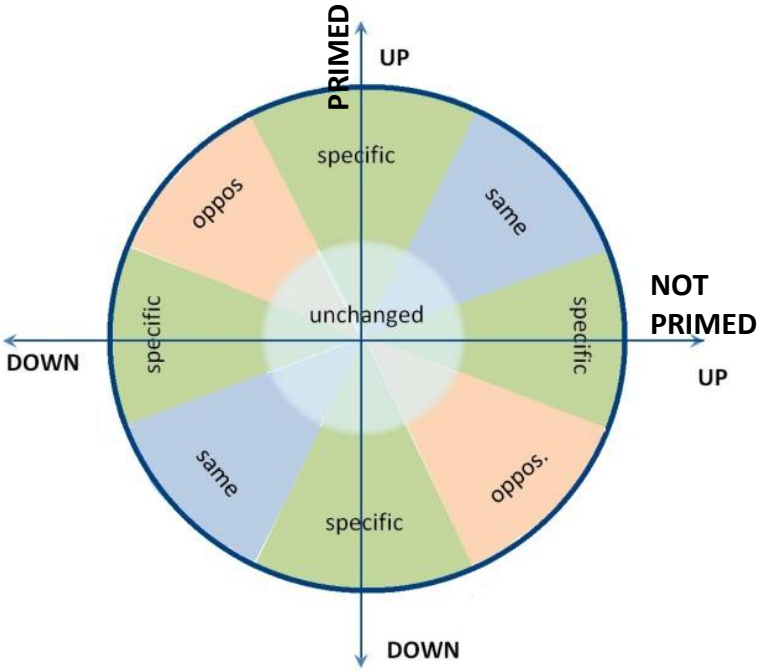


Fig. 4.1 Schematic representation of vector analysis.
On the two axes is reported the normalized fold change of transcript in response to salt in primed (y-axis) and non-primed (x-axis) plants. The plane can be systematically subdivided into sectors corresponding to the main behaviour types that are possible. In the centre, genes show very little response in either condition (white). Other genes respond about the same in both conditions (blue sector), are specifically changed in only one background (green), or are regulated in opposite directions in primed and non-primed plants (red).

Principal Component Analysis (PCA)

PCA is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set. The idea behind PCA is to identifying directions (principal components), along which the variation of the data is maximal (Ringnér, 2008). As a result, each individual sample can be represented by using a few components rather than values for thousands of variables. Samples can then be plotted for pairs of components, making it possible to visually assess similarities and differences between samples and to determine whether samples can be grouped (Ringnér, 2008). In other words the more the samples are separated along a component the more the samples differ from each other.

Analysis of Variance (ANOVA)

ANOVA (Zar, 1999) is a statistical technique which is useful to establish if significant differences occur between groups. The idea behind ANOVA is to divide the total variability into variability between groups and variability within groups. If the variability between groups is large compared to the variability within groups, as determined via a statistical test, conclude that there are significant differences between groups (Zar, 1999). The analysis was performed within the software Partek® Genomics Suite™.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

DAVID software (Huang et al., 2009) was used to generate functional annotation clusters which are enriched within a certain set of genes. The functional categories can be derived from various sources (i.e. Gene Ontology, BLAST result key words, KEGG database) and the detection algorithm will calculate the probability that the number of genes belonging to a cluster is random with the respect to the number in a chosen background e.g. the *Arabidopsis* genome. A p-value is extracted together with a fold discovery rate and an enrichment score.

4.3 Results

4.3.1 Quality dataset control and normalization

To compare transcriptional profiles, RNA samples were extracted from 15 plants grown under controlled conditions in three independent experiments. In brief, three weeks old (4L-stage) plants were primed for 24h (P) or not (C) and 10 days later exposed to 80 mM salt treatment for 4h (+) or not (-); samples were separated into shoots and roots and RNA was extracted, labelled and hybridized to Affymetrix® GeneChip™ ATH1 (see Chapter 2).

A total of 24 arrays (two tissues, four conditions C-, C+, P-, P+, three replications) were performed. The hybridization intensities of the probes were recorded in the form of Affymetrix® .CEL files by GCOS software used at the *Glasgow Polyomics Facility*. This software operates a preliminary correction for the background noise within the individual probes belonging to a probe set. The presence of outlier samples was identified using the software Expression Console (v.1.1) that contains graphic capabilities for visual inspection of the hybridization results. In Fig. 4.2 is reported a box plot created prior to any normalization, loading the Affymetrix® .CEL files obtained from the individual samples. The plot summarizes the distribution of the intensities from one array compared to the median probe intensity across all arrays. If individual arrays are different from the other replicates in the same group (in this case shoots and roots), the box will shift up or down with respect to the median value.

The arrays of the replicate E3 for both roots and shoots clearly showed lower probe intensities then the other arrays. The individual array sample E1RC- showed slightly higher intensities relative to its roots groups as well as E1SC- and E1SP+ in shoots group (Fig.4.2). This data demonstrates that in order to allow analysis without biases it is essential that all data are subjected to normalization. Expression Console uses the Robust Multichip Analysis (RMA) algorithm that provides a robust linear model at the probe level to minimize the differences of probe-specific affinity. Fig.4.3 shows the relative log probe cell intensities of roots and shoots samples after RMA normalization; all the values are now comparable in intensity.

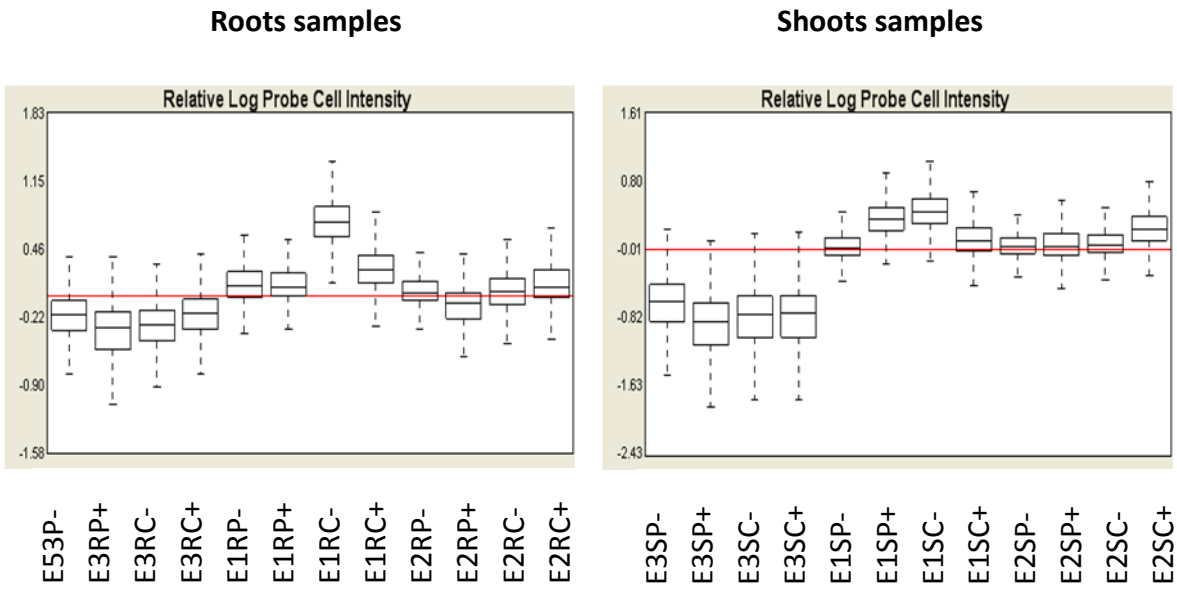


Fig. 4.2 Relative Log Probe Cell Intensities of root (left) and shoot (right) samples before RMA normalization.
Each box represents the distribution of the probe intensity values for each sample relative to the median of intensities of their replicate group (E1, E2, and E3). (E: replicate number, R: Roots, S: Shoots, p: primed, C: non-primed, +: salt applied, -: no salt applied).

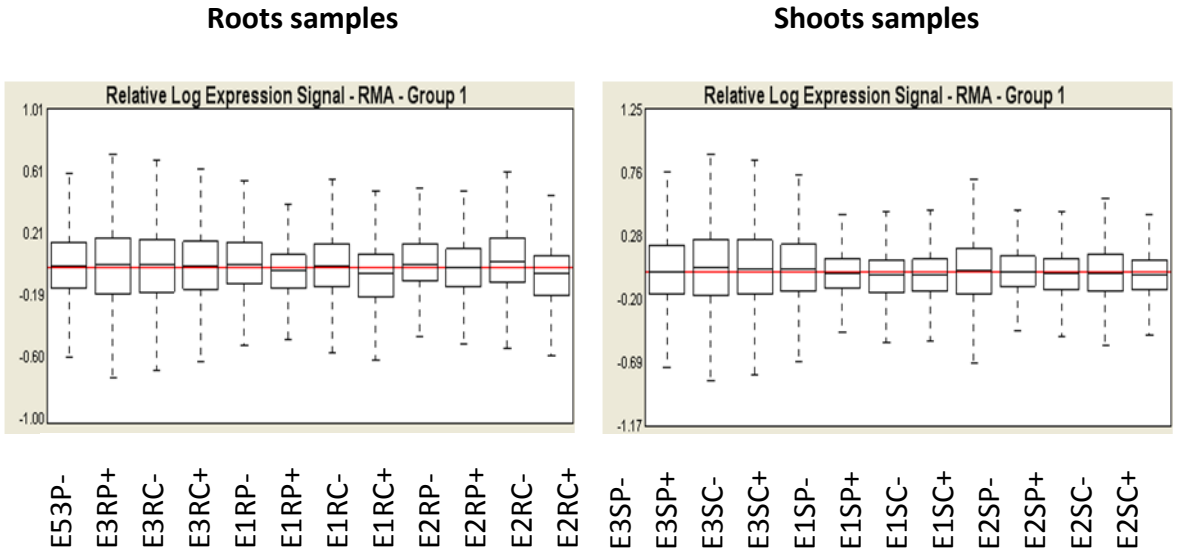


Fig. 4.3 Relative Log Probe Cell Intensities of roots (left) and shoots (right) samples after RMA normalization.
Each box represents the distribution of the probe set intensities for each sample adjusted to the median of intensity values of their replicate group after RMA normalization. (E: replicate number, R: Roots, S: Shoots, P: primed, C: non-primed, +: salt applied, -: no salt applied)

Fig. 4.4 shows the comparison of the distribution of signal intensities within the two groups of samples, generated by Expression Console™. The graphs were created by plotting the number of probe sets (y-axis) producing a certain signal intensity (x-axis) for each sample. Fig. 4.4 confirmed that samples originated from the same tissue presented a similar pattern of gene expression. In detail, the vast majority of genes showed low expression under all conditions, as represented in the graph by the highest peak at low signal intensities. The curve drops to moderate intensity values before peaking again at a lower level than the previous peak showing that a large number of genes were expressed at moderate levels. Finally after the second peak there is a slow decrease, indicating that there was a relatively small, but still a significant number of genes that were highly expressed in the samples.

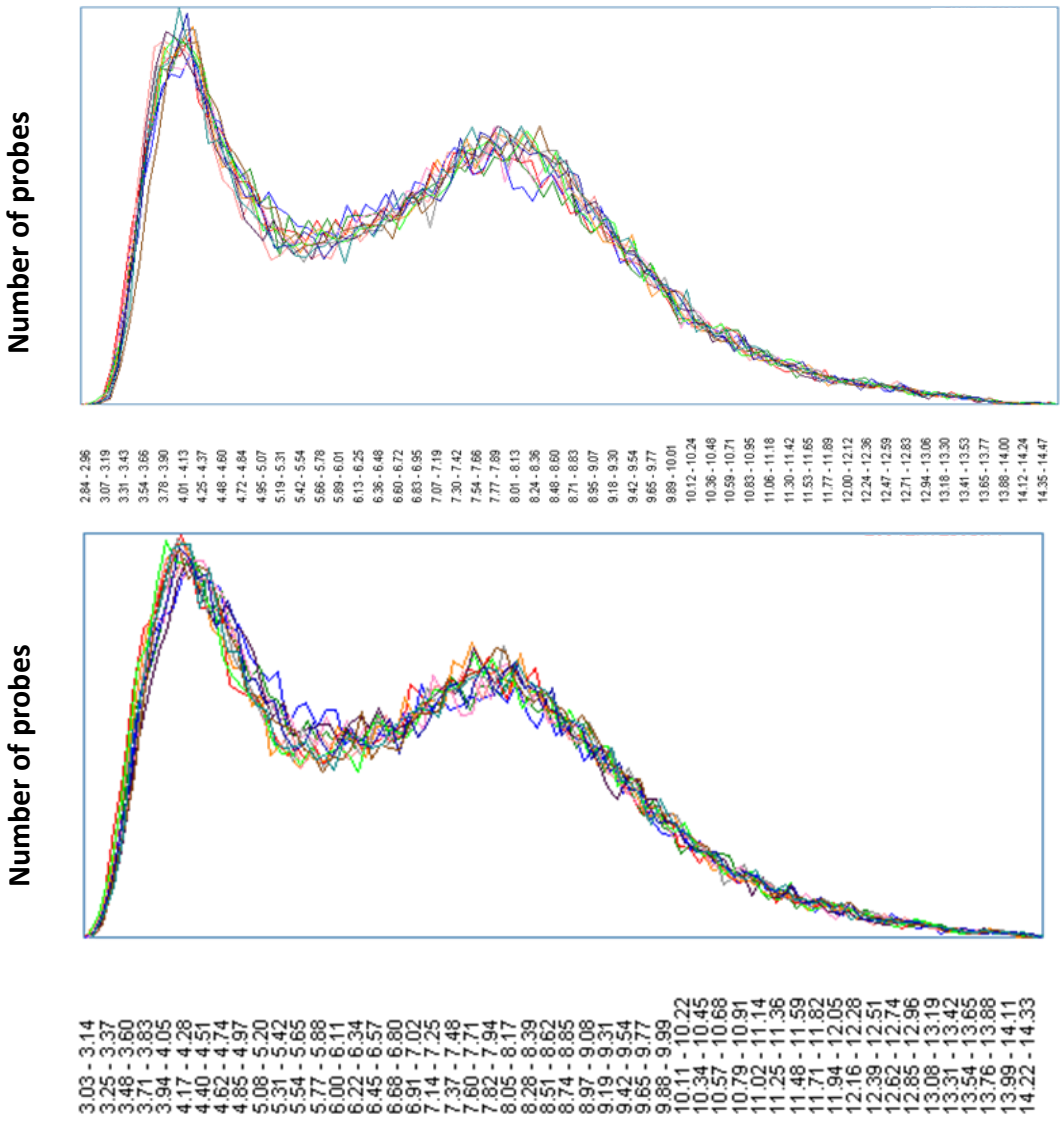


Fig. 4.4 Probe intensity distribution of root (top graph) and shoot (bottom graph) samples after RMA.
X-axis: intensities range, y- axis number of probes producing certain intensity.
Different coloured lines represent the different samples.

4.3.2 Priming induced changes in the transcriptional response

4.3.2.1 Data files

Raw data in the form of CEL files were loaded into the software Partek® Genomics Suite™ and subjected to RMA normalization along with a batch correction. Two text files (one for root and one for shoot samples) including the collection of the normalized probe signal intensities for all the four conditions and the three replicates were generated. Without any further filtering, all probe signal intensities were loaded into Microsoft Excel© (24 columns) together with Affymetrix probe identifier, AGI code and TAIR10 annotations, and subjected to different calculations.

4.3.2.2 Genes differentially expressed in primed and control plants without second stress

Fig.4.5 A-B shows the average signal intensities of the primed plants without salt exposure (P-) plotted against the average signal intensities of the control plants without salt exposure (C-). For both shoots (A) and roots (B) the vast majority of points were positioned close to a linear regression line with a slope of 1 representing equal x and y values. This indicated that the priming treatment did not have a strong influence on the transcriptional response 10 days later if the salt stress was not applied (Fig.4.5).

4.3.2.3 Genes differentially expressed upon stress in primed and control plants

Fig.4.5 C-D shows the average of the signal intensities of the primed plants exposed to salt (P+) plotted against the average of the signal intensities of the control plants exposed to salt (C+). For the roots samples (D), the scatter plot became clearly more spread indicating that the priming treatment had an impact on the gene transcription after exposure to the salt treatment 10 days later.

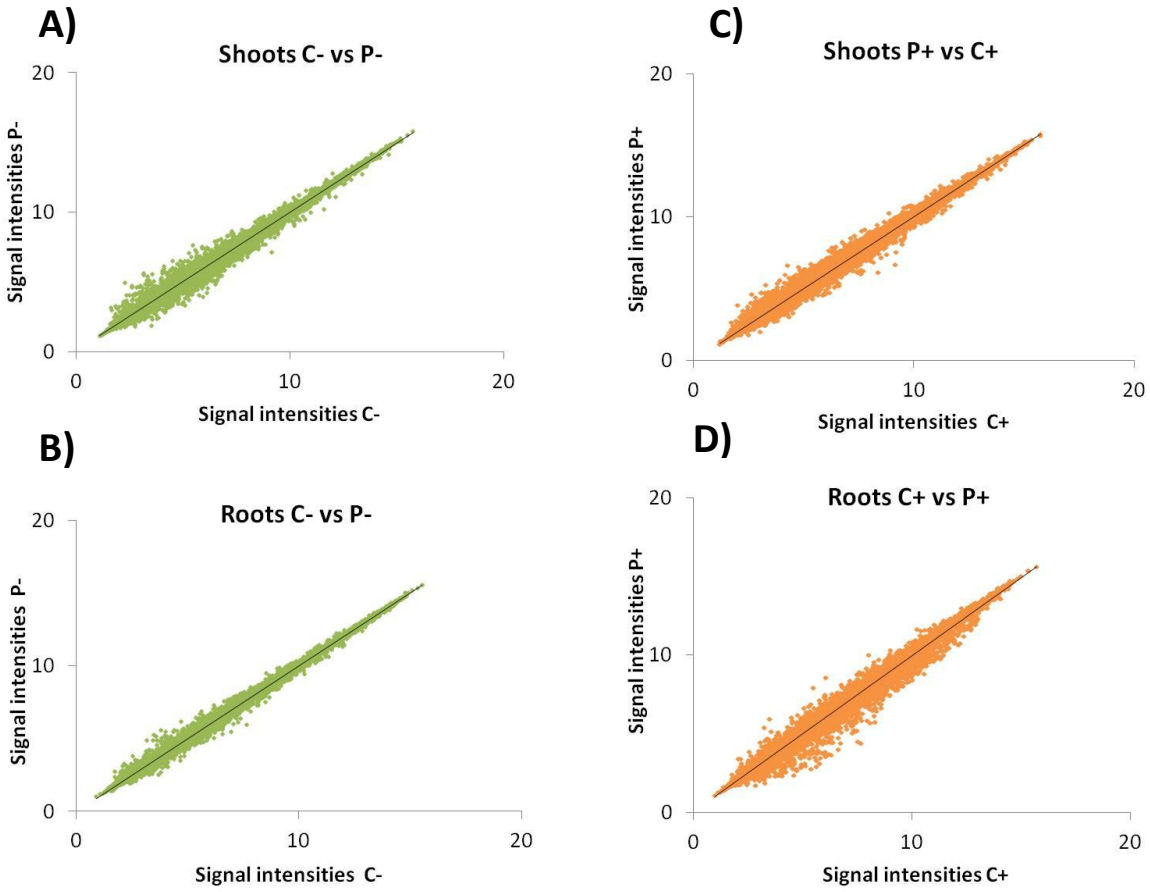


Fig. 4.5 Scatter plot of mean signal values from the Affymetrix gene chip showing the comparison between primed and non-primed plants under control without salt application (-, A shoots, B roots) and with salt applied (+, C shoots, D roots,).
Line indicates the position of the linear regression for the data. Left: Average of the log signal intensity from primed (P) and non-primed plants (C) not exposed to salt stress (-). Right: Average of the log signal intensity from primed (P) and non-primed plants (C) exposed to salt stress (+).

4.3.2.4 Response to salt in primed and control plants

Fig.4.6 shows the log response to salt of primed plants (P+/P-) plotted against the log response to salt of control plants (C+/C-). The response to salt was calculated for both shoots and roots, as the average of the +/- ratios of the absolute signal intensities in each replicate. Thus**:**

Equation 1

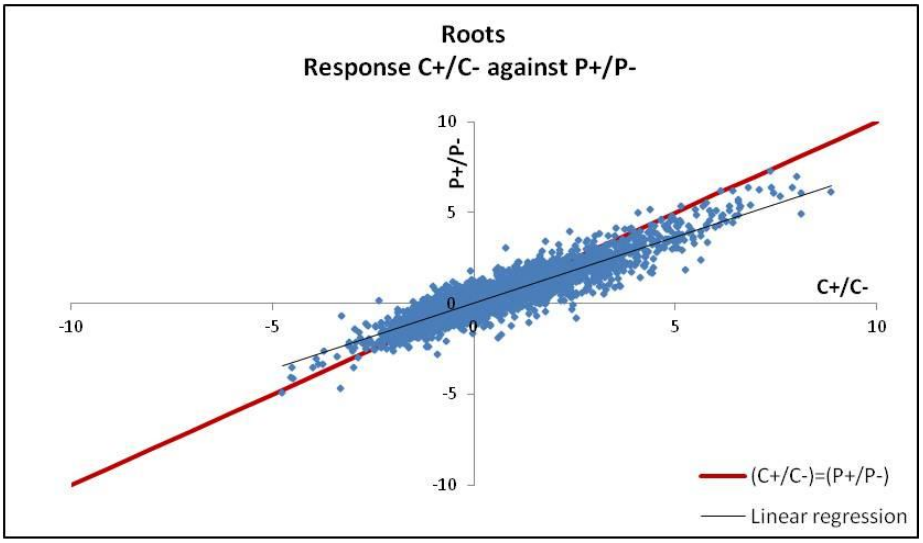
Response to salt in Control = [Average ((E_1C+/E_1C-), (E_2C+/E_2C-), (E_3C+/E_3C-))]

Response to salt in Primed = [Average ((E_1P+/E_1P-), (E_2P+/E_2P-), (E_3P+/E_3P-))]

A similar response to salt in primed and non-primed plants would result in a linear relationship with a slope of 1. As shown in Fig.4.6 in roots the slope is less than 1 (0.73), indicating that upon salt stress the vast majority of the genes responded less strongly to salt in primed than in non-primed plants (Fig.4.6 A).

Data from the shoots generated a significantly more condensed graph indicating an all-together weaker response than in roots. Nevertheless, similar to roots they showed less of a response in primed than non-primed plants (slope of response 0.69) Fig.4.6 B.

A)



B)

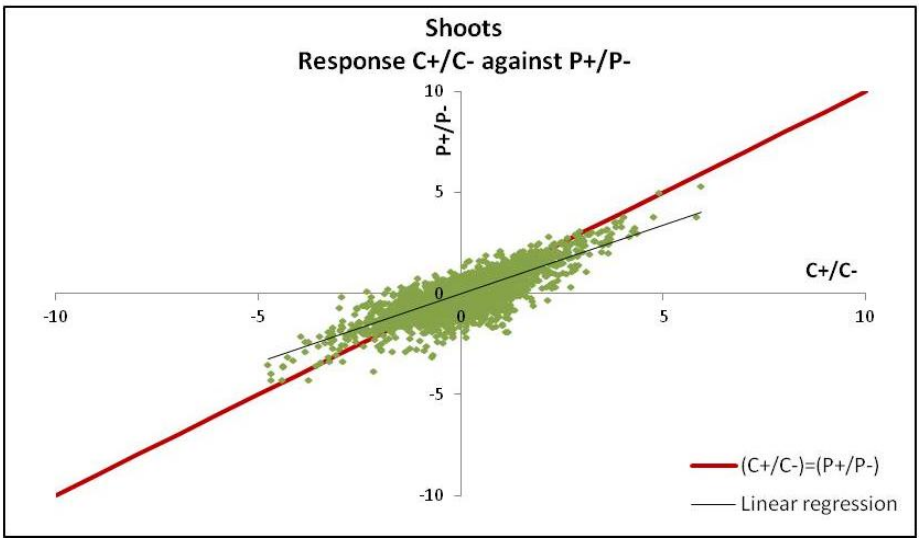


Fig. 4.6 Scatter plot showing the response to salt stress in primed and non-primed roots (A) and shoots (B).

The log of the response to salt calculated for primed plants (y axis, P+/P-) is plotted against the log of the response to salt calculated for control plants (x axis, C+/C-). Black line indicates the position of the linear regression for the data. Red line indicates y=x line; every point above this line represents a gene having a stronger response to salt in priming while every point below the line represents a gene having stronger response to salt in control.

4.3.2.5 Principal Component Analysis (PCA)

Partek® Genomics Suite™ was also used to perform PCA on the data set. In Fig. 4.7 is shown the position of the samples with respect to three components. In both tissues, the salt treated samples are clearly separated from the non-salt treated samples (Fig.4.7), indicating the strong effect of a 4 hours salt treatment on gene expression. Furthermore, the roots samples are also split into two clusters representing primed and non-primed plants (Fig.4.7A). This separation is only evident in the samples stressed with salt. This means that the factor Priming (P) presents a good source of variation between the samples but only in the stress factor group (+). In the shoot (Fig.4.7B), the samples are again separated in two main groups along the factor Stress, even though separation is less strong for roots samples. However, there was no clear separation of primed from non-primed samples for shoots unlike than roots.

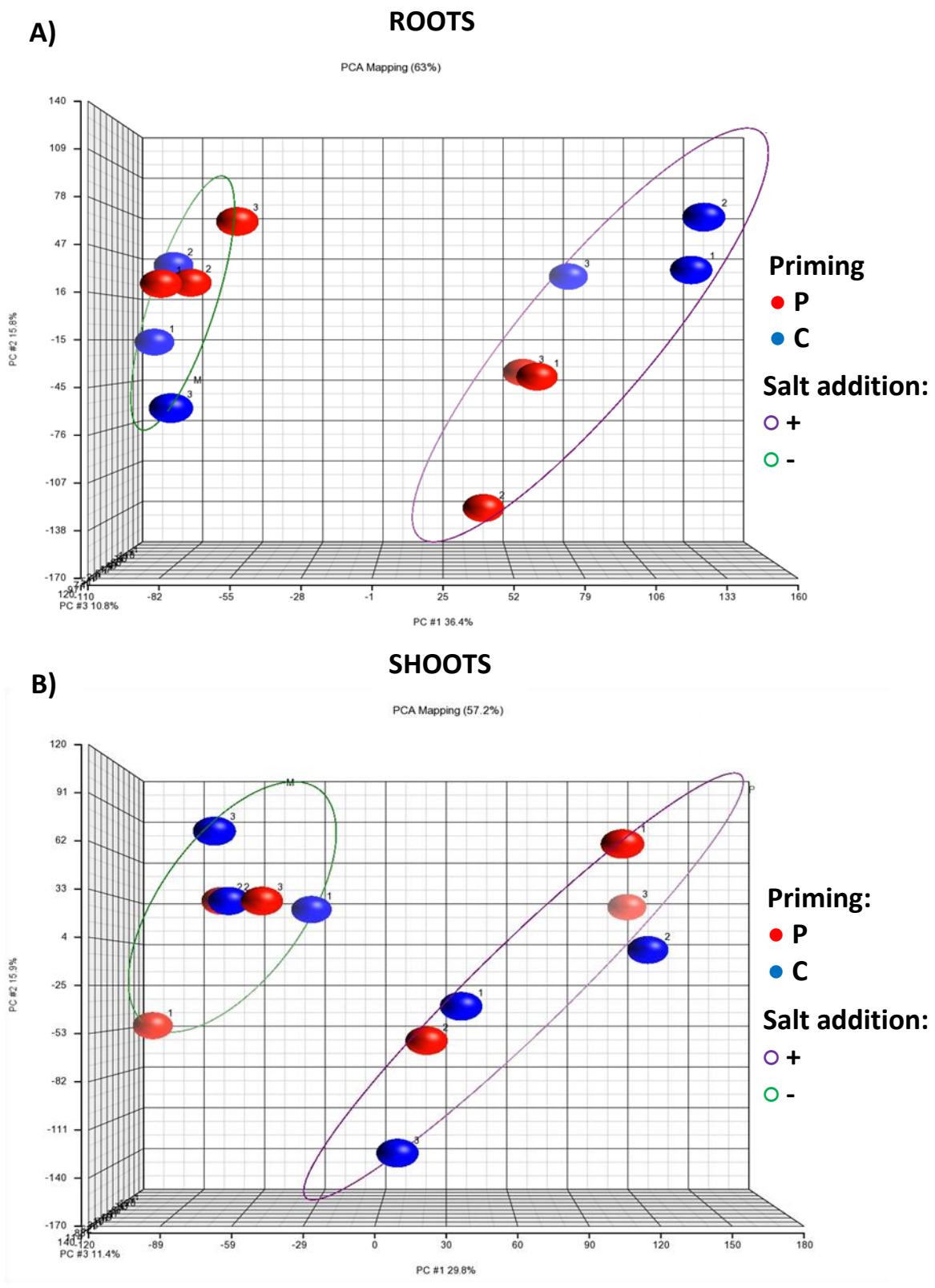


Fig. 4.7 PCA maps with roots (A) and shoots (B) samples plotted in three dimensions using their projections onto the first three principal components.
The different colours correspond to the factor Priming (Red: Primed, Blue: non-primed). Labels indicate the three different replicates (1: E1, 2: E2, 3: E3). The elliptical lines represent the factor Salt, + salt (purple) and - not salt (green).

4.3.2.6 Analysis of Variance (ANOVA)

Partek® Genomics Suite™ was used to perform ANOVA to investigate differential gene expression in the samples depending on the experimental conditions applied.
The ANOVA output is a table containing genes in 22,810 rows and columns containing p-values and fold changes for each of the contrasts presented in Tab.4.1.

Tab. 4.1 Factors used for PCA and ANOVA

FACTORS FOR PCA AND ANOVA ANALYSIS
Factor 1: Priming, Level: C, P
Factor 2: Stress, Level: -, +
Factor 3: Replication, Level: E1, E2, E3
Interaction Between Factors: Priming * Stress
Analysed contrast: P vs C, - vs +, P- vs P+, P+ vs C+, C+ vs C- and P- vs C-

The ANOVA performs a considerable high number of multiple tests due to the high number of genes, during which false positives may arise by virtue of performing many tests. In order to keep the overall error rate/false positives as low as possible, several statistic corrections have to be made. Therefore each contrast mentioned in Tab.4.1 was filtered several times according to different cut-off criteria for fold-change (FC), p-value and False Discovery Rate (FDR) values as presented in Tab.4.2.
One of the main properties of this dataset was that the changes in expression are predominant in the roots samples. In the roots of non-primed plants 3757 genes were identified as differentially expressed with FC ≥1.5, FDR ≤0.05 and p-value ≤ 0.05 after salt treatment. This number dropped of 25% when the plants had been previously primed. The analysis also identified 56 root genes as being differentially expressed in primed vs non-primed plants under salt + condition (P+ vs C+). This number decreased by about half when the fold change was raised to 2 (25 genes), and only one gene passed the filtering of a stringent p-value of 0.01. However, it is interesting to notice that even using less stringent criteria no genes entered the C-vs P- category in the roots samples.
In the shoots samples, with a cut off FC ≥1.5, FDR ≤0.05 and p-value ≤ 0.05 only 73 genes were identified as differentially expressed after salt treatment in non-primed plants (C+

vs C-) when exposed to a later salt stress in control condition. This number dropped to 3 genes when the plants had been previously primed (P+ vs P-). Also within these criteria none of 22,810 genes in total were differentially expressed in shoots of primed plants vs non-primed plants (P+ vs C+) exposed to salt stress. This was also the case even when the FDR was set to 0.1, although the number of genes differentially expressed under salt stress was now 648 in non-primed (C+ vs C-) and 281 in primed (P+ vs P-) shoots. To find any priming specific genes an unadjusted p-value had to be used, resulting in 28 genes entering in both (P+ vs C+) and (C- vs P)- categories.

Tab. 4.2 Number of genes differentially expressed in roots and shoots according to ANOVA analysis filtered by different criteria.
FC: Fold Change, FDR: False Discovery Rate. P: Primed, C: non-Primed, +: salt treated, -: not salt treated.

ROOTS	FC≥ 1.5 p-Value≤ 0.05 FDR≤ 0.05	FC≥ 2 p-Value≤ 0.05 FDR≤ 0.05	FC ≥1.5 p-Value≤ 0.05 FDR≤ 0.01
P-vsP+	2804	1387	1780
C-vsC+	3757	1925	2630
C-vsP-	0	0	0
P+vsC+	56	25	1

SHOOTS	FC≥ 1.5 p-Value≤0.05 FDR≤ 0.05	FC≥ 1.5 p-Value≤0.05 FDR≤ 0.1	FC≥ 1.5 Unadjusted p-Value FDR≤ 0.01	FC≥ 1.5 Unadjusted p-Value FDR≤ 0.05
P-vsP+	**3**	**281**	**862**	**1818**
C-vsC+	**73**	**648**	**1100**	**2149**
C-vsP-	**0**	**0**	**28**	**141**
P+vsC+	**0**	**0**	**28**	**159**

ANOVA Root lists P+ vs P- and C+ vs C- (FC≥ 1.5, p-value≤0.05, FDR≤0.05)

Within these criteria roots of non-primed plants strongly responded to the salt stress (C+ vs C-) differentially expressing a large number of genes. 2037 transcripts were up-regulated and 1721 were down-regulated. Shoots of control plants also responded to the treatment but in a minor way, 46 transcripts were up-regulated and 28 were down-regulated. Interestingly, in primed plants a considerably lower number of genes responded to salt stress (P+ vs P-): 1523 were up-regulated in roots (only 3 in shoots) and 1281 were down-regulated in roots, (only 1 in shoots). The weaker response in primed plants was not due to constitutively different expression levels in primed versus non-primed plants. At the given statistical criteria no gene was differentially expressed in primed (P-) versus non-primed plants (C-) that were not exposed to salt stress (Tab.4.2). However, after the salt treatment 11 transcripts were up-regulated and 45 transcripts were down regulated in primed plants compared to non primed plants (P+ vs C+).

The genes present in the roots lists P+ vs P- and C+ vs C- required a further examination. 628 out of 2804 genes present on the P+ vs P- were unique for the P list. 1581 out of 3757 genes present in the list C+ vs C-, do not overlap with the P list. This means a total of 2168 genes overlap between the two lists C+ vs C- and P+ vs P-. Functional annotated genes identified as differentially expressed by ANOVA will be analysed in section 4.3.3.2.

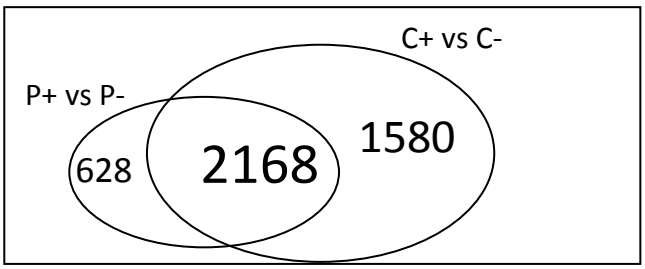


Fig. 4.8 Venn diagram representing the number of genes between the two root list P+ vs P- and C+ vs C-

4.3.2.7 Vector Analysis (VA)

Vector Analysis (VA) was used in order to visualize the differential response to the salt treatment of genes in primed and non-primed plants.

VA was achieved using an Excel file organized as detailed by Breitling et al., 2005, using an extended algorithm for 3 replicates. The number of genes in a given category was established according to the angle of the representative vector as specified in Tab.4.3.

Tab. 4.3 Angles that determine response categories in the VA.
The assignment of a particular gene to a response type was decided by calculating the angle between the representative vector and the various possible prototype vectors (Compare Fig. 4.1). Depending on the angle reported in the table a gene was categorized in one of the four categories. Same: same response to salt in control and priming, P specific: specific for primed plants, C specific: specific for non-primed plants, Opposite: opposite response in primed and non-primed plants.

GENE RESPONSE	VECTOR ANGLE	
Same	22.5°-67.5°	202.5°-247.5°
P specific	67.5°-112.5°	247.5°-292.5°
C specific	157.5°-202.5°	22.5°-337.5°
Opposite	112.5°-157.5°	292.5°-337.5°

Since the average length of the vector (L) indicates the average strength of the response, this parameter was used as a statistic correction to filter out genes that show a very low response together with the p-value obtained from the 3 replicates. One of the properties of this dataset was that genes showed the same response to salt treatment in primed and non-primed plants, in both roots and shoots. When (L) was set to greater than 1 (roughly corresponding to a two-fold expression change) the vast majority of the vectors of root genes resided at the periphery of the graph whereas many shorter vectors were found in the shoots, meaning that root responses in the replicates were more consistent (Fig.4.9). A larger number of genes appeared to have a specific response to salt in roots than in shoots, when the L-value is greater than 0.5 and a consistent p-value is set smaller than 0.05. From Fig.4.9 it also notable that a large number of genes are residing across the border of the established angle-categories and this makes an assignment to a response category problematic. Therefore safety margins for angles should be taken into account when individual genes are assigned to response categories.

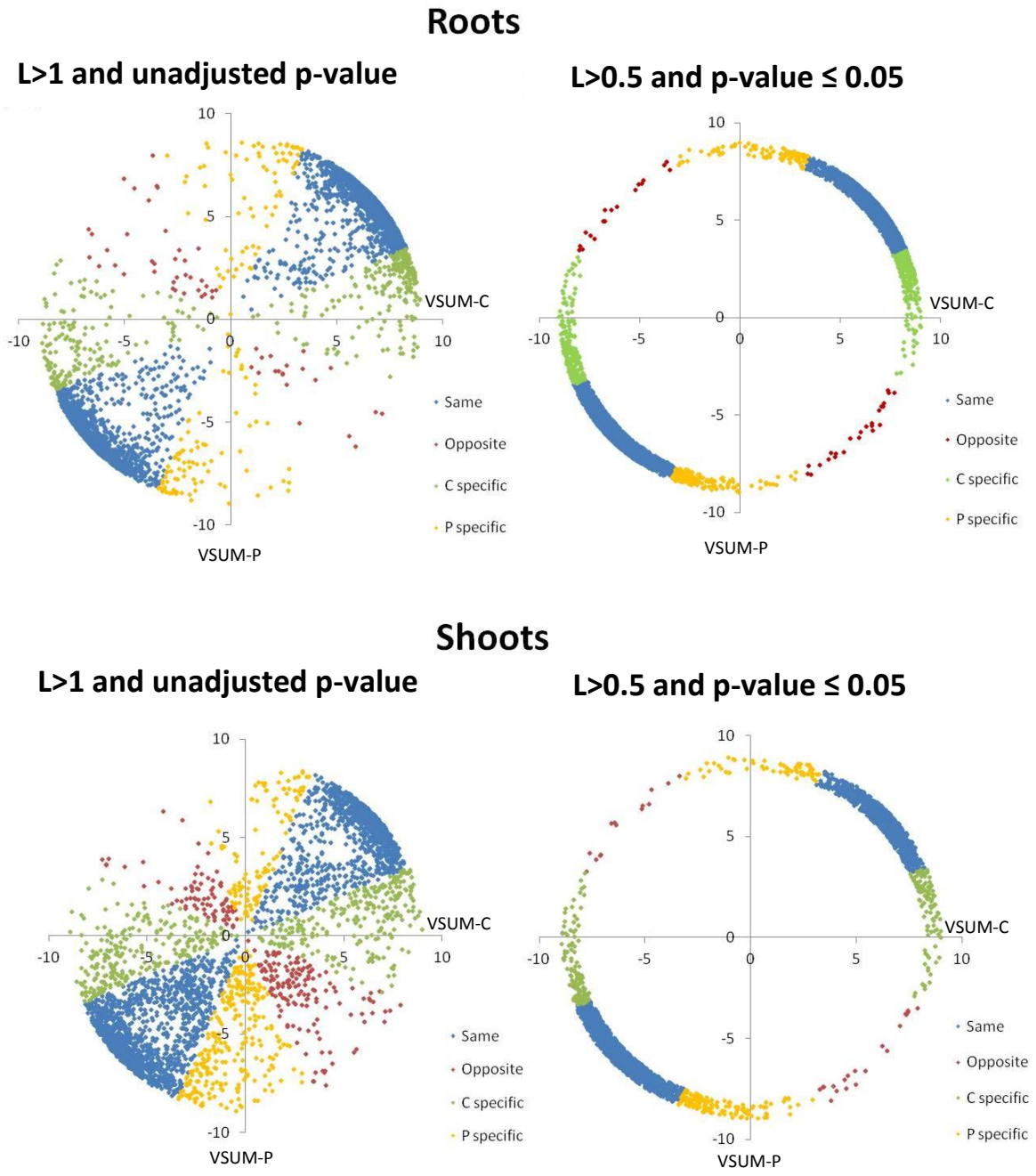


Fig. 4.9 Vectors representing gene responses to salt in root and shoot.
On the two axes are reported the sum vectors length for control and primed (x: VSUM-C control and y: VSUM-P primed). Two criteria were used to create the graphs: on the left average length vector (L) equal or larger than 1, on the right average length vector (L) equal or larger than 0.5 and p-value ≤ 0.05.
Each dot corresponds to a particular gene response in the form of a sum vector: the longer the vector then the more the response is consistent among replicates; the angle of the vector describes the gene response (angle types are specified in Tab. 4.3).
Genes responding similarly in primed and non-primed are shown in blue.
Genes that responded specifically in primed plants are shown in yellow.
Genes that changed specifically in non-primed plants are in green.
Genes that responded in opposite directions in primed and non-primed plants are shown in red.

As detailed in Tab. 4.4 vector analysis applied to shoots identified a large number of genes with specific responses at a consistent p-value smaller than 0.05 and L-value larger than 0.5. In particular 244 genes show a specific response in non-primed plants, 194 showed specific response in primed plants, 34 genes showed opposite responses in primed and non-primed plants. Among the 194 genes with P-specific responses more than half (134 out of 221) were down-regulated by salt (Tab.4.4). In contrast, vector analysis applied with the same statistical parameters to roots, identified an overall higher number of genes compared to the shoots VA. 454 genes showed a specific response in non-primed plants, 221 genes in primed plants with specific response in P and 42 showed opposite responses. Among the 221 root genes with P specific responses, more than half (132 out of 221) were down-regulated (as in shoots). Interestingly in the shoots, with L≥1 the number of genes that showed a specific response in non-primed plants was 513 while when the 0.05 p-value threshold was introduced the number dropped to 244 even though the (L) value was lowered to 0.5 (Tab.4.4). This reflects the representation in Fig.4.9 showing that lower consistency in the samples is due to the lower fold change of the response.

In summary VA revealed that primed plants responded less to the salt treatment.

Functional annotations of the genes assigned to different response categories are analysed in section 4.3.3.2.

Tab. 4.4 Number of genes in roots and shoots showing different types of prototypic behaviour in response to salt in two backgrounds of *Arabidopsis* plants (primed P, non-primed C), as identified by vector analysis respectively in roots and shoots.
The numbers of genes vary according to stringency of parameters selected, such as Vsum: sum vector, V length: average vector length from nine pairwise comparisons of the fold change. (C: control, P: priming down/up: up-/down- regulated gene).

Roots	no cut off	Vsum ≥3	p-value ≤0.05	V length ≤0.5	V length ≥1	V length≥0.5 AND p-value≤ 0.05
C specific up	1231	881	276	605	228	217
C specific down	3947	3210	937	773	168	237
TOT C specific	5178	4091	1213	1378	396	454
P specific up	1359	970	187	368	77	89
P specific down	1495	1130	224	597	88	132
TOT P specific	2854	2100	411	965	165	221
C and P up	4003	3496	2260	2918	1857	2104
C and P down	6715	5885	3250	4116	1308	2637
TOT Same	10718	9381	5510	7034	3165	4741
C up, P down	814	366	39	200	20	24
C down, P up	3246	2498	900	242	37	18
TOT Opposite	4060	2864	939	442	57	42

Shoots	no cut off	Vsum ≥3	p-value ≤0.05	V length ≥ 0.5	V length ≥1	V length≥0.5 AND p-value≤0.05
C specific up	1849	1149	193	881	254	120
C specific down	1811	1160	199	892	259	124
TOT C Specific	3660	2309	392	1773	513	244
P specific up	4062	2830	351	696	116	60
P specific down	1662	1030	205	929	273	134
TOT P Specific	5724	3860	556	1625	389	194
C and P up	5253	3915	1487	2566	1187	1216
C and P down	4699	3771	1619	3367	1359	1401
TOT Same	9952	7686	3106	5933	2546	2617
C up, P down	1237	531	56	537	147	20
C down, P up	2237	1221	136	409	81	14
TOT Opposite	3474	1752	192	946	228	34

4.3.3 Priming induces transcriptional changes in genes with specific functions

In the first part of this chapter several different algorithms were used to identify differentially expressed genes comparing primed and non-primed plants exposed to salt treatment or not. In the second part of this chapter the functions (known and predicted) of these genes are considered to establish the role of priming for plant stress responses.
In order to investigate the function of the genes, which were differentially expressed across the different experimental conditions, the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009) was used. This software permits one to identify genes within functional categories that were, as a group, differentially expressed between the priming (P) and control (C) condition, giving a p-value and a false discovery rate. Only roots samples were analyzed, since the filtered number of genes differentially expressed in shoots was too low.

Different gene lists were loaded into DAVID in order to answer the following questions:

- **Does priming introduce new functionalities into the salt stress response?**
  To answer this question functional annotation in the ANOVA gene lists P+ vs P-, C+ vs C- and P+ vs C+ were compared to functional annotations in the *Arabidopsis* genome background. This will indicate if new functional gene categories, normally not induced by salt, emerged upon priming.

- **Does priming regulate genes of specific functions in response to salt stress?**

  To answer this question I used 3 different types of gene lists obtained using the follow parameters:

  Gene List 1) "Ranked genes": the response to salt was calculated for each individual gene using the Equation1-Section 4.3.2.3. Genes were ranked according to their increased response to salt. Then, the top 300 genes (out of 712) were selected with expression ratio of P/C higher than 1.5 and the top 300 genes (out 1892) with expression ratio of C/P higher than 1.5. In particular these 300 selected genes showed expression ratios of primed/control ratio higher than 1.7 and

control/primed ratio higher than 2.2. This higher cut-off was used in an attempt to ensure that the selected genes were genuinely differentially responsive. Both lists were then compared against two backgrounds. Background 1 “roots transcriptome”: gene list with average absolute signal intensities greater than 10 and Background 2 “salt responsive genes”: list that combined genes with average ratio P +/- greater than 1.5, with genes of an average ratio C+/- greater than 1.5.

Gene List 2) “VA P specific-up” and “VA P specific-down” against Background 1 “Complete VA list” and Background 2 “salt responsive genes”: ANOVA combined lists P +/- AND C+/-”.

Gene List 3) “ANOVA P+/- list Unique P-up” and “ANOVA P+/- list Unique P-down” against the Background1 “complete ANOVA list P +/-” and against Background2 “salt responsive genes: ANOVA combined lists P +/- AND C+/-”.

Using these criteria I will show that the different comparisons identified similar functional clusters as being responsive to salt in primed and non-primed plants but also identified a specific subset of these clusters that responded more or less strongly to salt in primed plants.

Tab. 4.5 Schematic summary of gene lists compared with DAVID.

Does priming introduce new functionalities into the salt stress response?		
ANOVA LIST P +/-	**List loaded in DAVID**	**Background**
	Total list P+/-	Arabidopsis
ANOVA LIST C+/-	**List loaded into DAVID**	**Background**
	Total list C+/-	Arabidopsis
ANOVA LIST P+/C+	**List loaded into DAVID**	**Background**
	Total list P+/C+	Arabidopsis

Does priming regulate genes of specific functions in response to salt stress?			
VA LIST p-Value 0.05 l>0.5	**List loaded into DAVID**	**Background 1**	**Background 2**
	Specific for P UP	Total VA list	Salt responsive genes from ANOVA lists P +/- AND C+/- combined
	Specific for P DOWN	Total VA list	Salt responsive genes from ANOVA lists P +/- AND C+/- combined
RANKED RESPONSE LIST	**List loaded into DAVID**	**Background 1**	**Background 2**
	Top 300 genes which salt response P>C	Roots transcriptome with average signal intensities > 10*	Salt responsive genes: P +/- and C+/- (fold changed 1.5) combined
	Top 300 genes which salt response C>P	Roots transcriptome with average signal intensities > 10*	Salt responsive genes: P +/- and C+/- (fold changed 1.5) combined
ANOVA LIST P +/-	**List loaded into DAVID**	**Background 1**	**Background 2**
	Unique for P UP	ANOVA LIST P +/-	Salt responsive genes from ANOVA lists P +/- AND C+/- combined
	Unique for P DOWN	ANOVA LIST P +/-	Salt responsive genes from ANOVA lists P +/- AND C+/- combined

* Based on absolute average signal intensities of RMA normalised microarray data

4.3.3.1 Does priming induce an enrichment of new functional gene categories in response to the salt stress?

ANOVA gene lists P+ vs P- and C+ vs C-

To answer this question the ANOVA gene lists P+ vs P- and C+ vs C- (statistical criteria Tab. 4.2) were loaded into DAVID and functional annotations compared with the *Arabidopsis* genome background. In both lists the highest enrichment score was assigned to the category "GO: Response to organic substance" that included genes involved in the biosynthesis of plant hormones (e.g. ethylene, ABA, brassinosteroid, gibberellic acid). Other categories significantly enriched in both lists included: "Response to organic substance", "Toxin metabolic process", "Secondary metabolic process", "Response to abiotic stimulus", "Plant-type/cell wall" and "Transcription regulator" (Tab. 4.6). The results of this analysis showed that upon stress primed and non-primed plants activated the same functional gene categories.

Tab. 4.6 Enriched GO categories among genes responding to salt in primed and non-primed plants. Gene lists based on ANOVA list C+ vs C-; and list P+ vs P- (FDR ≤ 0.05, p-value ≤ 0.05 and FC ≥ 1.5).

P+ vs P-

Annotation Cluster	GOTERM	Enrichment Score	Count	%	p-Value	FDR
1	GO:0010033~response to organic substance	8.2	255	9.1	3.62E-24	6.11E-21
2	GO:0009407~toxin catabolic process	6.3	21	0.8	6.23E-08	1.05E-04
3	GO:0019748~secondary metabolic process	5.6	97	3.5	9.65E-11	1.63E-07
4	GO:0009628~response to abiotic stimulus	4.8	195	7.0	1.05E-06	0.001763
5	GO:0005618~cell wall	3.5	94	3.4	2.15E-04	0.281909
6	GO:0006955~immune response	3.1	69	2.5	2.11E-08	3.56E-05
7	GO:0015238~drug transporter activity	2.8	24	0.9	4.14E-05	0.064842
8	GO:0050662~coenzyme binding	2.5	63	2.3	0.001534	2.373695
9	GO:0005911~cell-cell junction	2.5	9	0.3	2.64E-04	0.347339
10	GO:0006979~response to oxidative stress	2.5	68	2.4	2.10E-08	3.54E-05

C+ vs C-

Annotation Cluster	GOTERM	Enrichment Score	Count	%	p-Value	FDR
1	GO:0010033~response to organic substance	11.9	283	9.5	4.185E-30	7.07E-27
2	GO:0009404~toxin metabolic process	7.2	22	0.7	3.119E-08	5.27E-05
3	GO:0019748~secondary metabolic process	6.1	105	3.5	4.296E-12	7.26E-09
4	GO:0003700~transcription factor activity	4.2	282	9.5	1.563E-08	2.46E-05
5	GO:0006955~immune response	4.2	77	2.6	2.523E-10	4.26E-07
6	GO:0009628~response to abiotic stimulus	3.9	222	7.4	4.819E-10	8.14E-07
7	GO:0009505~plant-type cell wall	3.5	53	1.8	7.945E-05	0.1051
8	GO:0006979~response to oxidative stress	3.1	68	2.3	2.420E-07	4.09E-04
9	GO:0009644~response to high light intensity	3.0	15	0.5	6.630E-04	1.1145
10	GO:0055114~oxidation reduction	3.0	198	6.6	1.160E-05	0.0195

ANOVA gene lists P+ vs C+

The list contains 56 genes differentially expressed between primed and non-primed plants under salt stress. Although the list contains a relative small number of genes 37 of them were still grouped into clusters by DAVID, however the FDR values obtained are very high (Tab.4.7). 23.3% of the genes are included in the category "intrinsic to membrane". This category contained genes involved in ionic and osmotic signalling together with transporters and ion channels, such *MPK3* (mitogen activated protein kinase 3), *CRK6* (RECEPTOR-LIKE PROTEIN KINASE 6), a member of the cyclic nucleotide gated channel family (ATCNGC13, CYCLIC NUCLEOTIDE-GATED CHANNEL 13) and a member of a family of proteins related to purine transporters (PUP14- PURINE PERMEASE 14). Also cell wall biosynthesis related genes are included in this category such as CELLULOSE SYNTHASE LIKE E1, a beta-glycan synthase that polymerizes the backbones of hemicelluloses. 18.3% of the genes were grouped in the cluster "response to organic substance" that included genes related to plant hormone pathways, in particular ERF019 (ethylene responsive element binding factor) and JAZ6 (jasmonate-ZIM-domain protein). Finally 10% of the genes were annotated as "immune response/defence" including genes from the salicylic acid signalling pathway such as *WRKY* transcription factors (*WRKY48*, *WRKY8*) and a gene from the cytokine signalling pathway, *ARR15* (RESPONSE REGULATOR 15), which encodes a nuclear response regulator acting as a negative regulator. In conclusion, these clusters are similar to the ones enriched in the ANOVA lists previously described confirming that the priming treatment did not alter expression of genes with new functions.

Tab. 4.7 Enriched GO categories for genes showing differential expression between primed and non-primed salt-treated plants based on ANOVA list P+ vs C+ (FDR ≤ 0.05, p-value ≤ 0.05 and FC ≥ 1.5).

Cluster 1	Enrichment Score: 1.9	Count	%	p-value	FDR
GOTERM_BP_FAT	GO:0006955~immune response	6	10.0	2.64E-03	3.35
Cluster 2	Enrichment Score: 1.3	Count	%	p-value	FDR
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	14	23.3	1.01E-03	0.908
Cluster 3	Enrichment Score: 1.07	Count	%	p-value	FDR
GOTERM_BP_FAT	GO:0010033~response to organic substance	11	18.3	4.19E-03	5.27

4.3.3.2 Does priming regulate genes of specific function in response to salt stress?

In order to answer this question a number of different approaches were used as summarized in Tab.4.5. All three lists loaded into DAVID identified similar functionality clusters however the “Ranked response list” was the only list showing consistently low FDR and p-values while both lists “ANOVA P+/- Unique P” and “VA- P specific” resulted in high FDR. Therefore, the “Ranked response list” was selected for further analysis but also subjected to further controls. To evaluate the incidence of false positive a random selection of 300 genes from all genes represented on the microarray was loaded into DAVID and this was repeated with three randomized lists. The obtained ‘enriched’ clusters were different for every random set of genes and had very high p-values and FDR. The “ranked response lists” Primed>Control and Control>Primed were loaded into DAVID. The analysis delineated a bias in function between the genes that responded more strongly to salt stress in primed than in non-primed plants and the genes that responded less.

4.3.3.3 Gene categories that responded more strongly to salt upon priming

- List: Top 300 genes Primed>Control
- DAVID analysis background: salt responsive genes/ roots transcriptome

Results from DAVID analysis reported in Tab. 4.8 showed the main categories with a FDR lower than 0.05 were *Ion binding related* (including GO:0005509~calcium, "GO:0020037~heme binding", "GO:0006800~oxygen and reactive oxygen species metabolic process", "GO:0034599~cellular response to oxidative stress") and *cell wall related* (including "GO:0005199~structural constituent of cell wall", "GO:0042545~cell wall modification", "GO:0009664~plant-type cell wall organization"). The enriched GO categories for "Ranked response genes" gave similar results when compared against the two different backgrounds (Appendix IV).

Because DAVID clusters are redundant (meaning that the same gene can be shown in two different clusters), redundant genes were manually excluded, then the genes underlying the enriched lists were compared resulting in a core set of functionally related genes underlying the difference in salt response between primed and non-primed plants, which is shown in Tab. 4.8. In accordance with the previously ANOVA and VA analysis, the vast majority of these genes had a higher expression level in the primed plants only after salt stress. Thus, if the stress did not reoccur, expression in primed and non-primed plants was the same.

Tab.4.8 Enriched GO categories for genes showing higher response to salt in primed plants compared to non- primed plants in the selected background (FDR≤0.05).
The top 300 genes from the list Primed>Control are compared against the roots specific background.

	GO:0020037~heme binding	GO:0046906~tetrapyrrole binding	GO:0042744~hydrogen peroxide catabolic process	GO:0070301~cellular response to hydrogen peroxide	GO:0004601~peroxidase activity	GO:0016684~oxidoreductase activity, acting on peroxide as	GO:0042743~hydrogen peroxide metabolic process	GO:0005506~iron ion binding	GO:0034614~cellular response to reactive oxygen species	GO:0034599~cellular response to oxidative stress	GO:0005509~calcium ion binding	GO:0016209~antioxidant activity	GO:0009055~electron carrier activity	GO:0006800~oxygen and reactive oxygen species metabolic	GO:0042542~response to hydrogen peroxide	GO:0000302~response to reactive oxygen species	GO:0055114~oxidation reduction	GO:0005199~structural constituent of cell wall	GO:0009664~plant-type cell wall organization	GO:0007047~cell wall organization	GO:0045229~external encapsulating structure organizatio	GO:0030599~pectinesterase activity	GO:0009664~plant-type cell wall organization
AT5G22410	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT5G66390	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G44970	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G68850	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT2G18980	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT4G26010	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT2G39040	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G30870	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT5G17820	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G05250	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT3G49960	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT5G08250	✓	✓						✓					✓				✓						
AT2G25160	✓	✓						✓					✓				✓						
AT5G06900	✓	✓						✓					✓				✓						
AT2G42250	✓	✓						✓					✓				✓						
AT4G12330	✓	✓						✓					✓				✓						
AT3G20110	✓	✓						✓					✓				✓						
AT4G31940	✓	✓						✓					✓				✓						
AT1G13710	✓	✓						✓					✓				✓						
AT3G10520	✓	✓						✓															
AT4G11230					✓	✓		✓			✓	✓	✓				✓						
AT4G25090						✓		✓			✓	✓	✓				✓						

	GO:0020037~heme binding	GO:0046906~tetrapyrrole binding	GO:0042744~hydrogen peroxide catabolic process	GO:0070301~cellular response to hydrogen peroxide	GO:0004601~peroxidase activity	GO:0016684~oxidoreductase activity, acting on peroxide as	GO:0042743~hydrogen peroxide metabolic process	GO:0005506~iron ion binding	GO:0034614~cellular response to reactive oxygen species	GO:0034599~cellular response to oxidative stress	GO:0005509~calcium ion binding	GO:0016209~antioxidant activity	GO:0009055~electron carrier activity	GO:0006800~oxygen and reactive oxygen species metabolic	GO:0042542~response to hydrogen peroxide	GO:0000302~response to reactive oxygen species	GO:0055114~oxidation reduction	GO:0005199~structural constituent of cell wall	GO:0009664~plant-type cell wall organization	GO:0007047~cell wall organization	GO:0045229~external encapsulating structure organizatio	GO:0030599~pectinesterase activity	GO:0009664~plant-type cell wall organization
AT1G80320								✓									✓						
AT4G19690								✓			✓												
AT1G52800								✓									✓						
AT1G06090								✓									✓						
AT1G06080								✓									✓						
AT4G22080											✓												
AT3G62950													✓				✓						
AT3G14415													✓				✓						
AT1G24620											✓												
AT1G11920											✓												
AT3G51860											✓												
AT5G46370											✓												
AT3G07490											✓												
AT4G13440											✓												
AT2G32300													✓										
AT5G50590																	✓						
AT4G29740																	✓						
AT4G33790																	✓						
AT5G03260																	✓						
AT1G54970																		✓					
AT4G08410																		✓	✓				✓
AT1G26240																		✓	✓				✓
AT3G20470																		✓					

	GO:0020037~heme binding	GO:0046906~tetrapyrrole binding	GO:0042744~hydrogen peroxide catabolic process	GO:0070301~cellular response to hydrogen peroxide	GO:0004601~peroxidase activity	GO:0016684~oxidoreductase activity, acting on peroxide as	GO:0042743~hydrogen peroxide metabolic process	GO:0005506~iron ion binding	GO:0034614~cellular response to reactive oxygen species	GO:0034599~cellular response to oxidative stress	GO:0005509~calcium ion binding	GO:0016209~antioxidant activity	GO:0009055~electron carrier activity	GO:0006800~oxygen and reactive oxygen species metabolic	GO:0042542~response to hydrogen peroxide	GO:0000302~response to reactive oxygen species	GO:0055114~oxidation reduction	GO:0005199~structural constituent of cell wall	GO:0009664~plant-type cell wall organization	GO:0007047~cell wall organization	GO:0045229~external encapsulating structure organizatio	GO:0030599~pectinesterase activity	GO:0009664~plant-type cell wall organization
AT3G19430																		✓					
AT3G28550																		✓	✓				✓
AT5G35190																		✓	✓				✓
AT5G06640																		✓	✓				✓
AT5G06630																		✓	✓				✓
AT1G12560																			✓	✓	✓		
AT2G20750																			✓	✓	✓		✓
AT1G62980																			✓	✓	✓		✓
AT5G56320																			✓	✓	✓		✓
AT1G48930																				✓	✓		
AT1G05650																				✓	✓		
AT2G43050																				✓	✓	✓	
AT5G04970																				✓	✓	✓	
AT4G28850																				✓	✓		
AT3G59850																				✓	✓		
AT2G15350																				✓	✓		
AT5G57530																				✓	✓		
AT1G53830																				✓	✓	✓	
AT4G15290																				✓	✓		
AT1G65570																				✓	✓		
AT5G51490																				✓	✓	✓	
AT5G04960																						✓	
AT2G23630																						✓	
AT5G51520																						✓	
AT4G25250																						✓	
AT4G37160																						✓	

In the following sections, individual functions within the enriched categories are described in more detail.

Ion binding and transporters

A higher response to salt upon priming was found for the gene *IRON-REGULATED TRANSPORTER 1* (AT4G19690, *IRT1*) which encodes Fe^{2+} transporter protein with broad specificity for divalent heavy metals, mediating the transport of zinc, manganese, cobalt and cadmium. It has also been recognized as the major transporter responsible for high-affinity metal uptake under iron deficiency (Vert et al., 2002).

Several calcium ion binding genes were also found be enriched. *CALMODULIN-LIKE 3* (*CML3*, AT3G07490) contribute into the calcium/calmodulin regulation network in the peroxisomes (Chigri et al., 2012). Calcium-binding EF-hand family protein genes (*AT4G13440* and *AT1G24620*) were found. In particular, it has been shown that mutation in the gene *AT1G24620*, led to longer root hairs under Pi-deficient conditions (Lin et al., 2011).

It was also found CATION EXCHANGER 3 (AT3G51860, CAX3), a Ca^{2+}/H^{+} tonoplast antiporter that mediates the sequestration of Ca^{2+} into the vacuole. *Arabidopsis* knockout mutants or over-expression of *CAX3* results in perturbations in ion homeostasis and altered responses to salinity and cold stresses (Zhao et al., 2008). Finally, a member of the *Arabidopsis thaliana* K+ channel family of AtTPK/KCO proteins (*AT5G46370*, *KCO2*), was also found. This gene is reported as being a Ca^{2+} activated outward rectifying K^{+} channel 2 and located at the vacuolar membrane.

Oxidation reduction

Genes in this class are involved in ionic and osmotic homeostasis signalling together with transporters and ion channels. 12 genes classified as “Peroxidase” have been found as particularly enriched in the category. The oxidoreductase *AtHSD4* (hydroxysteroid dehydrogenase 4) was 4.9 times higher expressed after the second stress in the primed plants but did not present any change after the priming without second salt exposure. Members of electron carriers involved in ion binding, *CYP93D1* and *CYP712A1* were respectively 3 and 2.5 times higher expressed in the primed plants after but not before

the second stress. These are presumably involved in oxidative signalling responding to altered levels of various reactive oxygen species (ROS).

Cell wall related proteins

Genes associated with cell wall biosynthesis important for correct cell developments were also found to respond stronger upon stress in primed roots. Among them were structural constituents of the cell wall such as *ATPRP1* (*PROLINE-RICH PROTEIN 1*, *AT1G54970*) or genes required for correct cell expansion such as *AGP30* (*ARABINOGALACTAN PROTEIN 30*) and *SP1L5* (*SPIRAL1-LIKE5*), which regulates cortical microtubule organization (Nakajima et al., 2004; van Hengel and Roberts, 2003). The *SKU5* and *SKS6* genes (*SKU5 Similar 15* and *SKU6 Similar 16*) encoding multiple-copper oxidases play an important role in regulating directional root growth (Sedbrook et al., 2002; Jacobs and Roe, 2005). *SKS16* was induced upon priming: in non-primed plants the level of transcription dropped when the salt was applied whereas in primed plants the level of expression is maintained high. *SKS15* was higher expressed in primed plants only upon salt.

Additional individual genes found more responsive to salt upon priming

Several individual genes were found among the ranked list as been most responsive but not among the enriched DAVID categories.

Numerous genes encoding disease responsive proteins and proteins involved in SA biosynthesis such as *ICS2* (*ISOCHORISMATE SYNTHASE 2*) were found to be more responsive to the salt treatment in primed plants compared to non-primed.

A member of glutathione transferase family GSTs (*ATGSTF13*) may be involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH).

The gene encoding the unique largest subunit of nuclear DNA-dependent RNA polymerase V (*AT2G40030*) was also differentially responsive to salt stress in primed and non-primed plants.

A member of the *SYP12* gene family *SYP123* (*SYNTAXIN OF PLANTS 123*) known to be involved in the tip growth of root hairs (Enami et al., 2009) was found to be more responsive to salt upon priming. It has been shown that this gene plays an essential role in the membrane fusion event that occurs at the final step of membrane trafficking; it is

specifically expressed in the root hair cells and showed focal accumulation to the tip region of the growing root hairs (Enami et al., 2009).

Enrichment of several functional categories was based on genes encoding ion transporters. Among these *SLAH1* (*SLAC1 HOMOLOGUE 1*, *AT1G62280*) was the only one that showed higher expression in primed roots even prior to application of salt. *SLAH1* encodes a root-specific anion channel protein similar to the guard-cell SLAC1 known to be involved in stomatal closure (Negi et al., 2008). All other transporter genes showed similar expression in primed and non-primed plants before application of salt but responded more strongly to the second stress in primed plants. *ATCHX16* (*CATION/H+ EXCHANGER 16*) belongs to a family of cation/H+ antiporters some of which have been shown to be involved in K(+) and pH homeostasis of distinct intracellular compartments (Chanroj et al., 2011). *AMT1-1* (*AMMONIUM TRANSPORTER 1-1*, *AT4G13510*) and *ATAMT1-2* (*AMMONIUM TRANSPORTER 1-2*, *AT1G64780*) encode proteins essential for ammonium uptake (Yuan et al., 2007). Ammonium can enter the symplastic route for radial transport toward the root stele via AMT1-1, AMT1-3, and AMT1-5, which are localized at the plasma membrane of rhizodermis cells, including root hairs. Ammonium can also bypass outer root cells via the apoplastic transport route and subsequently enter the root symplast by AMT1-2 mediated transport across the plasma membrane of endodermal (in the root hair zone) and cortical (in the basal root zones) cells (Yuan et al., 2007).

The expression profiles of selected genes were further confirmed using qPCR (Fig. 4.10).

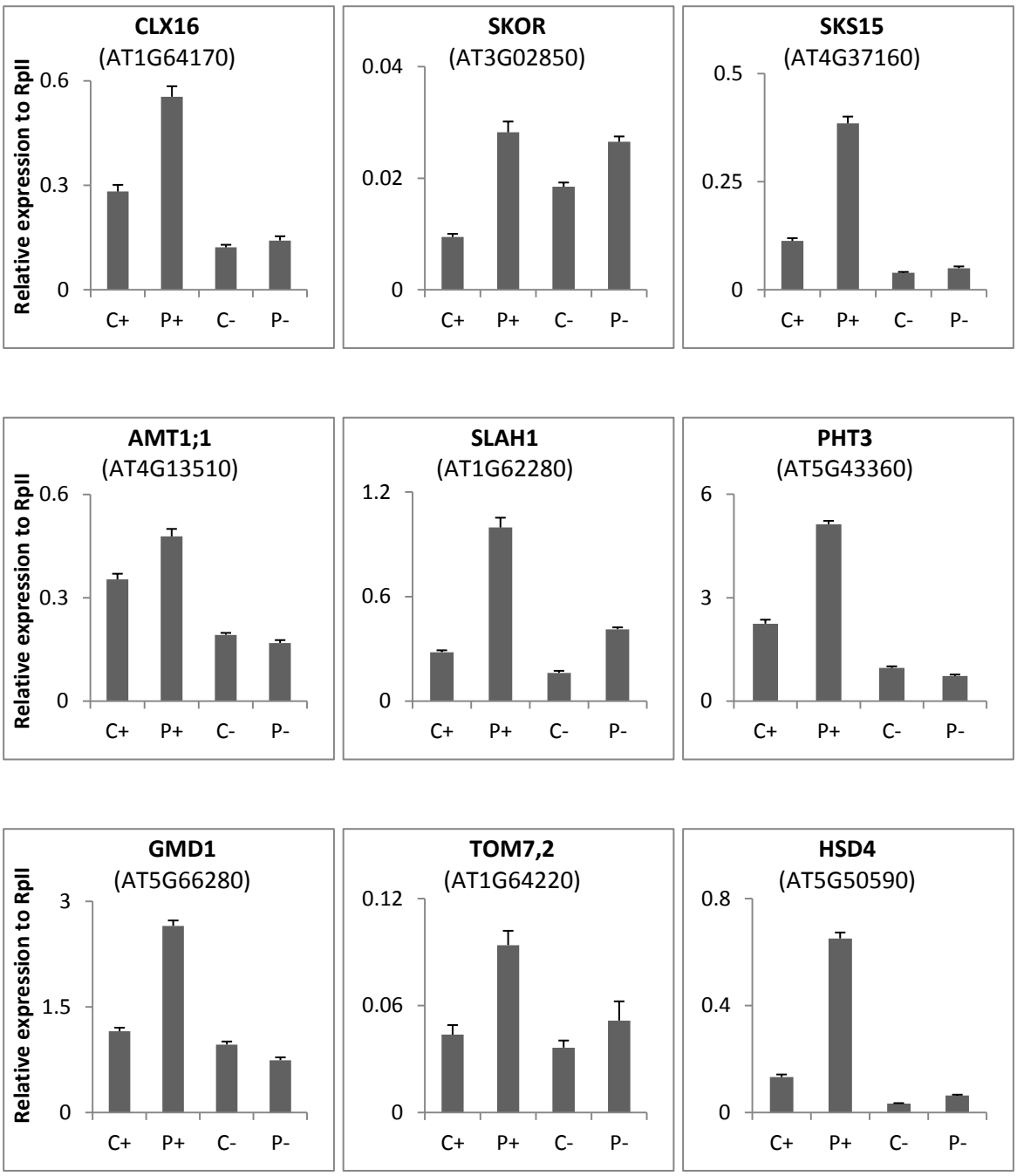


Fig. 4.10 Real time quantitative PCR (qPCR) analysis of relative expression of selected genes that responded higher to the salt in primed roots samples.
Total RNA was isolated from one experimental sample, reverse transcribed and cDNA was analyzed by qPCR using primers for genes that showed differential responses on the microarrays (see above). *RpII* was used as reference gene for the normalization. Standard errors are from four pairwise comparisons of technical duplicates.

4.3.3.4 Gene categories that responded less to the salt stress upon priming

- List: Top 300 genes Control>Primed
- DAVID analysis background: salt responsive genes/roots transcriptome

DAVID analysis reported in Tab. 4.9 shows that the enriched GO categories for "Ranked response genes" and results were similar independent of the chosen background (Appendix V). The program identified three main enriched categories with a FRD lower than 0.05: transcription factors (including GO:0003700~ transcription factor activity, GO:0006350~ transcription, GO:0030528~transcription regulator activity, GO:0006355~regulation of transcription, DNA-dependent, GO:0051252~regulation of RNA metabolic process, GO:0045449~ regulation of transcription), hormonal response genes (including GO:0010033~ response to organic substance, and GO:0009723~response to ethylene stimulus), and oxidative stress related genes (GO:0009644~response to high light intensity).

The vast majority of the genes in the list showed a higher transcript level in the non-primed plants only after salt stress as predicted by the previous ANOVA and VA analysis. Thus, when the stress did not occur the gene expression was the same in primed and non-primed plants.

Tab.4.9 Enriched GO categories for genes showing lower response to salt in primed plants compared to non-primed plants in the selected background (FDR≤0.05).
The top 300 genes from the list Control>Primed are compared against roots specific background.

	GO:0009723~response to ethylene stimulus	GO:0010033~response to organic substance	GO:0009719~response to endogenous stimulus	GO:0003700~transcription factor activity	GO:0009873~ethyleneediatalg pathway	GO:0000160~two-component signal transduction system	GO:0030528~transcription regulator activity	GO:0006350~transcription	GO:0006355~regulation of transcription, DNA-dependent	GO:0051252~regulation of RNA metabolic process	GO:0009725~response to hormone stimulus	GO:0045449~regulation of transcription	GO:0003677~DNA binding	GO:0009644~response to high light intensity	GO:0009642~response to light intensity	GO:0009628~response to abiotic stimulus	GO:0009644~response to high light intensity	GO:0009642~response to light intensity	GO:0009408~response to heat
AT3G23230	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT5G13330	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT3G23250	√	√	√	√	√	√	√	√	√	√	√	√	√			√			
AT1G74430	√	√	√	√			√		√	√	√	√	√						
AT5G40990	√	√	√		√	√	√	√	√	√	√	√	√						
AT1G43160	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT4G11280	√	√	√								√					√			
AT2G44840	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT5G51190	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT3G23240	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT5G61890	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT3G50060	√	√	√	√			√		√	√	√	√	√			√			
AT4G05100	√	√	√	√			√		√	√	√	√	√			√			
AT5G64900	√	√	√								√								
AT3G47600	√	√	√	√			√				√	√	√			√			
AT1G28370	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT3G06490	√	√	√	√			√		√	√	√	√	√			√			
AT2G47520	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT1G19210	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT1G22810	√	√	√	√	√	√	√	√	√	√	√	√	√						
AT3G23220	√	√	√	√	√	√	√	√	√	√	√	√	√						

	GO:0009723~response to ethylene stimulus	GO:0010033~response to organic	GO:0009719~response to endogenous	GO:0003700~transcription factor activity	GO:0009873~ethyleneediatalg pathway	GO:0000160~two-component signal	GO:0030528~transcription regulator	GO:0006350~transcription	GO:0006355~regulation of transcription,	GO:0051252~regulation of RNA metabolic	GO:0009725~response to hormone	GO:0045449~regulation of transcription	GO:0003677~DNA binding	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009628~response to abiotic stimulus	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009408~response to heat
AT1G21910	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT5G07310	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G15520	✓	✓	✓								✓								
AT2G47190	✓	✓	✓	✓			✓				✓	✓	✓			✓			
AT5G47220	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT5G61600	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G74930	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT4G34410	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓						
AT1G21250		✓																	
AT1G27730		✓	✓	✓			✓				✓		✓	✓	✓	✓	✓	✓	
AT5G13930		✓	✓													✓			
AT4G30270		✓	✓								✓								
AT1G17380		✓	✓				✓				✓								
AT5G49620		✓	✓	✓			✓		✓	✓	✓	✓	✓			✓			
AT5G10380		✓																	
AT5G59820		✓		✓			✓						✓		✓	✓		✓	✓
AT1G72450		✓	✓					✓				✓							
AT1G17420		✓	✓											✓	✓	✓	✓	✓	
AT3G26830		✓	✓								✓								
AT5G09980		✓	✓																
AT2G26740		✓	✓								✓					✓			
AT1G80840		✓		✓			✓	✓	✓	✓		✓	✓						
AT1G56150		✓	✓								✓								
AT5G66700		✓	✓	✓			✓	✓	✓	✓	✓	✓	✓						
AT1G70700		✓	✓				✓				✓								
AT4G26120		✓																	
AT3G16530		✓																	
AT1G20823		✓																	
AT5G22570		✓		✓			✓	✓	✓	✓		✓	✓						
AT3G56400		✓	✓	✓			✓	✓	✓	✓		✓	✓						
AT2G24850		✓	✓																

	GO:0009723~response to ethylene stimulus	GO:0010033~response to organic	GO:0009719~response to endogenous	GO:0003700~transcription factor activity	GO:0009873~ethyleneediatalg pathway	GO:0000160~two-component signal	GO:0030528~transcription regulator	GO:0006350~transcription	GO:0006355~regulation of transcription,	GO:0051252~regulation of RNA metabolic	GO:0009725~response to hormone	GO:0045449~regulation of transcription	GO:0003677~DNA binding	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009628~response to abiotic stimulus	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009408~response to heat
AT2G46400		✓		✓			✓	✓	✓	✓		✓	✓						
AT5G57560		✓	✓								✓					✓			✓
AT1G28480		✓	✓																
AT5G13220		✓	✓					✓				✓							
AT2G17040		✓		✓			✓						✓						
AT4G37850				✓			✓	✓				✓	✓						
AT5G54470				✓			✓					✓	✓						
AT1G01720				✓			✓	✓				✓	✓						
AT3G63350				✓			✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓
AT5G05410				✓			✓	✓	✓	✓		✓	✓			✓			✓
AT3G01970				✓			✓	✓	✓	✓		✓	✓						
AT4G25490				✓			✓	✓	✓	✓		✓	✓			✓			
AT1G52890				✓			✓	✓				✓	✓			✓			
AT5G22380				✓			✓	✓				✓	✓						
AT1G80590				✓			✓	✓	✓	✓		✓	✓						
AT2G38250				✓			✓					✓	✓						
AT1G12610				✓			✓	✓	✓	✓		✓	✓			✓			
AT3G11580				✓			✓	✓	✓	✓		✓	✓						
AT3G11020				✓			✓	✓	✓	✓		✓	✓			✓			✓
AT2G40740				✓			✓	✓	✓	✓		✓	✓						
AT5G46350				✓			✓	✓	✓	✓		✓	✓						
AT2G18550				✓			✓	✓	✓	✓		✓	✓						
AT2G22760				✓			✓	✓				✓	✓						
AT1G30135				✓								✓							
AT3G44260				✓								✓							
AT5G10760												✓							
AT4G25380												✓							
AT1G52560														✓	✓	✓	✓	✓	✓
AT5G12030														✓	✓	✓	✓	✓	✓
AT1G54050														✓	✓	✓	✓	✓	✓

	GO:0009723~response to ethylene stimulus	GO:0010033~response to organic	GO:0009719~response to endogenous	GO:0003700~transcription factor activity	GO:0009873~ethyleneediatalg pathway	GO:0000160~two-component signal	GO:0030528~transcription regulator	GO:0006350~transcription	GO:0006355~regulation of transcription,	GO:0051252~regulation of RNA metabolic	GO:0009725~response to hormone	GO:0045449~regulation of transcription	GO:0003677~DNA binding	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009628~response to abiotic stimulus	GO:0009644~response to high light	GO:0009642~response to light intensity	GO:0009408~response to heat
AT5G59720														✓	✓		✓	✓	✓
AT2G29500														✓	✓		✓	✓	✓
AT2G05620														✓	✓	✓	✓	✓	
AT5G20230															✓	✓		✓	
AT1G53540																✓			✓
AT5G12020																✓			✓
AT1G07400																✓			✓
AT3G46230																✓			✓
AT3G25760																✓			
AT3G51240																✓			
AT3G01500																✓			
AT3G55120																✓			
AT1G56600																✓			
AT5G07990																✓			
AT3G57260																✓			
AT5G62520																✓			
AT2G37970																✓			
AT1G75040																✓			
AT5G11210																✓			

In the following sections, the individual functions within the enriched categories are described in more detail.

Transcription factors

Transcription factors are well known to be primarily involved in the initiation stage of RNA transcription and they are the key factors that regulate gene expression. A large number of transcription factors in the roots responded to the NaCl treatment. Many of these were less responsive to salt upon priming, including key regulatory gene families involved in response to abiotic and biotic sources of stress such as *WRKY* (*WRKY38*, *WRKY70*, *WRKY46*, *WRKY45*, *WRKY66*, *WRKY55*, *WRKY8*, WRKY40), *DREB2B* (*DRE/CRT-BINDING PROTEIN 2B*, *1F*, *2A*, *1B*), *ANAC (ANAC6, ANAC019,* Arabidopsis NAC domain containing protein), *MYB* (*MYB108*, *MYB2*, *MYB15*, *MYB95*, *MYB77*, *MYB74*, *MYB94*, *MYB108*, *MYB78*). Others identified are related to hormone signalling such as JASMONATE-ZIM-DOMAIN PROTEIN (JAZ8, JAZ10, JAZ5, JAZ6, JAZ9, JAZ11) proteins acting as repressors of jasmonate (JA) and ethylene response factors (*ERF98, ERF113, ERF13, ERF105, ERF114, ERF71, ERF11, ERF17, ERF19, ERF95, ERF12, ERF115, ERF2, ERF104, ERF18, ERF109*). Also found was a kinase (ERS2-ETHYLENE RESPONSE SENSOR 2), as well as auxin-inducible AtHB53 and ARGOS (Auxin-Regulated Gene Involved in Organ Size). Finally, several disease resistance-responsive family proteins (AT1G75040, AT5G40990, AT5G09980, AT5G64900) were also less responsive to salt in primed plants.

Kinase and cell wall related genes

A lower response to salt upon priming was found for key factors in promoting formative cell divisions in the pericycle *CCR4* (*ARABIDOPSIS THALIANA CRINKLY4 RELATED 4*, *AT3G44260*) and for *BGLU2* (*BETA GLUCANASE 2*, *AT3G57260*). In addition, cell wall enzymes were also found such as wall-associated receptor kinase 1 (AT1G21250), ABC40 transporter family (AT1G15520), two Xyloglucan endotransglucosylase/hydrolase proteins (AT5G57560, AT4G30270), GDSL esterase/lipase 1 (AT5G40990) and a Lectin like protein (AT3G16530).

Ion binding and oxy/reduction signalling

Several genes involved in the oxy/reduction pathway were identified as being less responsive to stress upon priming: Chalcone--flavonone isomerase 1 (*AT3G55120*), Chalcone synthase (*AT5G13930*), *Flavanone hydroxylase* (*AT3G51240*), Flavonoid 3'-monooxygenase (*AT5G07990*), Carbonic anhydrase (*AT3G01500*), lipoxigenase

(*AT1G17420*, *LOX3*), Glutaredoxin-C9 (*AT1G28480*), 1-aminocyclopropane-1-carboxylate synthase 6 (*AT4G11280*). Electron carrier iron binding proteins such as CYP715A1 (CYTOCHROME P450 715A1) were also identified. Finally, two cation transporters were also identified in this category such as Blue copper protein (AT5G20230) and PROTON GRADIENT REGULATION 5 (AT2G05620).

Chaperones and Heat Shock genes

Numerous chaperones were found to be less responsive upon priming these included HSP18 (AT5G59720), HSP16.4 (AT5G12030), HSP17.6 (AT5G12020), HSP17.4 (AT3G46230), (AT3G63350, A-7b), HSP20 (AT1G52560) and many HSP20-like (AT2G29500, AT1G53540, AT1G07400, AT1G54050).

The expression profiles of selected genes were further confirmed using qPCR (Fig. 4.11).

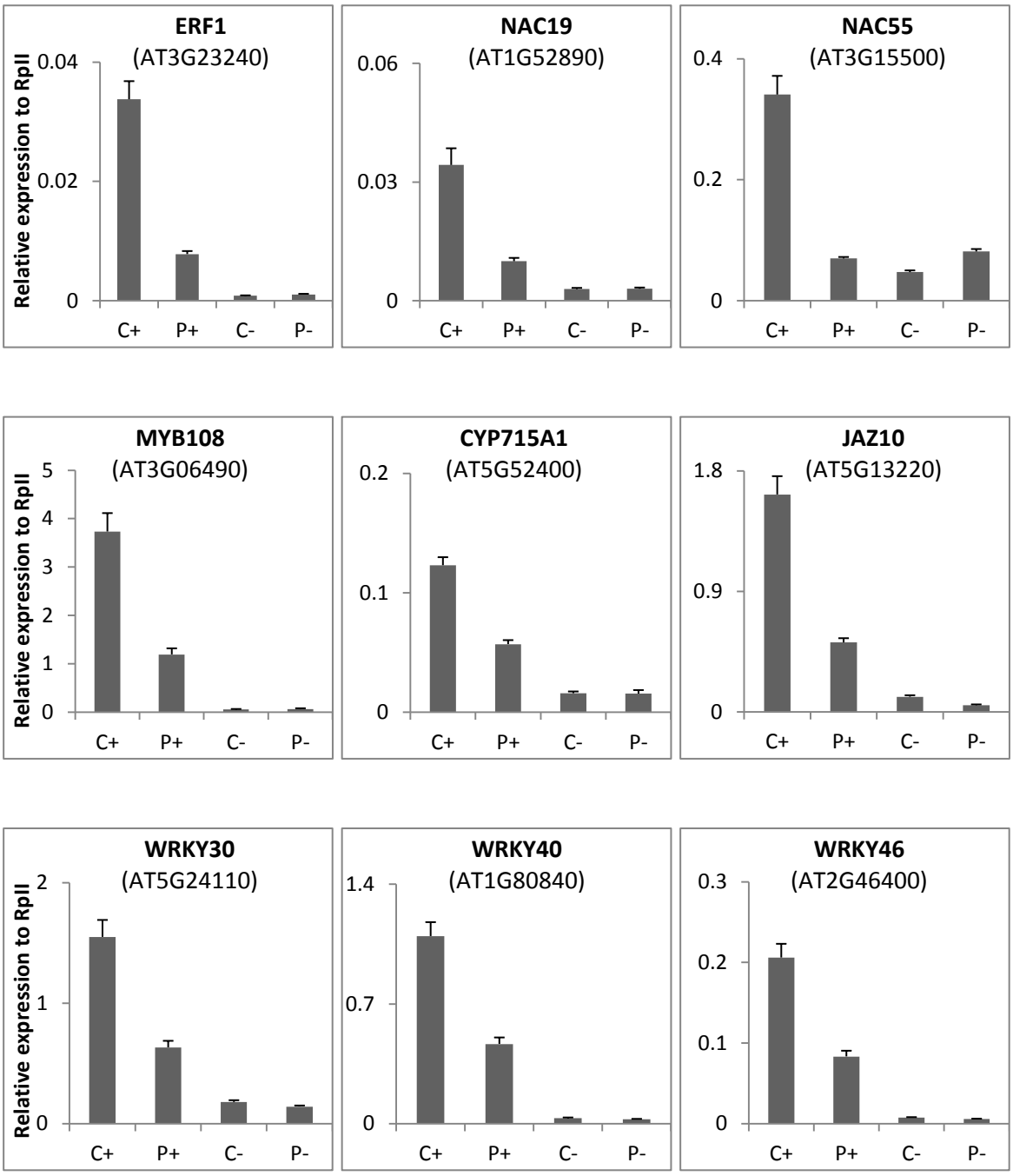


Fig. 4.11 Real time quantitative PCR (qPCR) analysis of relative expression of selected genes that responded less to the salt in primed roots samples.
Total RNA was isolated from one experimental sample, reverse transcribed and cDNA was analyzed by qPCR using primers for genes that showed differential responses on the microarrays (see above). *RpII* was used as reference gene for the normalization. Standard errors are from four pairwise comparisons of technical duplicates.

4.4 Discussion

4.4.1 Pros and cons of transcriptomics

The work presented herein generated a genome-wide dataset that identified and quantified the differences occurring at the transcriptional level between primed and non-primed plants exposed to a salt stress at a later stage in development. Furthermore, differences were identified at the organ specific level, as shoots and roots were analysed and compared separately. A particular effort was made to attest quality, comparability and reliability of the data by using several different statistical approaches. However, statistics are limited by the background variability (noise) of the data and therefore small changes that potentially could have a significant impact on the overall biology might not be detected. Furthermore, microarray technology is based on a one-to-one (non-amplifying) hybridisation process between probes and samples making the detection of low abundant transcripts difficult. This is particularly true for genes that are transiently expressed and/or only in a specific location because the temporal window or specific cell type may not be well represented in the experimental sample tested. For example, qPCR found significant changes in the expression of *HKT1*, a sodium transporter that is specifically expressed in root xylem parenchyma cells and was not detected by the microarray. For these reasons, it cannot be excluded that other changes occurring in individual transcripts upon stress/priming will be revealed by a more detailed analysis. Nevertheless the genome-wide results obtained here provided an excellent tool not only to identify candidate target genes of priming but also to reveal the overall effect of priming on transcriptional regulation and functional trends therein.

4.4.2 Primed plants show a different response to salt between roots and shoots

One of the new finding from this work was that primed and non-primed plants responded differently to the salt stress. This was demonstrated by a slope significantly smaller than 1 on the scatter plot of the transcript response to salt in primed and non-primed plants (Fig.4.6), by a smaller number of differentially expressed genes in primed and non-primed plant for a given p-value and FDR cut-off (Table 4.2), and by principal component

separation of primed from non-primed root samples after salt treatment (Fig.4.7). Because the roots were in direct contact with the applied salt, the fact that the priming effect was more pronounced in the roots is more likely to be a consequence of the way the treatment was applied than a strictly tissue-specific response. The short-term treatment is not long enough for the salt (or downstream signals) to accumulate in the aerial part of the plants and perhaps a longer treatment could herald more significant responses in the shoots. As a consequence, only a few genes were found to be differentially responsive in the shoots. These genes were reported as typically activated during the early phase of stress response signalling (i.e. response to unbalanced osmotic potential, detoxification pathways and ROS protection) (Tab.4.2).

4.4.3 Primed plants respond to salt differentially from non-primed plants by regulating less genes and/or evoking lower responses within the same genes

Primed plants showed a vast spectrum of genes being differentially regulated when exposed to the latter salt stress. However, compared to non-primed plants, the primed plants responded differentially regulating a lower number of genes or regulating the same genes less strongly, in fact the curve returned a slope lower than 1. (Fig. 4.6). In fact, according to ANOVA, 25% of the genes responding to salt in non-primed plants did not respond in primed plants (Tab.4.2). One possible explanation for the finding is that the plants were “less stressed” (e.g. contained less Na^+). Another possibility is that they did not sense the stress (‘desensitization’), and a third possibility is that primed plants focussed the response. Our finding that primed plants accumulated less Na^+ (Fig. 3.9) could argue for the first explanation as far as shoot genes are concerned. However, the roots of primed and non-primed plants were exposed to the same sodium concentration. The second possibility is supported by the finding that many genes were still responsive but showed a lower response. The third possibility is supported by the fact that *A. thaliana* is a salt-sensitive species that induces many genes that do not necessarily help with adaptation (‘panic’) response. It has been argued that a close salt-tolerant relative of *Arabidopsis* has a more focussed response to salt in fewer genes (Wong et al., 2006; Gong et al., 2005). If this was the case, primed plants should also show some genes with a

higher response to salt. Indeed this was the case (Tab. 4.2 and 4.4) and upon priming treatment a more focused response occurred in a determinate group of genes (Tab.4.8). Interestingly, no differences were detected in primed plants either in the overall transcripts profile (Fig.4.5 and 4.7) or at the vast majority of individual genes (Tab.4.2) 10 days after priming plants without the additional stress exposure. This demonstrated that the priming treatment influenced the transcriptional regulation of genes without interfering with the basal level of expression but only when salt is applied. For these reasons, it was decided to further investigate the differences occurring at the chromatin level and these data will be presented in Chapter 5.

4.4.4 Gene functions and signalling pathways affected by the priming treatment

To obtain clues about the downstream pathways and physiological processes that were affected by differential transcriptional responses in primed plants, several gene lists were subjected to analysis of functional enrichment (DAVID). The analysis did not reveal any new salt-responsive functional clusters as being specifically regulated in primed plants but identified a subset of salt-responsive functional clusters that were further enriched in primed plants. The genes underlying this enrichment (Tab. 4.8 and 4.9) along with individual genes found particularly to be more responsive upon priming compared to non-primed plants represent the different strategies plants use for coping with salt stress and are further described in the following text.

Strategy 1: Avoid toxic ions

One of the strategies that a plant can employ in order to improve its chances of surviving salt stress is to keep sodium out by activating an active transporter. Ion transporters are essential in order to detoxify the cell when elevated concentrations of sodium are reached. Interestingly functional categories identified by DAVID as being more salt-responsive upon priming included regulators of ion homeostasis, transporters and channels.

Other genes were also found individually to be very highly responsive to salt upon priming. Among those, two chloride channels *CLC-B* (AT3G27170) and *SLAH1*

(AT1G62280) have been found to show a higher response to salt upon priming. CLC-B is localized at the tonoplast and was identified as a proton-coupled anion transporter, involved in anion release from the vacuole (Von der Fecht-Bartenbach et al., 2010). The chloride transporter SLAH1 has been found to be strictly localized to the plasma membrane of lateral root primordia and in the vascular systems of root. SLAH1 has similarity to the SLAC1 protein involved in ion homeostasis in guard cells where it controls the extrusion of chloride (Negi et al., 2008). Although it is not naturally expressed in guard cells, SLAH1 (At1g62280) can complement the ion accumulation phenotypes of slac1-2 mutants when expressed under the control of the SLAC1 guard-cell-specific promoter, suggesting that it performs a similar function but in a particular tissue specific localization (Negi et al., 2008).

Strategy 2: Maintain a good balance in the ion and water content

In the mature root, the Casparian strip present at the endodermis blocks diffusion into the stele (Pitman, 1982). Therefore, root epidermal and cortical cells mediate the net uptake of ions into the root symplasm, whereas the stelar cells of the root mediate the net passage of ions from the root symplasm into the xylem vessels (Cellier et al., 2004). Spatial differences in expression could explain why two members of the same family show an opposite response to the priming treatment and also shed light on the specificity of priming in gene regulation. For example, *AtCHX17* was found to be less up-regulated to salt upon priming whereas the *AtCHX16* was more up-regulated. *AtCHX16* is described as having a function in pH homeostasis and K^+ uptake in yeast. When *AtCHX16* was expressed in a K^+ uptake-deficient yeast mutant the gene restored the ability of the cells to grow at alkaline pH (Chanroj et al., 2011). AtCHX17 has also been described as important in ion transport (Chanroj et al., 2011). Intriguingly, according to the Efp browser (Winter et al., 2007) AtCHX16 is localized in the inner layer of the roots (endodermis and perycicle) whereas AtCHX17 was found to be strongly expressed in zones (epidermis and cortex) that are in apoplastic contact with the external medium (Cellier et al., 2004). This brings about the possibility that the sodium is localized in some cells and therefore some genes are more responsive than others because they are located in cells that are in contact with the Na^+ stress while others are not.

Salt stress lowers the external water potential and hence the driving force for water influx into root cells. It was found here that priming abolished the down-regulation of the water channel TIP4;1 (tonoplast intrinsic protein 4;1) upon salt exposure. Aquaporins facilitate the uptake of water and regulate root hydraulic conductivity in response to environmental stresses (Boursiac et al., 2005). TIP4;1 expression is restricted to the root epidermis and cortex, with no signal detectable in the inner layers (Gattolin et al., 2009). Also, the expression is in the main root at the base of the elongation zone whereas in lateral roots it is localized in the differentiation zone (Gattolin et al., 2009).

This brings about the possibility that the priming treatment can potentially lead to an advantage in stress adaptation by an improved uptake of water during the over accumulation of ions, a feature that becomes essential during osmotic stress where the ion inbalance simulates a drought stress.

Strategy 3: Modify cell wall components

Along with transporters cell wall related genes are the other category identified by DAVID as enriched among genes differentially regulated in primed and non-primed plants. Cell wall processes are likely to play an important role in several responses to salt, such as growth inhibition, ROS detoxification and pH homeostasis (Hamann, 2012; Hossain et al., 2012). Two multicopper oxidase genes targeted to the cell wall *SKS16* (*SKU5 Similar 16*) and *SKS15* (*SKU5 Similar 15*) were found to be highly expressed upon priming during salt stress. *SKS6* expression in roots changed when root seedlings were brought into direct contact with auxins and ACC indicating that the hormones could enhance *SKS16* expression directly or through a root-delimited signalling cascade (Jacobs and Roe, 2005). The *SKS15* gene plays an important role in regulating directional root growth (Sedbrook et al., 2002). The gene product localizes to the cell wall and plasma membrane in all the expanding plant parts, which suggests a role in plant growth processes, possibly through cell wall remodelling (Sedbrook et al., 2002). *AnnAt7* encoding an annexin protein was more responsive to salt upon priming. Annexins form a multifunctional gene family whose members have been found in many cellular locations and are involved in a number of diverse physiological processes, including the secretion of new cell wall materials during growth and development (Laohavisit et al., 2012; Laohavisit and Davies, 2011). *AnnAt7* has been shown to be highly expressed under salt stress and to be specifically localized in

the roots (Cantero et al., 2006). The actions of these genes suggest that priming can stimulate mechanisms that regulate the root growth and consequently plants can either avoid the stress or grow quicker to combat the stress.

Very few genes showed higher fold changes in response to priming without salt application; among them members of Cytochromes P450 (P450s) and the hydroxysteroid dehydrogenase AtHSD4. P450s are hemethiolate mono-oxygenases involved in the synthesis of backbone structures of different classes of primary and secondary metabolites and are highly conserved throughout the plant kingdom. *AtHSD4* encodes a protein involved in sterol biosynthesis. Sterols are lipids of biological membranes that contribute to membrane compartmentalization, such as lipid and protein segregation in the secretory or endocytic pathways along with providing precursors for biosynthesis of plant brassinosteroids (Li et al., 2007b). The end product of the brassinosteroid biosynthesis pathway is synthesized by sequential hydroxylation reactions catalysed by P450 enzymes (Morant et al., 2007). Interestingly, the administration of epibrassinolide has been shown to enhance the tolerance to drought in seedlings of *A. thaliana* and *B. napus* as well as to help in overcoming the inhibition of germination under high salt conditions (Kagale et al., 2007).

Chapter 5: Epigenetic profile

5.1 Introduction

5.1.1 Background

Epigenetic changes can be defined as modifications to the genome that do not alter the nucleotide sequence. Examples of such changes are DNA methylation and histone modifications, both of which regulate gene expression without altering the underlying DNA sequence. Some histone modifications, such as acetylation of histones H3 and H4 and trimethylation of H3 lysine 4 (H3K4me3), are known as euchromatic marks and are often associated with increased transcription whereas other modifications, such as methylation of H3K9 and H3K27, are known as heterochromatic marks and related to gene transcription repression (Jenuwein and Allis, 2001; Li et al., 2007a).

Several pieces of evidence suggest that exposure of plants to stressful conditions modifies their epigenetic landscape (Luo et al., 2012). Studies investigating the effects of mutation in histone modifying enzymes found altered abiotic stress response and tolerance. For example, mutations of the histone methyltransferase Trithorax-like Factor (ATX1), which tri-methylates H3K4me3, caused higher sensitivity to dehydration stress (Ding et al., 2011), while mutation of the members of the histone de-acetylases caused altered freezing tolerance and higher sensitivity to ABA and NaCl (Zhu et al., 2008; Luo et al., 2012b; Chen et al., 2010; To et al., 2011).

However, mutant studies have their limitations: most of the histone modifying enzymes are still unknown and often the mutations have a severe effect on the plant health and can disrupt several signal transduction pathways making it difficult to pin-point primary effects. A more detailed analysis of the impact of environmental stress on histone modification can be achieved using Chromatin immuno-precipitation (ChIP) associated with next generation sequencing or hybridization with tiling array (respectively ChIP-Seq or ChIP-on-ChIP). In these methods, the modified histone proteins are immuno precipitated using an antibody that specifically recognizes the histone carrying a

particular modification at its tail. The isolated modified histones can then be separated from the associated DNA and the obtained DNA is sequenced or hybridized to DNA microarrays.

When this project started no studies had used ChIP-Seq or ChIP-on-ChIP to investigate the effect of abiotic stress on the histone modification landscape. Since then, a small numbers of studies have been published, in which genome wide approaches have been applied to investigate changes in the chromatin structure upon abiotic stress exposure of plants. For example, van Dijk et al., (2010) compared genome wide histone 3 lysine 4 methylation in *A. thaliana* plants exposed to dehydration stress against normally watered plants. To address the role of chromatin modifications in salt stress response and adaptation we carried out a comparative analysis of the epigenetic landscape of *A. thaliana* plants after exposure to a short salt priming treatment.

The large set of data obtained provides new experimental evidences for the hypothesis that epigenetic changes caused by a priming treatment reduce the need for great transcriptional changes upon later stress exposure ("plant stress memory") (Bruce et al., 2007).

5.1.2 Aims of the chapter

In this chapter I describe the genome wide epigenetic landscape of *A. thaliana* plants after exposure to a short (24h) priming treatment with 50mM NaCl. Chromatin immuno-precipitation sequencing (ChIP-Seq) was used to create genome-wide maps of H3K4me2, H3K4me3, H3K9me2 and H3K27me3 in primed and non-primed A. *thaliana*. Differences in epigenetic markers were detected using statistical tools and further confirmed by qPCR. A second set of ChIP-Seq experiments investigated the H3K27me3 profile 10 days after the priming treatment to test whether priming induced changes were maintained over periods of extensive growth and hence can act as a somatic memory of stress events.

5.1.3 Workflow of data analysis

Analysis of ChIP-Seq data has been carried out at the Glasgow Polyomics facility in the Glasgow University. In particular ChIP-Sequencing, reads aligment and mapping has been carried out by Dr. Pawel Herzyk at the Glasgow Polyomics facility**.** The complete analysis workflow is summarised in the Tab 5.1.

The obtained sequences determined by Illumina sequencing were firstly filtered from the identical sequences. The remained sequences were aligned against the *Arabidopsis* genome (version TAIR9) and from among those, only the ones which aligned to a determinate number of genome locations were considered.

To build quantitative landscapes for the individual histone modifications, the number of reads was counted within 200 bp windows along the genome.

In a next step, the whole *Arabidopsis* genome model from TAIR together with the datasets derived from the sequencing of different ChIP samples was loaded into the Integrated Genome Browser (IGB) browser. IGB is a free-access software that permits the analysis of the distribution and exploration of genome-scale datasets in an easy visual format (Nicol et al., 2009). Furthermore, the nucleotide sequences are loaded into the browser, which allows the design of specific primers that are needed to confirm the level of modification in particular areas of the gene by PCR amplification (Fig.5.1). Once these lists were generated I performed a data mining analysis in an attempt to explore and extrapolate biological meaning from this vast dataset.

Tab. 5.1 Workflow of ChIP-Seq analysis.

SEQUENCING AND READS ALIGNEMENT					
Input	**Activity**	**Software**	**Output**	**File**	**Reference**
DNA from ChIP	Sequencing	Illumina GAIIX	Reads (Redundant/non redundant)	Fastq	
Reads	Align to *A. thaliana* genome TAIR 9	Bowtie	Aligned and non redundant reads	.bam	(Langmead et al., 2009)
Aligned reads	Select reads that align to a pre-specified number of genome location UNIQUE: only single alignment permitted ALL: every alignments permitted	Assembly	Aligned reads	.bam	
Aligned reads	1. Aligned reads within bins of 200 bp size.	SICER	Number of aligned reads per bin. = "bar" in profile.	.wig	(Zang et al., 2009)
	2. Identify continuous stretches of eligible bins. Gap optimized for histone modification: H3K4me2, gap=0 H3K4me3, gap=0 H3K9me2, gap=3 H3K27me3, gap=2		Islands of methylation or "Islands".	.bed	
Aligned reads from 2 samples	Find significant differences	ChIP-Diff	Differences	.bed	Xu et al., 2008

MAPPING					
Input	**Activity**	**Software**	**Output**	**File**	**Reference**
Islands	Map to coding regions	Bowtie	Mapped islands	.bam	
Differences	Map to coding regions		Mapped differences	.bam	

GRAPHICAL VISUALIZATION					
Input	**Activity**	**Software**	**Output**	**File**	**Reference**
Number of aligned reads per bin	Load into IGB genome browser	IGB	Visualised profiles ('landscape').		
Islands			Visualised islands		
Differences			Visualised differences		

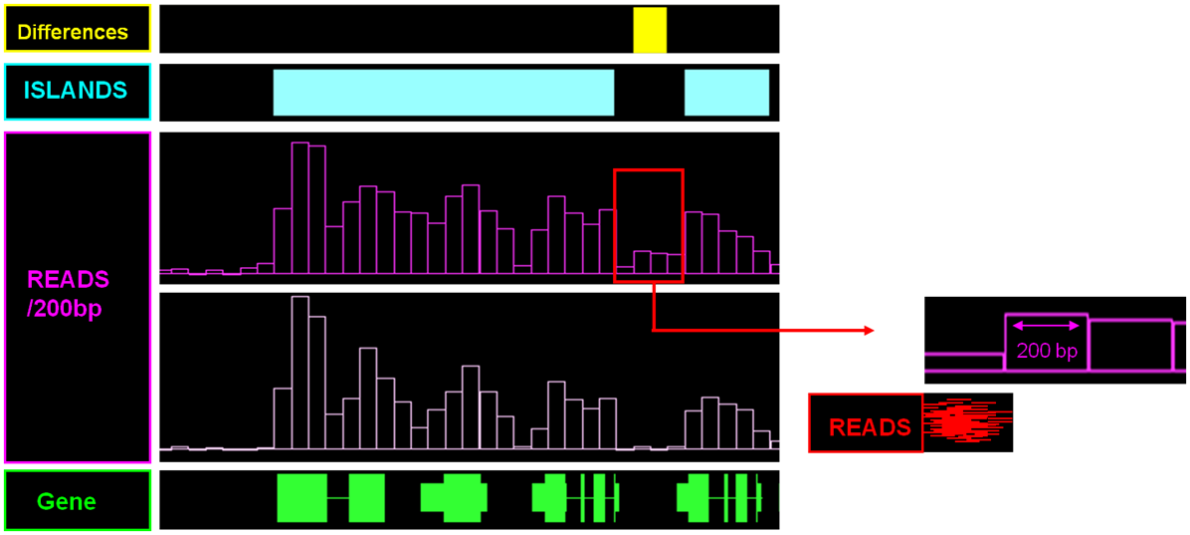


Fig. 5.1 Data overview as appear in the IGB browser.
READS: Number of reads over 200 bp window. ISLANDS: consecutive region with a significant enrichment of reads by SICER (Zang et al., Bioinformatics, 2009). DIFFERENCES: significant difference between control and primed calculated with ChIP-Diff (Xu et al., Bioinformatics, 2008).

5.2 Results

5.2.1 Development of an effective and reproducible Chromatin Immuno-precipitation protocol

In order to investigate whether salt priming induces changes in the chromatin state of plants, Chromatin Immuno-Precipitation (ChIP) was carried out on root and shoot samples from primed and non-primed plants harvested directly after a 24 hours exposure to priming (50mM NaCl) or control solution (0mM NaCl) (see Chapter 2 for methods).

Since several different antibodies against histone modifications were used, a particular effort was made to optimize the ChIP protocol such as by adjusting the stringency of the conditions used (e.g number of washes, amount of antibody) and identifying the appropriate time of sonication necessary to obtain fragments of the correct length.

Once an efficient protocol was generated, the analysis was focused on quantifying the methylation level of different lysine residues in the tail of the histone H3, such as: di- and tri-methylation of Lysine 4 (K4me2, K4me3), di-methylation of Lysine 9 (K9me2) and tri-methylation of Lysine 27 (K27me3). Material from three independent priming treatments was used for all ChIP experiments. In each replicate all antibodies were applied to samples originating from the same population of plants. DNA samples obtained from each ChIP were tested for enrichment of the particular marker (e.g H3K27me3) in specific regions of the *Arabidopsis* genome previously shown to be enriched (Zhang et al., 2009; Zhou et al., 2010; Zhang et al., 2007b). Fig. 5.2 shows PCR amplification of these regions used as quality control to confirm the expected absence/presence pattern for the different samples. Finally, after passing the quality control, the three replicate ChIP samples were pooled together and subjected to sequencing with the Illumina technology.

Fig. 5.2 DNA fragments amplified with different sets of primers designed against genome regions known to be associated with (Positive Control) or lacking (Negative Control) a particular marker.

(CS) Non-primed Shoots, (PS) Primed Shoots, (CR) Non-primed Roots, (PS) Primed Roots. I: Total genomic DNA (ChIP input), NA: ChIP without antibody, A: ChIP with antibody specified in the figure, M2: ChIP with antibody against anti di-methyl H3K4, M3: ChIP with antibody against anti tri-methyl H3K4.

In A-B) the sets of primers were designed in order to distinguish between di- (Positive Control), tri- (Positive Control) or un- (Negative control) methylated H3K4. In (C) the sets of primers were designed in order to distinguish between di- (Positive Control), or un- (Negative Control), methylated H3K9. In (D) the sets of primers were designed in order to distinguish between tri- (Positive Control), or un- (Negative Control), methylated H3K27.

5.2.2 Genome wide histone modification landscape

Chip sequencing was used to identify and quantify genome regions associated with the follow histone modifications: H3K4me2, H3K4me3, H3K9me and H3K27me3. Quality controlled samples from *Arabidopsis* shoots and roots, primed or not with 50 mM NaCl for 24h (see previous section) were used for this analysis. In order to confirm that the sequencing process produced consistent results, the total number of sequences (reads) obtained was compared between samples. As shown in Fig.5.3, the total number of sequences obtained from the ChIP-Seq was very similar independent of antibody, sample, treatment and tissue.

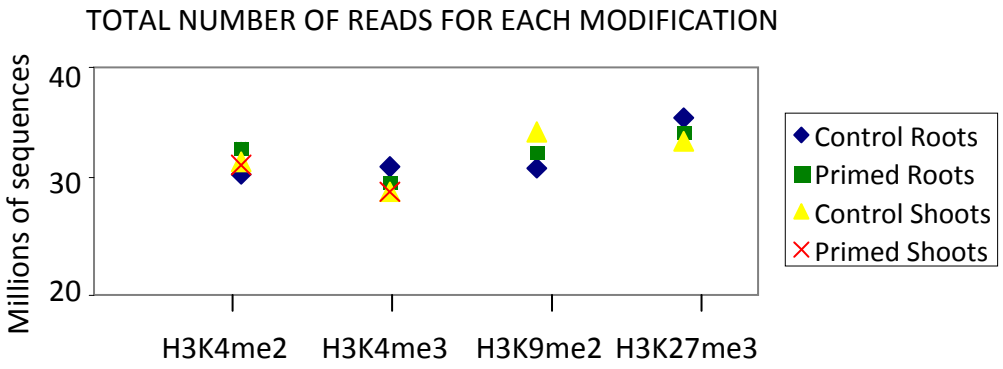


Fig. 5.3 Total number of reads for different histone modifications.
Plotted are the total numbers of redundant sequences obtained from individual Illumina sequencing for each modification (H3K4me2, H3K4me3, H3K9me2, H3K27me3), tissue (shoots/roots) and treatment (control/primed).

In the next step identical sequences were removed from the dataset and the non-redundant reads were aligned against the *A. thaliana* genome (version TAIR9). Initially only unique alignments were allowed. Modifications generally described as being localized in euchromatic areas such as H3K4me2, H3K4me3 and H3K27me3, showed a good percentage of alignment (approximately of 80-90%) (Tab.5.2). In contrast, for H3K9me2, known to be associated with transposons and highly repetitive sequences (Zhou et al., 2010), less than half of the sequences aligned to a unique position in the genome. When multiple alignments were allowed, a high percentage of alignment for H3K9me2 was restored. This demonstrates that depending on the targets of the modification different alignment parameters have to be applied. Therefore, in our

analysis, unique alignment was used for H3K4me2, H3K4me3 H3K7me3, while for H3K9me2 all possible alignments were used.

Tab. 5.2 Percentage of sequences that aligned to the genome for each individual modification sequenced (H3K4me2, H3K4me3, H3K9me2, H3K27me3).
Shown are shoots and roots in control conditions.

SHOOTS	Max number of alignments	% Aligned	ROOTS	Max number of alignments	% Aligned
CSH3K4me2	1	84.1	CRH3K4me2	1	75.6
CSH3K4me3	1	94.3	CRH3K4me3	1	87.5
CSH3K9me2	1	41.7	CRH3K9me2	1	43.4
CSH3K27me3	1	90.4	CRH3K27me3	1	87.3
CSH3K4me2	50	94.5	CRH3K4me2	50	84.2
CSH3K4me3	50	97.5	CRH3K4me3	50	91.4
CSH3K9me2	50	78.3	CRH3K9me2	50	71.6
CSH3K27me3	50	96.7	CRH3K27me3	50	96.7
CSH3K4me2	All	95.4	CRH3K4me2	all	85.0
CSH3K4me3	All	97.7	CRH3K4me3	all	91.7
CSH3K9me2	All	82.2	CRH3K9me2	all	74.6
CSH3K27me3	All	97.0	CRH3K27me3	all	97.5

SHOOTS	Max number of alignments	% Aligned	ROOTS	Max number of alignments	% Aligned
CSH3K9me2	1	41.7	CRH3K9me2	1	43.4
CSH3K9me2	50	78.3	CRH3K9me2	50	71.6
CSH3K9me2	All	82.2	CRH3K9me2	all	74.6

A general overview of the epigenetic landscape of roots from primed and non-primed plants using IGB is shown in Fig.5.4. These data confirm the known association of particular histone modifications with specific regions along the chromosome (Zhang et al., 2009; Zhou et al., 2010; Zhang et al., 2007b). H3K4me2, H3K4me3 and H3K27me3 were widely spread along the euchromatic arms of the chromosomes, while H3K9me2 was localized in and around the centromeric regions which are rich in hetero-chromatin. Fig.5.4 also shows that the overall methylation landscape of control and primed roots were very similar. Therefore, the mild priming treatment did not lead to dramatic changes in the overall epigenetic landscape.

Validity of the data was also evaluated by observing gene tracks with an expected methylation patterns shown by a previous study (Brusslan et al., 2012). Fig.5.5 shows the constitutive gene ACTIN2 (At3g18780) as being highly associated with H3K4me3 marks but lacking in H3K27me3, while the floral gene FLC (At5g10140) shows significant level of H3K27me3 but no H3K4me3 except for a small peak that spans the first exon.

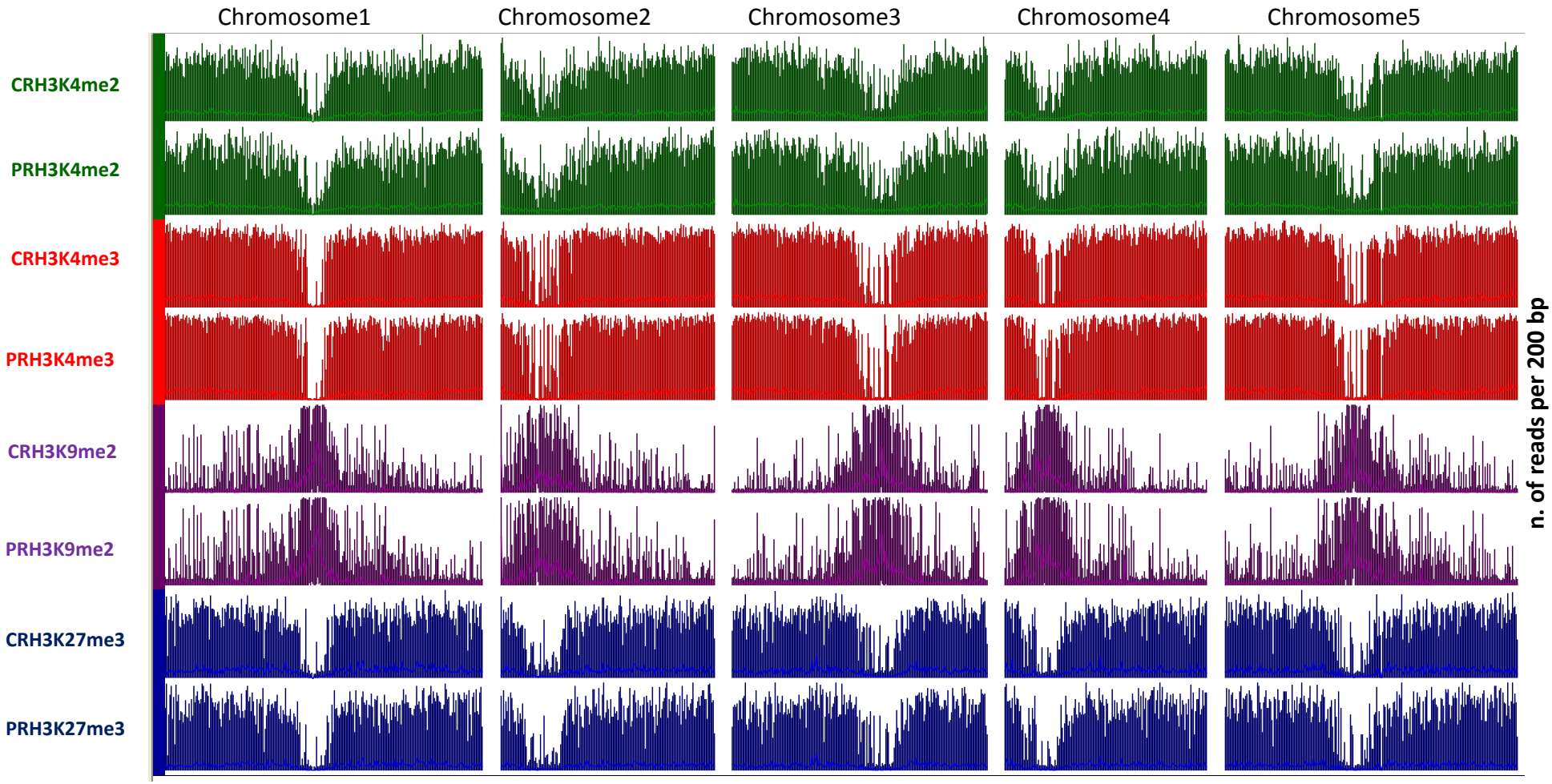


Fig. 5.4 Genome-wide landscapes visualized with IGB of H3K4me2 (green), H3K4me3 (red), H3K9me2 (purple), H3K27me3 (blue) modifications along the five *Arabidopsis* chromosomes in primed (PR) and control (CR) roots samples.
Bars represent the number of non-redundant sequences from the ChIP-Seq in a 200 bp window aligned against the *Arabidopsis* genome.

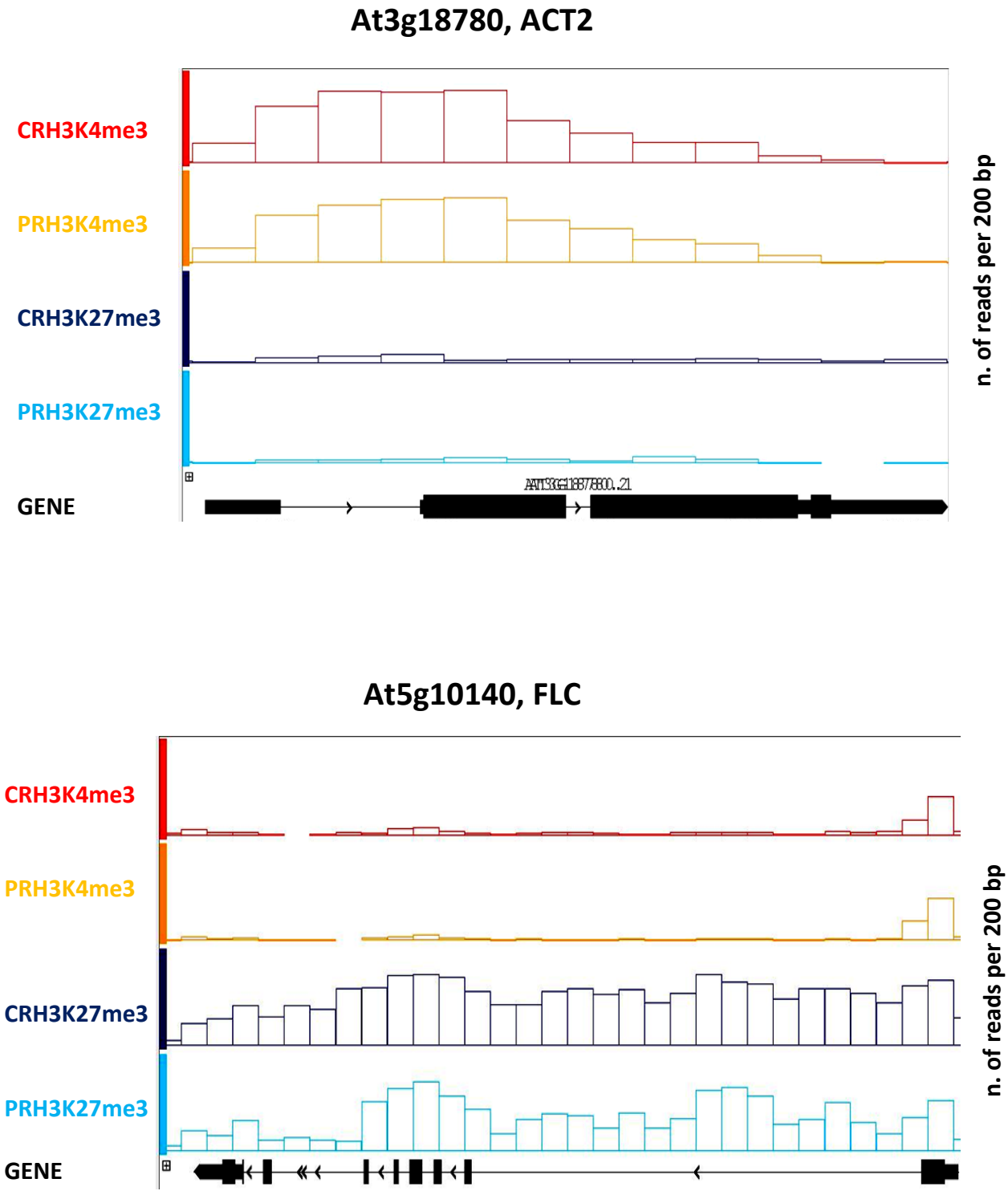


Fig. 5.5 Expected pattern of H3K4me3 and H3K27me3 histone modification on two representative genes in primed (PR) and control (CR) roots samples.
Genes are shown at the bottom of this Genome Browser image, exons are represented in black as thick lines and introns are shown as black thin lines. The black arrows indicate the direction of transcription. Bars represent the number of non-redundant sequences from the ChIP-Seq in a 200 bp window aligned against the *Arabidopsis* genome.

5.2.3 Priming affects histone modifications in a tissue specific manner

Root and shoot profiles were further analysed in order to evaluate the effect of the priming treatment on specific tissues. For this purpose we initially compared the whole genome wide profile of two modifications (H3K4me2 and H3K4me3) between root and shoot of the same plants.

Firstly, continuous stretches of significant number of reads (islands) were identified using SICER (Zang et al., 2009) (Ref. Tab.5.1). Results reported in Fig.5.6A, show that in neither root or shoot the number of H3K4me2 and H3K4me3 islands changed upon priming. However, the total coverage of the genome with H3K4me3 showed a slight decrease by 1.1% in the roots upon priming, while there was no difference in the shoots (Fig.5.6B). In addition, comparison of the island length distribution for H3K4me3 between primed and non-primed roots, revealed a slight shift towards shorter island (Fig.5.6C). Once more no such difference was seen in the shoots.

Positions displaying significant differences in histone modifications between primed and non-primed plants were identified by ChIP-Diff (Xu et al., 2008a) and displayed in the IGB browser. Fig.5.7 shows differences in H3K4me2 and H3K4me3 of at least 1.2 fold between primed and non-primed plants. A large number of differences were identified in roots while hardly any differences were identified in shoots of the same plants.

This finding indicates that the differences observed in the roots, even if they are small, are not random fluctuations. Several of these differences were subsequently confirmed by qPCR (Fig.5.8). On the basis of these results, it was decided to focus solely on root tissue and here extend the analysis to other histone modifications.

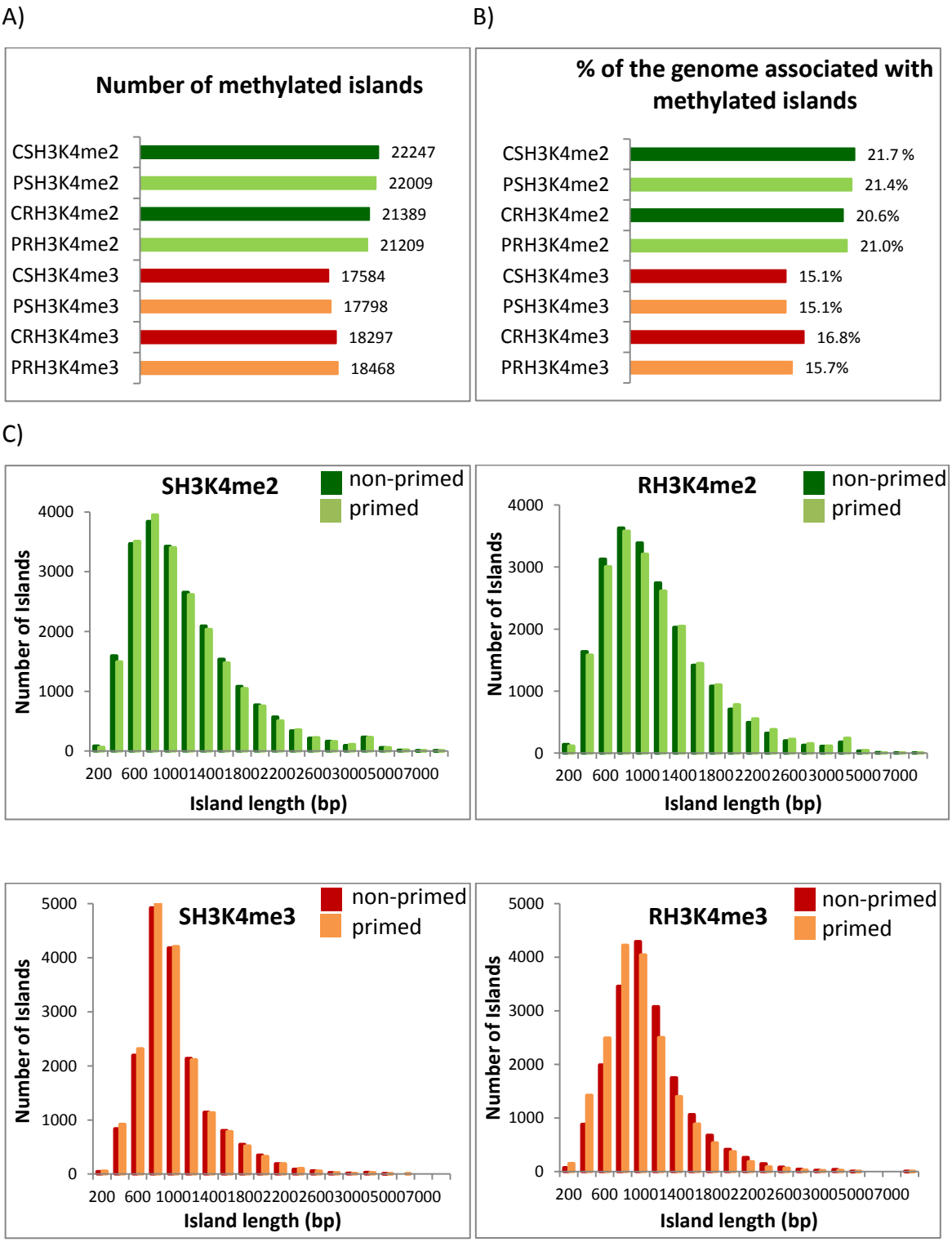


Fig. 5.6 Number of methylated islands and percentage of genome coverage for H3K4 di- and tri-methylation in roots (R) and shoots (S), non-primed (C) and primed (P).
A) Total number of Islands. B) Percentage of genome coverage with islands. C) Island length distribution in primed (light colour) and non-primed (darker colour) samples. The islands were divided into bins of a given length increasing by 200 bps and the number of islands in each bin was counted.

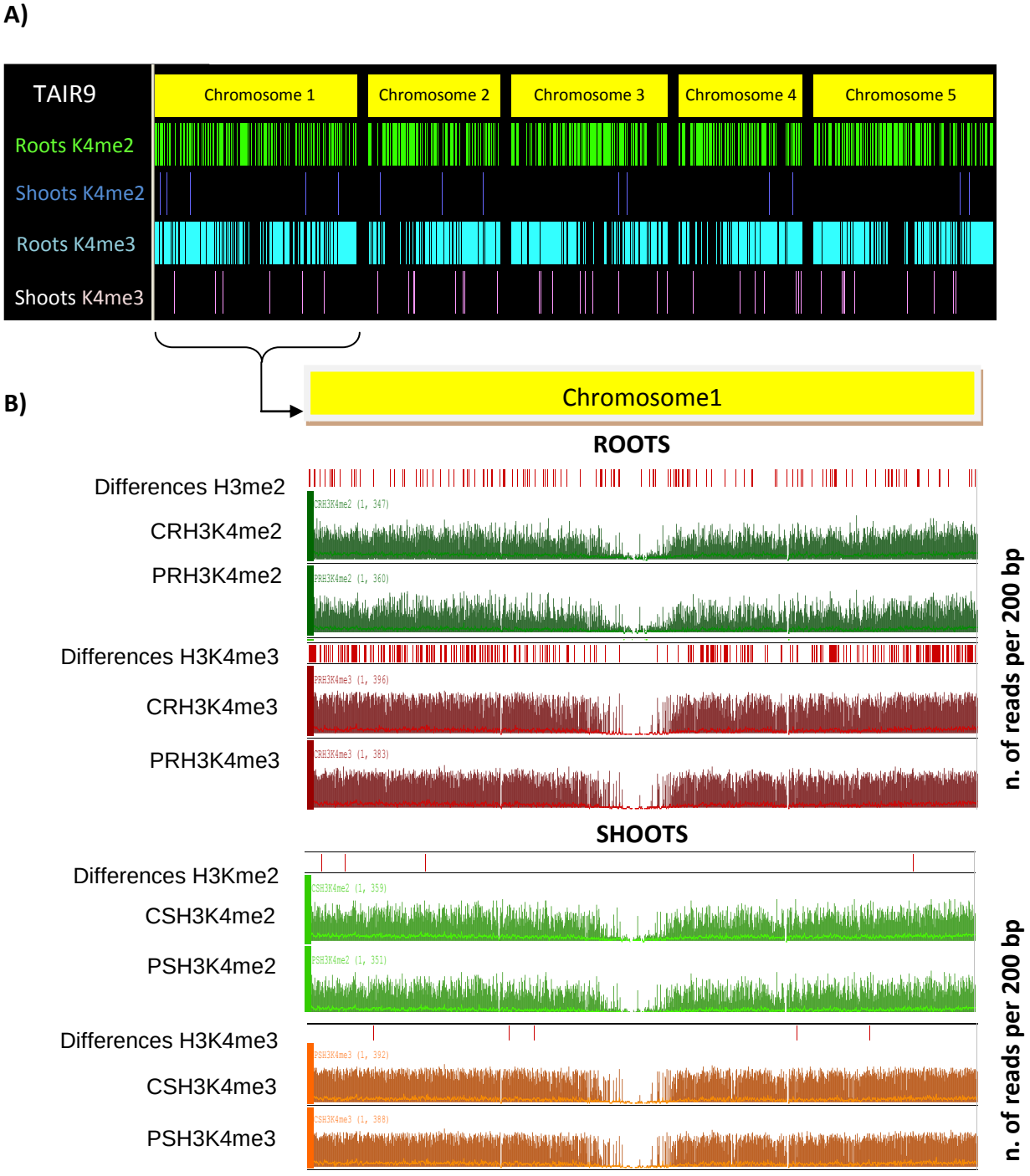


Fig. 5.7 A) Distribution of differences of H3K4me2-me3 in roots and shoots of primed and non-primed plants along all five *A. thaliana* chromosomes.
Bars represent differences in the level of H3K4me2 and -me3 between primed and control with a change of at least 1.2 fold.
B) Distribution of differences of H3K4me2-me3 in roots and shoots of primed and non-primed plants along Chromosome 1 of *A. thaliana*. Top panel: profile of H3K4me2 in roots of primed (PRH3K4me2) and non-primed (CRH3K4me2) plants; profile of H3K4me3 in roots of primed (PRH3K4me3) and non-primed (CRH3K4me3) plants.
Lower panel: profile of H3K4me2 in shoots of primed (PSH3K4me2) and non-primed (CSH3K4me2) plants; profile of H3K4me3 in shoots of primed (PSH3K4me3) and non-primed (CSH3K4me3) plants. Red bars represent differences in H3K4me2 –me3 level between primed and non-primed plants with a change of at least 1.2 fold.

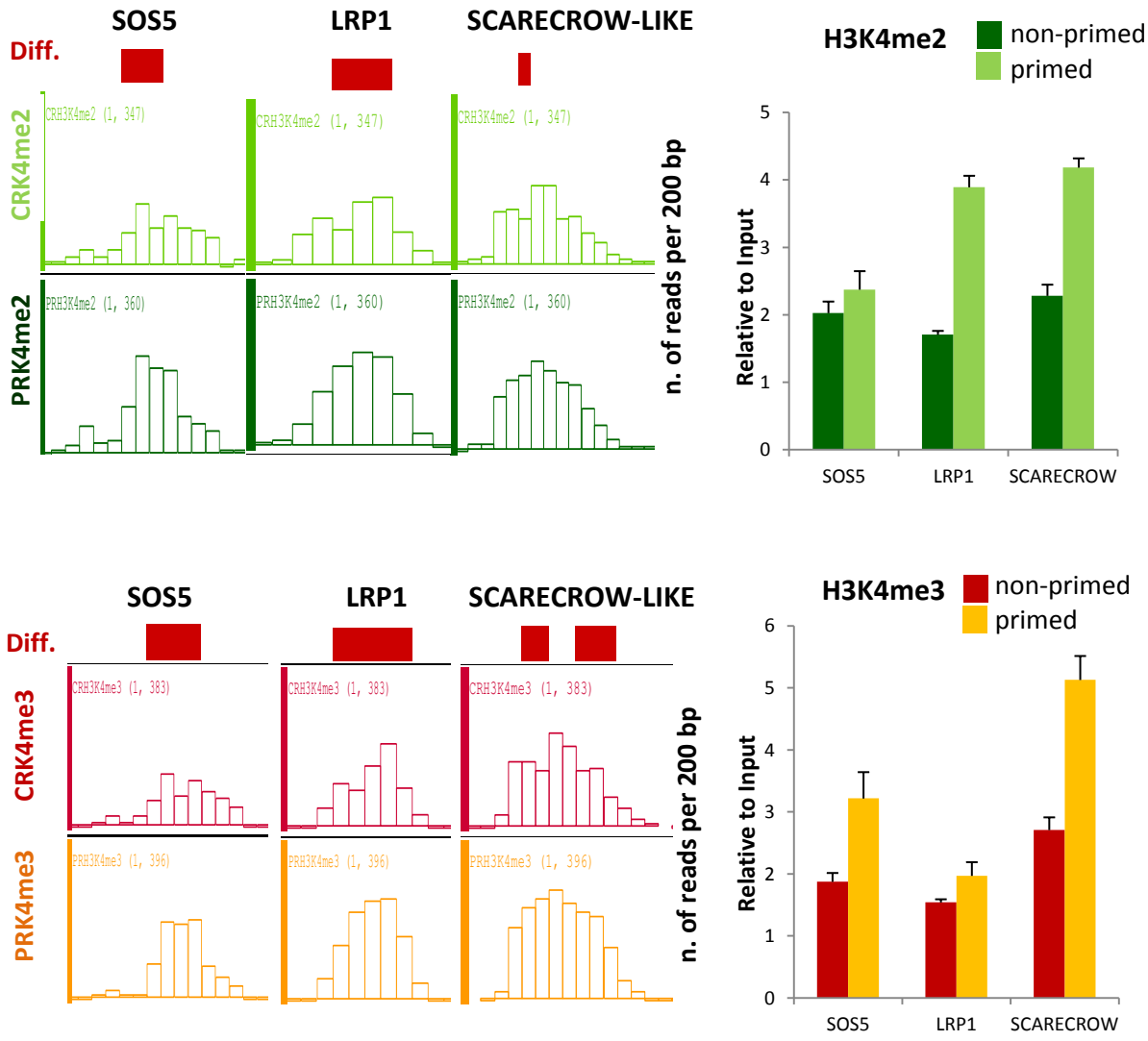


Fig. 5.8 Confirmation of individual differences of H3K4me2 and H3K4me3 between primed and non-primed roots.

On the left are shown selected differences determined by ChIP-Seq displayed in the IGB browser: in the top panel are reported the differences between priming and control determined by ChIP-Diff (fc ≥1.2) and in the lower panel are the H3K4me2 and –me3 profiles where every bar corresponds to the read number over a 200 bps window.

Primers were designed against the border of the identified differences.

On the right the qPCR results are shown in a graph displaying enrichment of H3K4me2—me3 relative to Input. Standard errors are derived from four pairwise comparisons of technical duplicates.

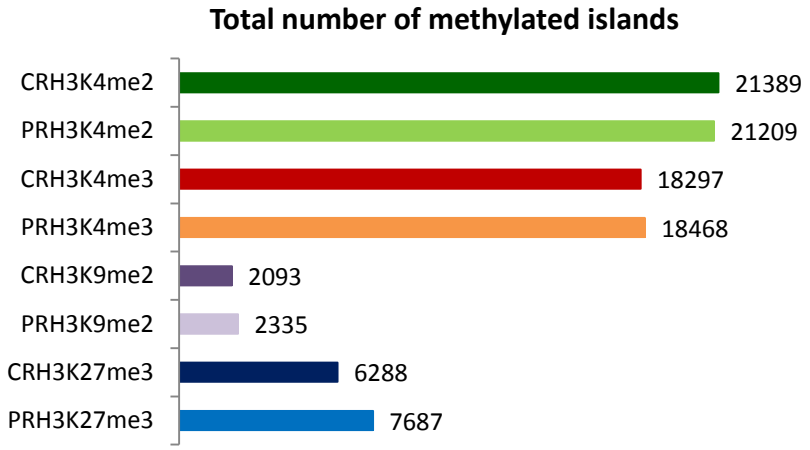
5.2.4 Effect of the priming treatment on different histone modifications in the roots

A comparative analysis between control and primed roots was carried out to show differences in various histone modifications. An initial analysis was made using the list of all islands and differences generated by SICER and ChIP-Diff in order to investigate priming induced changes for all the histone modifications considered. Then I performed a more detailed analysis looking at differences that could be mapped to genes in order to investigate which type of gene functions were associated with the differences.

5.2.4.1 Genome coverage, island number and island length for different histone modifications

In Fig.5.9 is shown the total number of methylation islands for all four modifications as well as percentage of genome coverage. In the primed samples the genome coverage with H3K27me3 dropped by 2.8% and the genome coverage with H3K4me3 dropped by 1.1%, respectively. Genome coverage with H3K9me2 and H3K4me2 slightly increased by 0.9% and 0.4%, respectively (Fig.5.9A). Intriguingly, these changes in the total amount of methylation were not positively correlated to the total number of islands. In fact the number of methylated islands was very similar in primed and non-primed samples for all the modifications apart from H3K27me3, which showed an increase by 20% in islands number in the primed sample (Fig.5.9B). Accordingly, for this modification the distribution of the island length also dramatically changed upon priming, showing a higher number of short islands and a smaller number of large islands. This suggests that the priming treatment divides the existing methylated islands into smaller islands (Fig.5.10). From these results H3K27me3 emerged as the most affected modification by the priming treatment; the priming treatment decreased the overall coverage of the genome with H3K27me3 and generated a higher number of shorter H3K27me3 islands.

A)



B)

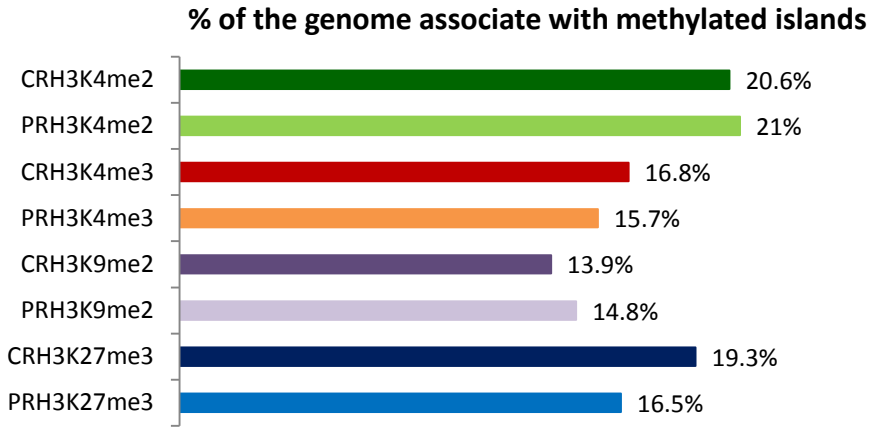


Fig. 5.9 Number of islands for H3K4 di- and tri-methylation, H3K9 di-methylation and H3K27 tri-methylation in roots of non-primed (CR) and primed (PR) plants.
A) Percentage of genome; B) Number of Islands.

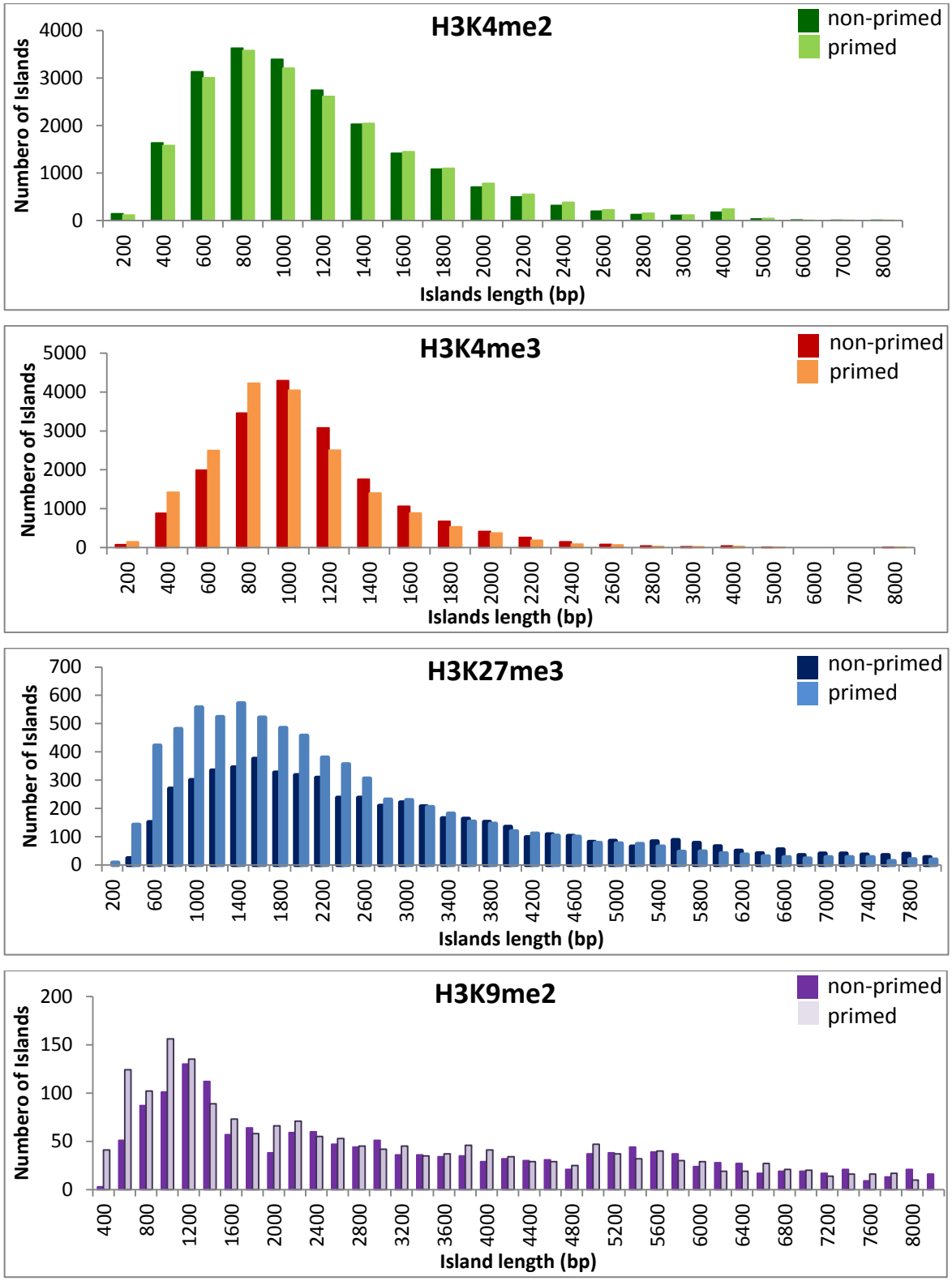


Fig. 5.10 Island length distribution in primed and non-primed roots analysed for the four histone modifications.

The islands were divided into bins of a given length increasing by 200 bp and the number of islands contained in each bin was counted.

5.2.4.2 Identification of genome regions associated with changing in histone methylation upon priming

ChIP-Diff was used to identify methylated regions that significantly differed between control and primed samples. ChIP-Diff works by partitioning the genome into bins then computing the fold-change of the number of ChIP fragments in each bin and finally taking into account the correlation between consecutive bins (Xu et.al 2008).

The total number of differences identified by ChIP-diff is reported in Fig. 5.11A. When differences of at least 1.2 fold were included, the program detected 7733 differences for H3K27me3, 744 for H3K4me2, 1500 for H3K4me3 and 509 for H3K9me2. When the cut-off was increased to 2 fold, H3K27me3 was the only modification that passed this criterion with 1679 differences being detected. When the direction of the differences (more or less methylation upon priming) was taken into account, it was found that the priming had a different influence on the level of methylation depending on the modification considered (Fig. 5.11A). For H3K4me2 and H3K4me3 the majority of differences (87% and 97% respectively) resulted in an increase of the modification in primed plants. In contrast, the majority of differences consisted in a decrease (84 %) for H3K27me3. Only for H3K9me2 half of the differences resulted in an increase of the modification in primed plants (52%) and consequently half decreased.

Fig.5.11B relates the number of differences to the total number of islands. The island number of H3K4me2 –me3 is large (almost 20000) while the number of differences is small. In contrast, the number of H3K27me3 islands is smaller (almost 8000) but a similar number of differences were identified (an average of one difference per island). Figure 5.11B also shows the number of mapped regions that had a different level of methylation upon priming, including a change of at least 1.2 fold.

These results demonstrate that the effect of priming differed among the different modifications analysed.

The opposite effect on H3K4me3 (increased) and H3K27me3 (decreased) is particularly interesting, because it suggests that priming acts on the chromatin configuration in a manner that opens the chromatin at specific loci.

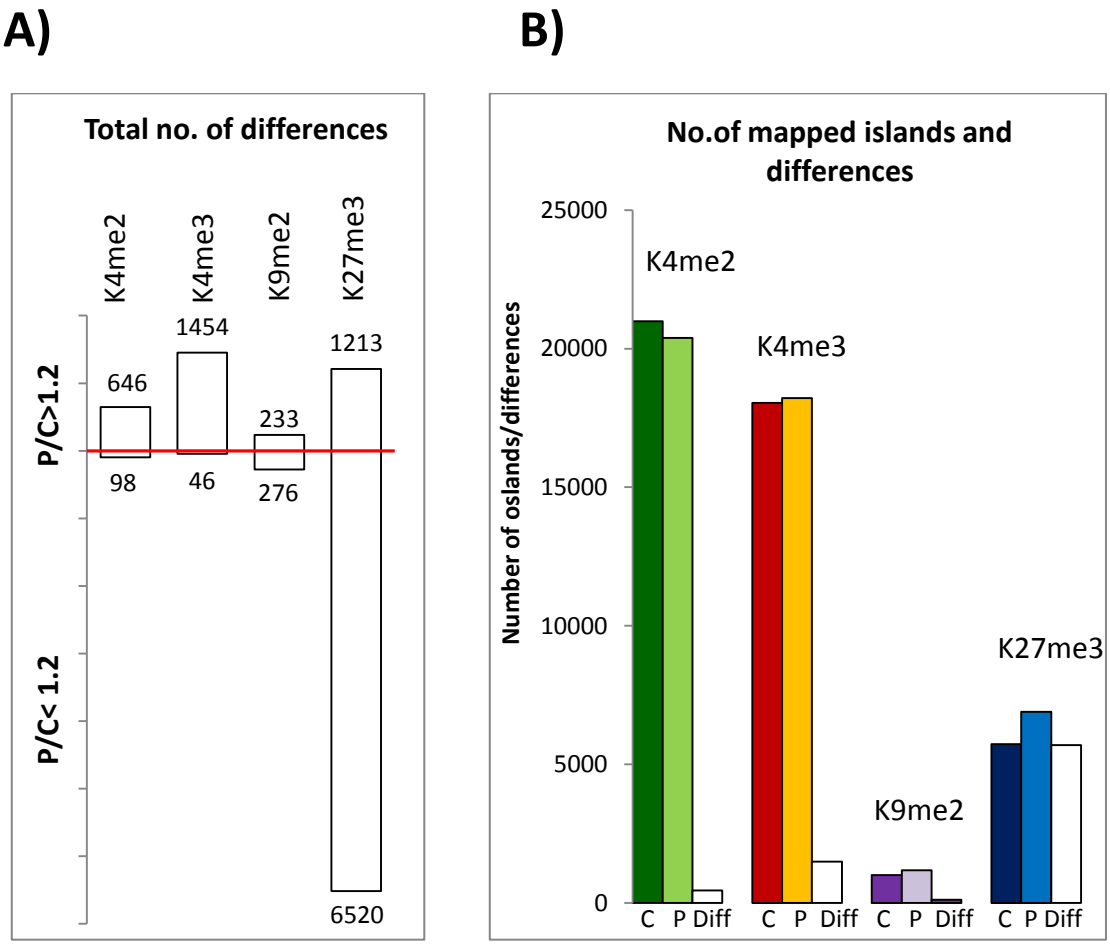


Fig. 5.11 Total number of regions that differ in the level of methylation between control and primed roots in all the modifications considered.

A) Number of differences higher than 1.2 fold. Bars above the red line give the number of sequences with an increase in the mark after priming; bars below the red line give the number of sequences with a decrease in the mark after priming.

B) Total number of mapped islands. Non-primed samples are in darker colours and primed samples are in lighter colours. The total number of differences higher than 1.2 fold change is shown in open bars.

5.2.4.3 Spectrum of genes with identified functions changed at the epigenetic level upon priming

The type of genes which were associated with different histone modifications and priming induced changes was further investigated by generating lists of islands and differences that were mapped to genes (criteria reported in Tab.5.1). The lists containing the TAIR ID of individual genes that showed a difference detected by ChIP-Diff were further analysed using DAVID. This software identified groups over-representing GO-terms and displayed them together with an enrichment score, a false discovery rate and a p-value (Huang et al., 2009). The results from this analysis are summarized in Tab. 5.3.

To exclude an enrichment in categories that were simply more associated with the specific modification, the appropriate list of genes resulting from mapped islands was used as background.

Interestingly, genes associated with priming-induced differences in histone modifications showed a bias in function. In particular, the most enriched categories were: “cell wall related” for H3K4me2, “transcriptional factors related” for H3K27me3 and “response to organic substance (hormones) for H3K4me3. DAVID analysis of differences for H3K9me2 was not possible due to their low number (resulting in high FDR).

Considering all these findings together, it is clear that priming has an effect on different histone modifications and in specific gene categories.

H3K4me2 Total DAVID ID 428	**Enrichment score 5.9**	**Count**	**P-Value**	**Fold Enrichment**	**FDR**
	Cell wall	**28**	**2.2E-07**	**3.1**	**2.56E-04**

H3K4me3 Total DAVID ID 1480	**Enrichment Score: 9.4**	**Count**	**P-Value**	**Fold Enrichment**	**FDR**
	Transcription factor activity	**165**	**2.1E-14**	**1.8**	**3.14E-11**
	Response to organic substance	**124**	**9.7E-10**	**1.7**	**1.58E-06**

H3K27me3 Total DAVID ID 3375	**Enrichment Score: 17.2**	**Count**	**P-Value**	**Fold Enrichment**	**FDR**
	Transcription factor activity	**188**	**2.8E-30**	**2.3**	**4.11E-27**
	Enrichment Score: 7.3	**Count**	**P-Value**	**Fold Enrichment**	**FDR**
	Secondary metabolites	**38**	**1.1E-12**	**3.5**	**8.19E-10**
	Enrichment Score: 7.1	**Count**	**P-Value**	**Fold Enrichment**	**FDR**
	Signal	**88**	**1.2E-09**	**1.9**	**1.55E-06**

Tab. 5.3 Functional categories enriched among genes showing differences in histone modifications between primed and non-primed plants.
The DAVID analysis was performed using the list of mapped islands as background for the respective modification.

5.2.5 Genome wide profiles of histone H3K27me3 10 days after priming

It was shown in the previous section that the histone modification most affected by the priming treatment in terms of islands length, number of differences and fold change was H3K27me3. Based on these observations, H3K27me3 was selected to investigate if these changes were maintained over time. In order to explore this possibility, additional experiments were performed on plants (primed or not) that had been grown in hydroponic culture without salt for a further 10 days after the 24h priming treatment. Plants were harvested and ChIP was performed on roots samples using the antibody against H3K27me3. Three independent experiments were carried out and each independent immuno-precipitated DNA sample was quality tested before the samples were pooled, sent for sequencing and analysed as before.

Fig.5.12 shows the genome wide epigenetic landscape of primed and non-primed roots after 24h and 10 days. Considerably less DNA was recovered from the 10 days samples. However the overall profiles were the same in all four samples.

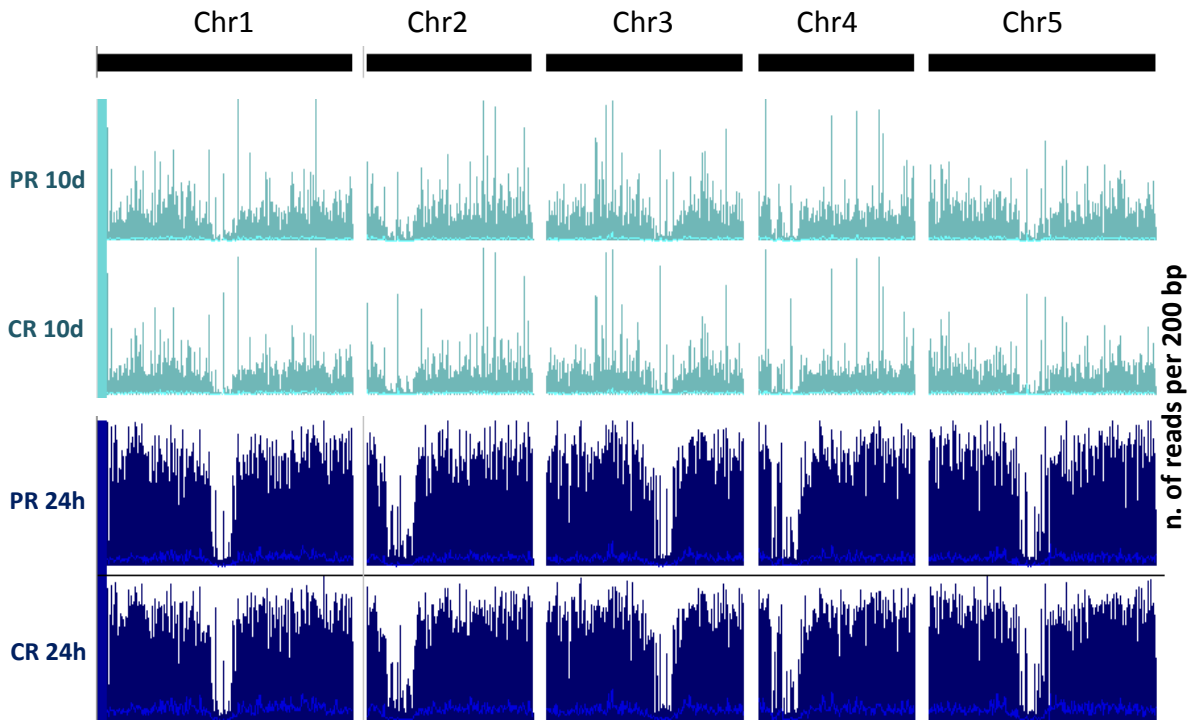


Fig. 5.12 Genome-wide landscapes visualized using IGB of H3K27me3 modifications along the five *Arabidopsis* chromosomes in roots samples obtained 24 hours and 10 days after priming.
H3K27me3 associated reads are shown in dark blue for roots samples harvested after 24h and in light blue for 10 days samples harvested 10 days after the priming treatment. Samples from non-primed plant roots at the same times are shown in darker shades.

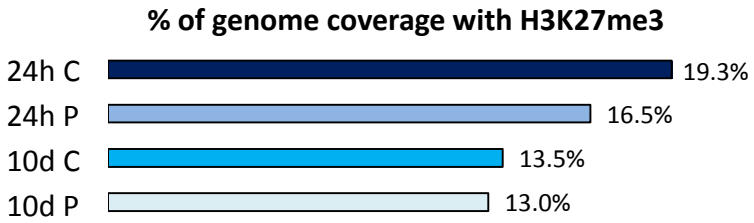
5.2.5.1 Methylation coverage 10 days after the priming treatment

Fig.5.13 shows the percentage of genome coverage, number of islands and island length distributions of H3K27me3 in samples harvested 10 days after the priming treatment compared to the same parameters in samples harvested after 24h (same data as Fig.5.9). H3K27me3 coverage had dropped from 19.3% to 16.5% (2.8% difference) 24h after priming. 10 days later the difference in coverage between primed and non-primed samples was much smaller (13.5 and 13% respectively, 0.5% difference), but coverage was still lower in the primed samples (Fig.5.13A).

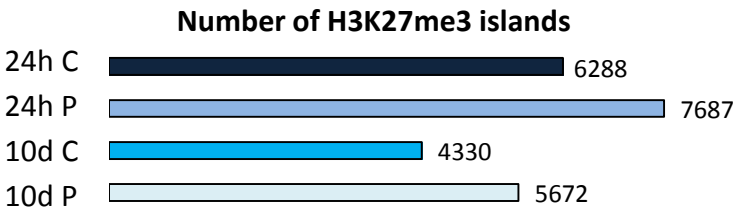
Fig.5.13B shows that immediately after 24h priming there was an increase in the number of H3K27me3 islands from 6288 detected in the non-primed to 7687 detected after priming (+1399 islands). The number of H3K27me3 islands was still greater in primed with 4330 in control and 5672 in primed (+1342 islands) 10 days after the treatment (Fig.5.13B).

Analysis of the distribution of the island length has shown that 24 hours after priming the island length had been shifted towards shorter islands. This shift was still apparent 10 days after the treatment (Fig.5.13C). In summary, these data show that in the older plants there was an all together lower coverage of the genome with H3K27me3 and a lower number of islands in older than in younger plants (Fig.5.13A-B). However, 10 days after priming there were still consistent differences to non-primed plants that mirrored those observed immediately after priming although these differences were smaller. The data suggest that priming induced changes are retained through cell division and plant development.

A)



B)



C)

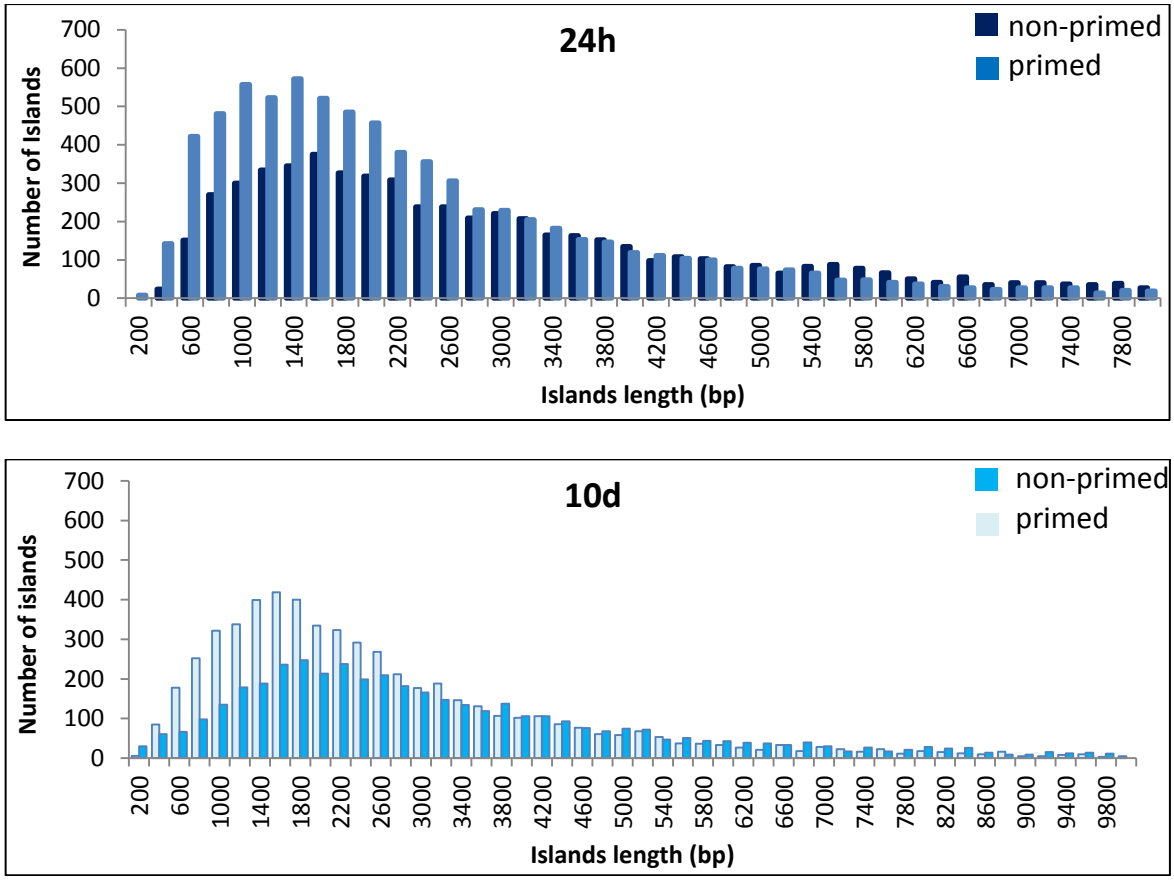


Fig. 5.13 Comparison of priming induced changes of H3K27me3 in roots 24h and 10 days after the transient priming treatment.

A) Percentage of genome coverage with H3K27me3. B) Number of H3K27me3 islands.
C) Island length distribution before and after priming treatment.
C: non-primed, P: primed samples.

It was then investigated, if the methylated islands detected in the primed samples were the same ones as those detected in the control samples for both the 24h and 10 days time points. For this purpose, only the islands that occurred within genes were analysed. The 24 hours samples showed 3680 mapped islands overlapping between non-primed and primed plants while 2677 islands were unique for the primed and 1745 were unique for non-primed plants (Fig.5.14). The 10 days samples showed 1411 islands overlapping between non-primed and primed plants while 2499 were unique for primed and 1572 for non-primed plants (Fig.5.14).

Finally, I wanted to establish if the islands detected 24h after priming were the same as the ones that were detected 10 days after the priming treatment or if new islands were generated once the stress was ended. In non-primed plants 1117 islands overlapped between the 24 hours and the 10 days sample, while 1866 were unique in the 10 days sample and 4308 in the 24 hours sample. In primed plants 3102 islands overlapped between 24 hour and 10 days samples, while 808 were unique in the 10 days sample and 3244 in the 24 hours sample (Fig.5.14).

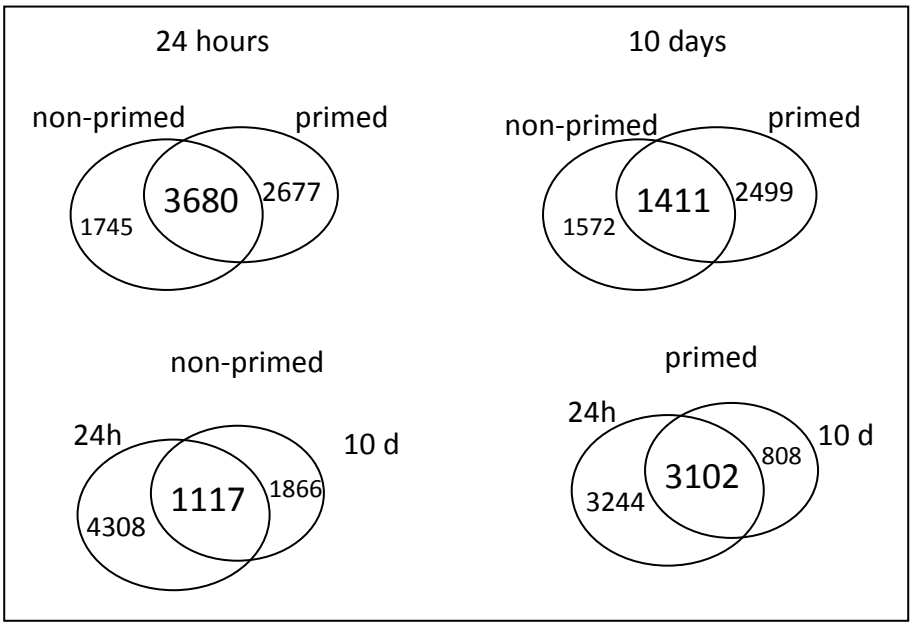


Fig. 5.14 Number of H3K27me3 islands mapped to genes in roots of non-primed and primed plants harvested 24h or 10d after the priming treatment.

5.2.6 Differences upon priming 10 days after the treatment

To investigate whether the individual changes in H3K27me3 that occurred after 24 hours of priming were still maintained 10 days later, the total number of differences identified by ChIP-Diff (FC> 1.2) was compared between the two time points. A general overview of differences in H3K27me3 along the five *Arabidopsis* chromosomes shows that the number of differences 10 days after priming is significantly reduced, passing from ~8000 differences to ~500. Of these differences 70% are represented by a decrease in H3K27me3 in the primed plants (Fig.5.15B). When only the differences which occurred within genes were considered (370 differences in total), 80% were already different after 24h of priming exposure (Fig.5.15C).

These results demonstrate that differences in H3K27me3 level, which were established by the priming treatment, are still retained 10 days after the exposure although the level is still predominantly lower. More intriguingly, most of the differences detected within genes had also been previously detected in the 24 hours sample, indicating that there is gene specific retention of methylation. Among these genes there were ion transporters such as *HAK5*, *ATCHX5, ATCHX10, ATCHX17, ATCHX24*, lipid transporter (*TGD1*), genes involved in the defence responses (*AZI1*). Furthermore genes encoding transcriptional factors (*AP2-like, MYB47, MYB112*) and a hormonal regulator (*ARR11)* were found. In particular, among the transcriptional factors one of the most predominant categories was involved in the cell cycle and cell fate determination (*JACKDAW, APELA, CAULIFLOWER* and *SUPERMAN*). The complete list is reported in Appendix VI.

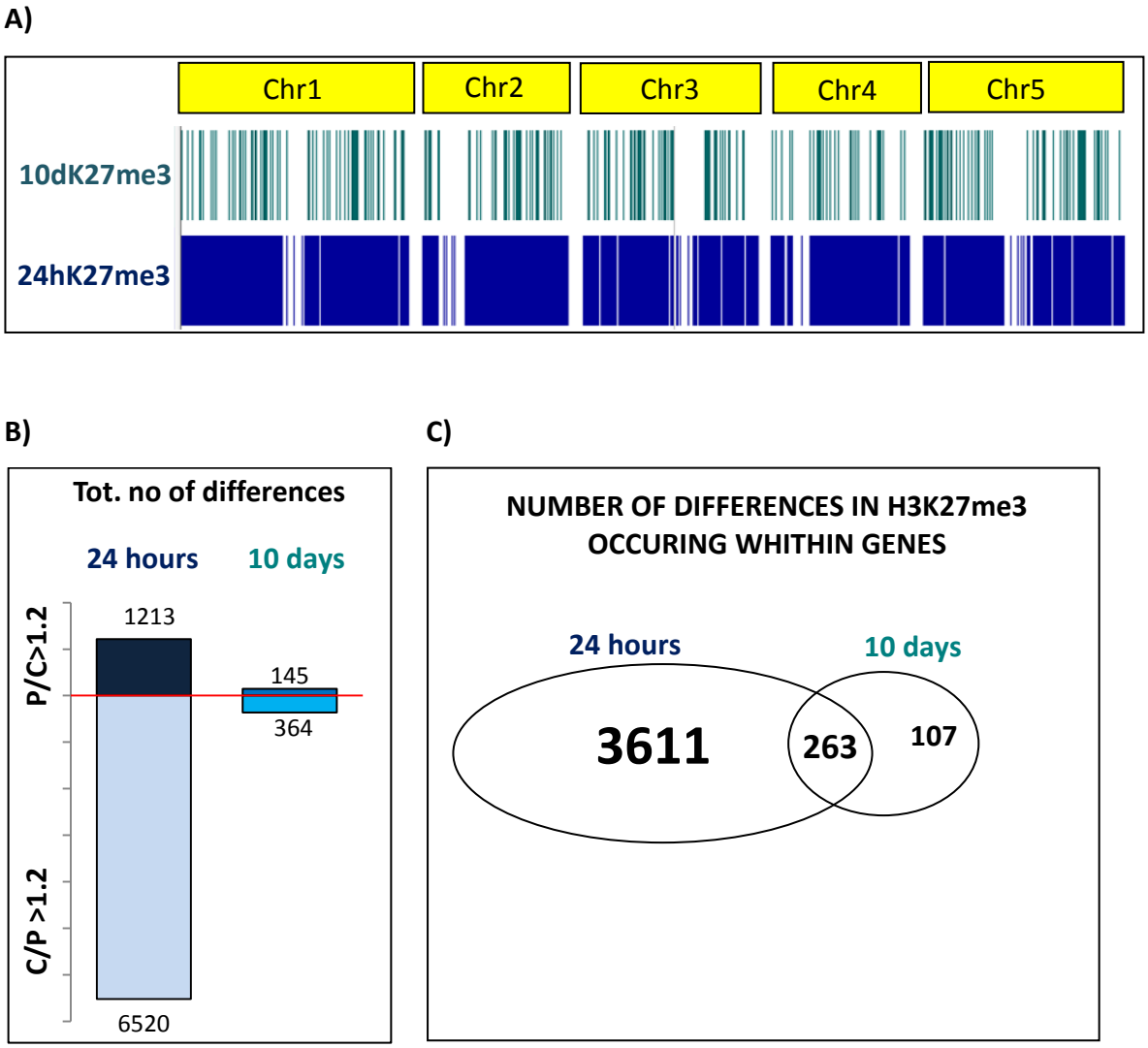


Fig. 5.15 Differences in H3K27me3 level between primed and non-primed plants at 24h and 10d after priming treatment.

A) Differences plotted along the five *Arabidopsis* chromosomes as visualized in IGB. Bars indicate differences in the level of H3K27me3 between primed and non-primed roots with a fold change higher than 1.2.

B) Total number of differences higher than 1.2 fold detected by ChIP-Diff. Lighter bars represent the number of sequences with a lower level of H3K27me3 in primed than in non-primed plants and darker bars represent the number of sequences with a higher level of H3K27me3 in primed than in non-primed plants.

C) Number of differences higher than 1.2 fold detected by ChIP-Diff within genes. The number of differences higher than 1.2 fold mapped against TAIR9 genes is detected in samples taken after 24h after priming (left circle), 10 days after priming (right circle) or both (overlap).

To assess whether individual differences in H3K27me3 between primed and non-primed plants detected 24 hours after priming were still maintained 10 days later, several genes were selected for which significant differences had been found after 24 hours.

Primers were chosen using the sequences visualized on IGB and designed on the borders of differences identified by ChIP-Diff and used to amplify the respective regions by quantitative real-time RT-PCR (Fig.5.16).

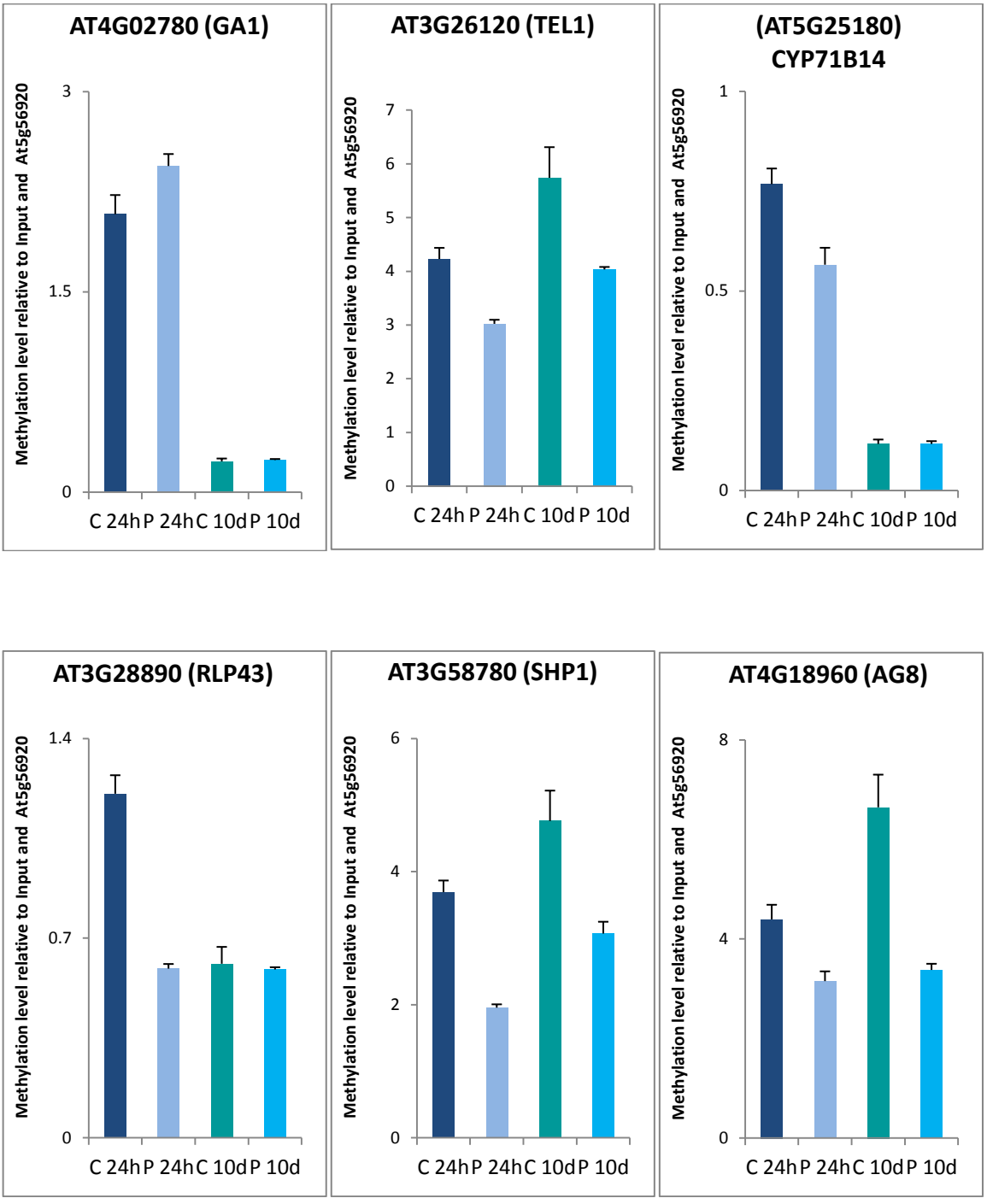


Fig. 5.16 H3K27me3 levels determined by qPCR in roots of primed (P) and non primed (C) plants harvested 24h or 10d after the transient priming treatment.
Primers were designed on the border of the identified differences. The percentage of enrichment was calculated relative to the reference gene At5g56920 (C: ChIP K27me3 control, P: ChIP K27me3 Primed) for both time points (24h and 10 days). Standard errors are calculated from four pair wise comparison of technical duplicates.

5.3 Discussion

5.3.1 Novelty and robustness of the experimental design

In the last few years several studies have investigated, on a genome wide scale, the correlation between different epigenetic markers, gene expression and gene function.
The approach used here differed from previous studies in several aspects. Firstly, in previous studies the plant material used was whole seedlings (Charron et al., 2009) or leaves (Van Dijk et al., 2010), which means a lack of consideration of differences between aerial and underground parts of the plant. This is very important when considering that stress responses may occur at an organ specific level and might result in a completely different epigenetic landscape for different tissues or cell types. Secondly, rather than combining Chromatin Immuno-Precipitation (ChIP) with microarray hybridization (ChIP-on-chip), I used every single DNA molecule pulled down with an antibody to be sequenced by Illumina technology (ChIP-Seq). While ChIP-on-chip measures relative signal strength, ChIP-Seq provides an absolute measure with every single sequence aligning to a genome region being counted, and is therefore much less prone to errors (Ho et al., 2011). Finally, for the first time a dual-factor comparative analysis was carried out to investigate the differences between primed and non-primed plants and between roots and shoots. The ChIP experiment itself is a methodology that can lead to variability in the results, because the recovered DNA is detected by an indirect method: a specific antibody recognizes a specific modification on the histone tail associated with a precise part of the DNA sequence. This indirect determination makes the quality of a ChIP experiment largely dependent on the antibody's affinity and specificity which in turn depends on the exact environment and timing during the process and hence can lead to variation between IP assays from the same material ('technical replicates'). Furthermore, as in all biological experiments, conditions of plant growth and treatment applications cannot always be fully controlled leading to variation between plant batches and replicate experiments ('biological replicates'), which adds to the phenotypic plasticity within an isogenic population caused by variation between individual plants. Due to the high costs of Illumina sequencing and the antibodies used, I could not quantify all of these variations. Instead I have taken the approach to pool tissue from a large number of individual plants

for a particular developmental stage (300 plants at 4-leaf stage per biological replicate) from three independently treated plant batches. The technical quality of the ChIP performed was checked by PCR for each of the three replicate samples before pooling. Several antibodies were used to explore the effect of salinity priming on the chromatin structure including antibodies against di- and tri-methylation of Lysine 4 (H3K4me2 and me3), di-methylation of Lysine 9 (H3K9me2) and tri-methylation of Lysine 27 (H3K27me3) of the histone tail H3. The quality of the sequencing was very good; in particular the number of fragments was similarly high for each sample, independent of experiment, condition or tissue (Fig.5.2). The sequences obtained were aligned against the *Arabidopsis* genome (version TAIR9). Interestingly, a comparable level of alignment for H3K9me2 was only reached if multiple alignments were allowed (Tab.5.2). If multiple alignments were not allowed, a severe loss of information occurred for this particular modification, because H3K9me2 is a heterochromatic marker typically associated with transposons and highly repetitive sequences. Therefore to retain this information, multiple alignments were allowed for this modification. However, this leads to uncertainty as to which of the identical sequences in the genome really carry the modification.

5.3.2 Priming does not radically disrupt the epigenetic landscape

The overall patterns of the four histone modifications analysed in this study were found to be in agreement with previous studies (Fig.1.4): H3K4me2-me3 and H3K27me3 occur with high frequency in euchromatic arms and rarely in heterochromatin (Zhang et al., 2009, 2007b), while H3K9me2 shows the opposite pattern (Bernatavichute et al., 2008). Furthermore, these overall genome-wide signatures were also found unaltered when the priming treatment was applied (Fig.5.4). It has been shown that one of the roles of the epigenetic marks is to define gene expression in a particular cell line (Lafos et al., 2011). For example, genes associated with H3K27me3 were found to be associated with tissue-specific repression and are highly enriched in transcription factors and DNA binding proteins that are important to developmental regulation (Ha et al., 2011) . Thus, it is more likely that during environmental perturbation the overall patterns of methylation are robust but can be fine-tuned. The finding that changes in the histone modification patterns were small is in accordance with this reasoning.

5.3.3 Biological meaning of the measured parameters

Significant differences between primed and non-primed plants were detected by comparing the reads obtained from the two ChIP samples. Overall, primed plants were not found to gain (or loose) entire islands along the chromosomes. Instead, the variation was in the number of reads at the same position. Within a given DNA molecule a specific genomic position can either be associated with a histone modification or not. Within a mixed cell sample a change in reads at a given position can be due to several reasons: I) Only a specific cell type reacts to the stress changing its contribution to the overall profile. II) The stress enhances the probability of the modification to occur in several or all cells types in the sample. The first possibility is supported by the fact that some cell types carry specific histone modification patterns (Lafos et al., 2011). It is therefore possible that some cell types are more prone to epigenetic changes upon stress than others and this is detected as a small change in a particular position in the overall profile.

The second possibility is supported by a recent study that investigated the vernalization process involving the negative regulator of flowering gene *FLC* (*FLOWER LOCUS C*). The authors found that during cold exposure the H3K27me3 levels progressively increase spreading through the *FLC* locus, until the overall coverage is reached and thus prevents the plants from flowering (Angel et al., 2011). Furthermore, it was shown that cold exposure increased the number of cells carrying H3K27me3 in the *FLC* gene (Angel et al. 2011). Thus, during repetitive cold treatments the silencing marker appeared associated with different cell types in random positions within the root, suggesting a change in overall methylation probability rather than targeting of a specific cell type. This probability model could explain small changes observed after priming; perhaps not all the cells are carrying the modification because a single priming treatment is not sufficient to further stimulate the switch in the vast majority of the cells.

5.3.4 Are the observed priming induced changes in epigenetic modifications significant?

As mentioned above, ChIP sequencing is a very expensive technique, therefore the number of replicates is a limiting factor.

It was decided to pool 3 ChIP replicates into one sequencing sample meaning that it was not possible to test the significance of the observed changes. However, several observations indicate that the identified differences between primed and non-primed plants were significant even if they only showed a small difference in fold change.

Firstly, root and shoot of the same plant were compared and this allowed us to use the shoot data as a proof for the soundness of the design of the experiments. For example the two profiles of H3K4me2 and H3K4me3 showed that the shoot profiles of primed and non-primed plants were almost identical, whereas considerable variation was found between the root profiles (Fig.5.4). Secondly, differences in island number, percentage of coverage and length distribution upon priming were only found in roots. It should be noted that even though roots and shoots were harvested separately the obtained profiles are still likely to be a combination of profiles overlaid from different cell types. As a consequence changes that are strongly cell type specific will be detected as small differences because they are diluted.

Taking all these observations into account, it is possible to conclude that the small differences observed in the roots are real and significant.

5.3.5 Observed differences between primed and non-primed samples were specific for the particular histone modification measured

Interestingly, no major changes upon priming were detected in the total methylation coverage for H3K4me2-me3 and H3K9me2. The number of islands and the island lengths for these modifications was also relatively constant (Fig.5.9 and Fig.5.10). In contrast, H3K27me3 showed a 3% decrease in the general methylation coverage when priming treatment was applied (Fig.5.9). These results indicate that rather than creating new methylation islands, priming tunes the level of histone methylation in specific sequence positions. Interestingly, a similar conclusion was reached by van Dijk et al., (2010) who compared patterns of H3K4me3 methylation of *Arabidopsis* leaves between plants that were watered normally and plants that had undergone a prolonged dehydration stress. Even though the authors found that dehydration stress increased the H3K4me3 gene coverage by 4.8%, the location of islands did not change. The data obtained in my work also indicated that priming increased the number of H3K27me3 islands while shortening

the length of the islands. This could be explained by a small decrease of H3K27 in a position within an island that already has a low probability for the modification. As a result islands would be interrupted ('chopped'). Closer inspection of individual islands proved that this was indeed the case.

5.3.6 Individual priming induced changes

It was found that the direction of priming-induced changes differed for different modifications, in particular the level of H3K27me3 mostly decreased after priming, while the level of H3K4me3 increased (Fig.5.11). Furthermore, H3K27me3 was by far the most responsive modification to the priming treatment (Fig.5.11). This suggests that priming acts most strongly by releasing silencing in specific loci (through a decrease of H3K27me3), and in a moderate way by activating specific genes (through an increase in H3K4me3).

It was also investigated which type of genes were associated with the priming-induced differences in methylation levels. Results from this analysis showed that all four histone modifications targeted specific functional gene families: including categories related to stress responses, transcription factor activity and cell wall remodelling (Tab.5.3). This bias in function among the different histone modifications suggests that priming has a specific effect on particular gene families. In addition, other studies indicate that, in *Arabidopsis*, H3K27me3 targets are enriched for genes with tissue-specific expression patterns or are induced by abiotic or biotic stresses. This suggests that H3K27me3 is dynamically regulated in response to developmental or environmental cues as suggested by previous studies (Lafos et al., 2011; Zhang et al., 2007b).

5.3.7 Do the observed changes in H3K27me3 constitute somatic memory?

H3K27me3 was identified as the modification most responsive to the priming treatment in terms of the total number of sequences and fold change (Fig. 5.11). These results made H3K27me3 the ideal candidate to further explore the persistence of these priming induced changes. A further ChIP-Seq of H3K27me3 was carried out using plants exposed to priming treatment, then removed from the stress and left to grow for another 10 days

in hydroponics. The results showed that the overall level of H3K27me3 after 10 days appeared to be slightly lower than after 24 hours, even in control conditions. However, despite the fact that 10 days after priming the difference in methylation coverage between primed and non-primed samples dropped by 2.3% (Fig. 5.13A), a difference in the number of islands (+1342) between primed and non-primed plants was still detected suggesting that overall the changes in methylation are retained (Fig. 5.13B). The data also reflected another exciting finding: the distribution of the island length was found not only changed after 24 hours of priming but still continued to be changed even after 10 days of priming (Fig. 5.13C).

In addition, 509 individual differences were still detected 10 days after the priming (Fig. 5.15B) and more than half of them were present within genes (270). Out of these, more than a third occurred in the same genes (Fig. 5.15C). Taken together these results suggest that priming-induced changes of H3K27me3 are maintained in the same genes 10 days after the priming. It does not, however, clarify whether and how this mechanism takes part in the complex network of signalling pathways that regulate stress responses. These observations certainly raise the question of how this retention of histone methylation is possible through replication or cell division. It has to be noted that plants older than 10 days are not dividing anymore. Instead, they are entering in a phase of expansion and maturation (after 21 days), where endoreduplication is only occurring in the cells (Beemster et al., 2005). During this process the cells often become polyploid (i.e up to 16C genomic) (Beemster et al., 2005). It has been shown that osmotic stress (i.e salt, mannitol) stops both cell division and endoreduplication (Skirycz et al., 2011). However, it is very unlikely that this delay causes the observed differences in H3K27me3 because the stop of cell division and endoreduplication is transient. Skirycz et al. (2011) have shown that stressed cells were undistinguishable from non-stressed cells for a period of three days after the stress was removed (Skirycz et al., 2011).

The fact that the changes in H3K27me3 are still detectable after 10 days could have two interpretations: I) The methylation marks are actively maintained and deposited during replication; II) The methylation marks are diluted during the replication process but remain and are still detectable even after 10 days.

In the first hypothesis, the low level of H3K27me3 could be explained by the fact that the modification primarily occurs in specific cell types, which are outgrown by other cell types during the 10 day period.

In the second hypothesis, what is seen here, is the end of the priming effect: there are still traces of the marks put into place by the priming treatment but these will be eventually removed through the action of de-methylase enzymes. It should be interesting to find out whether repeated treatments prevent the marks from being lost, and what is the required length of time between two treatments to fully maintain marks.

How histone methylation marks are maintained through the DNA replication is still under debate. Currently two models have been proposed for *Drosophila* (Abmayr and Workman, 2012).

The first model proposes that histones carrying the modification pass through the replication fork, but not the histone methyltransferase complex (MTCs) that is associated with them. Upon replication, histones are inherited by the daughter strands in a semi-conservative way and new MTCs are recruited to re-establish the methylated domain (Corpet and Almouzni, 2009).

In the second model, the modifications are removed from the histone prior to the passage through the replication fork. The MTCs methylation machinery is distributed onto the daughter strands where it re-methylates the parental nucleosomes and methylates the new nucleosomes. This leads to a recruitment of new MTCs to fully complete the methylation domain (Petruk et al., 2012).

However, the process of how histone modifications are inherited still awaits detailed study in plants.

Chapter 6: Comparative analysis between epigenetic and transcriptional profiles

6.1 Introduction

6.1.1 Background

The relationship between particular histone modifications and gene expression is well documented and well conserved among organisms: H3K4me3 is positively correlated with gene expression meaning that high levels of methylation at Lysine 4 are usually associated with actively transcribed genes. On the contrary H3K27me3 is often associated with non transcribed genes (Roudier et al., 2009).

However, the question whether specific histone modifications and transcription are still correlated during or after a salt stress, has to my knowledge, never been investigated.

One question is whether changes in histone modifications observed after 24 hours (as shown in Chapter 5) merely reflect transient changes in gene expression. Another question is whether differential transcriptional responses after the second treatment are related to priming induced changes in histone modifications.

6.1.2 Aims of the chapter

In order to investigate the relationship between epigenetic changes and transcriptional differences, the gene-specific epigenetic differences between primed and non-primed plants detected after 24 hours of priming were integrated with transcript levels in the same samples determined by RNA sequencing.

Furthermore, the differences in transcriptional responses of primed and non-primed plants detected after the second stress exposure (described in Chapter 4) were compared with the differences in H3K27me3 at 10 days after the priming.

6.2 Results

6.2.1 Analysis of transcriptome immediately after priming

To investigate the relationship between changes occurring in histone modifications and changes in gene expression after priming, complete mRNA sequencing (RNA-Seq) was carried out on roots and shoots samples from primed and non-primed plants harvested directly after exposure for 24 hours of priming solution (50mM NaCl). The sequencing data were recorded at the *Glasgow Polyomics Facility*. Reads obtained from Illumina sequencing were aligned to *A. thaliana* genome and cumulative read values were assigned to each transcript (mRNA level). To provide a first representation of the data, all mRNA levels were used to generate scatter plots. Fig.6.1, shows the mRNA level for primed plants (P) plotted against the mRNA level for non-primed plants (C). As expected the priming treatment influenced the gene expression (Fig.6.1). In shoots, RNA-Seq detected a total number of 22787 significantly expressed genes, of which 1248 (~5%) were differentially expressed with a fold change higher than 2 (634 were up-regulated, 614 down-regulated). In roots, RNA-Seq detected a total number of 23222 significantly expressed genes, of which 2987 (~13%) were differentially expressed with a fold change higher than 2 (1689 up regulated, 1298 down regulated). The differentially expressed gene lists (Fc> 2) were loaded into DAVID and functional annotation compared against the *Arabidopsis* genome background. The results of the DAVID analysis showed that the functions of the genes that changed in expression upon priming were the same as those identified by the Affymetrix array as responding to the second salt exposure (Tab.6.1, compared with Tab.4.6).

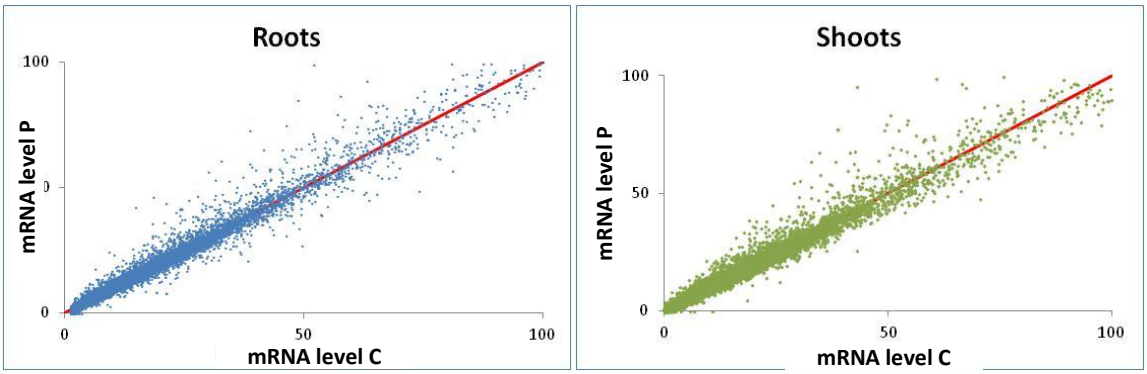


Fig. 6.1 Scatter plot of mRNA levels in roots (left) and shoots (right) of non-primed (C) and primed (P) plants from the RNA-Seq.
Red line indicates the position of the linear regression for the data.

Tab. 6.1 Enriched GO categories for genes showing differential expression upon priming in roots (blue) and shoots (green). (UP: up-regulated genes where P>C with Fc>2; DOWN: down-regulated genes where C>P with Fc>2).

ROOTS

UP	Cluster1 Enrichment Score: 6.0	Count	%	P-Value	FDR
	GO:0045449~ regulation of transcription	71	11.7	1.0E-09	1.5E-06
	Cluster 2 Enrichment Score: 2.9	Count	%	P-Value	FDR
	GO:0006350 ~transcription	48	7.9	4.2E-07	6.1E-04
	Cluster 3 Enrichment Score: 1.5	Count	%	P-Value	FDR
	GO:0009695~jasmonic acid biosynthetic	5	0.8	5.4E-04	8.0E-01
DOWN	Cluster 1 Enrichment Score: 3.5	Count	%	P-Value	FDR
	GO:0030528 ~transcription regulator activity	47	9.2	2.2E-05	2.8E-02
	GO:0051252 ~regulation of RNA metabolic process	31	6.0	5.5E-05	7.6E-0.2

SHOOTS

UP	Cluster 1 Enrichment Score: 3.2	Count	%	P-Value	FDR
	GO:0020037~heme binding	22	3.4	1.5E-05	2.0E-02
	GO:0009055~electron carrier activity	27	4.1	4.0E-04	5.3E-01
	Cluster 2 Enrichment Score: 2.7	Count	%	P-Value	FDR
	GO:0005199 ~structural constituent of cell wall	9	1.4	8.2E-07	1.1E-03
	Cluster 3 Enrichment Score: 2.4	Count	%	P-Value	FDR
	GO:0004601 ~peroxidase activity	10	1.5	7.5E-04	9.9E-01
DOWN	Cluster 1 Enrichment Score: 4.4	Count	%	P-Value	FDR
	GO:0003700 ~transcription factor	60	8.8	1.2E-07	1.5E-04
	Cluster 2 Enrichment Score: 2.5	Count	%	P-Value	FDR
	GO:0009055 ~electron carrier	25	3.6	3.6E-04	4.7E-01

6.2.2 Localization of epigenetic differences

To enable comparison between priming induced changes in mRNA levels and the level of histone modifications the latter had to be mapped to genes. The total number of differences in histone modifications detected by ChIP-Diff (fc>1.2) was compared to the number of differences localized within genes and to the number of differences in mRNA levels. 51% of the total number of differences detected in H3K27me3 could be mapped to genes (3981 out of 7733). Furthermore, more than half of the genes that harboured differences (2600) were expressed (as detected by RNA-Seq). The vast majority of differences in H3K4me3 were localized in genes (1438 out 1500) most of which were expressed (1381) (Fig.6.2). Similarly, 53% of the total number of differences detected in H3K4me2 could be mapped to genes (449 out of 744) most of which were expressed (400).

On the contrary, H3K9me2 presented very few differences which were localized in expressed genes (50 out of 118 mapped differences, out of a total of 509 total differences detected along the genome) (Fig.6.2).

I then compared the changes in individual genes with changes for histone modifications in the same genes (Fig.6.3). As shown in Fig.6.3, 11.2% of the expressed genes (detected by RNA-Seq) harboured changes in H3K27me3 upon priming, followed by H3K4me3 (5.9%), H3K4me2 (1.7%) and H3K9me2 (0.2%). However, if only the differentially expressed genes were considered, these values doubled for H3K27me3 and H3K9me2 (22.1% and 0.4% respectively), but decreased by half for H3K4me3 and H3K4me2 (2% and 1% respectively).

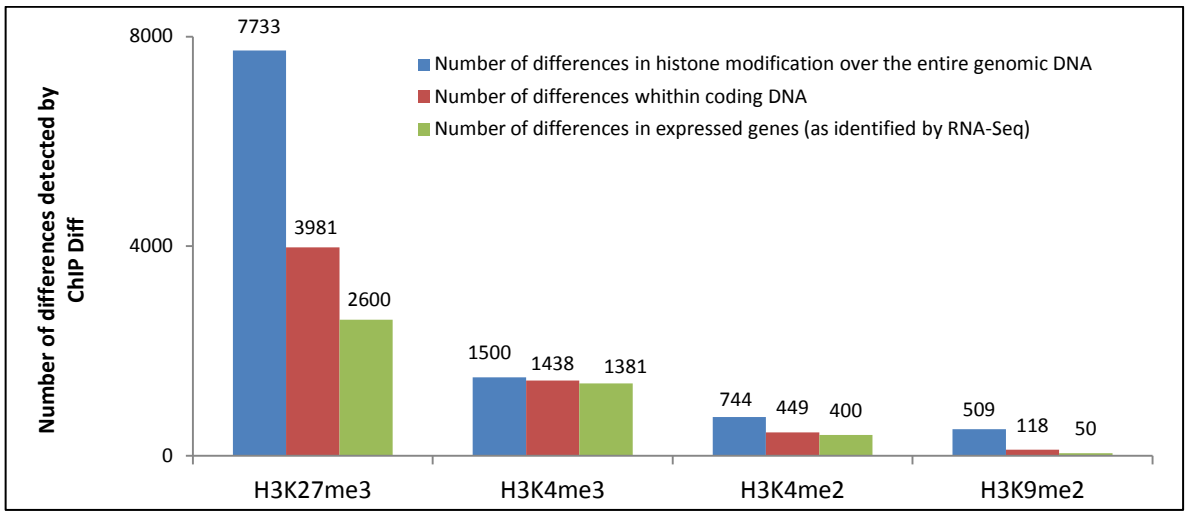


Fig. 6.2 Number of priming induced histone modifications related to the genome and the transcriptome.
Blue: total number of differences occurring over the entire genomic DNA.
Red: number of differences occurring exclusively in coding DNA (mapped using TAIR9).
Green: number of differences occurring in expressed genes detected by RNA-Seq.

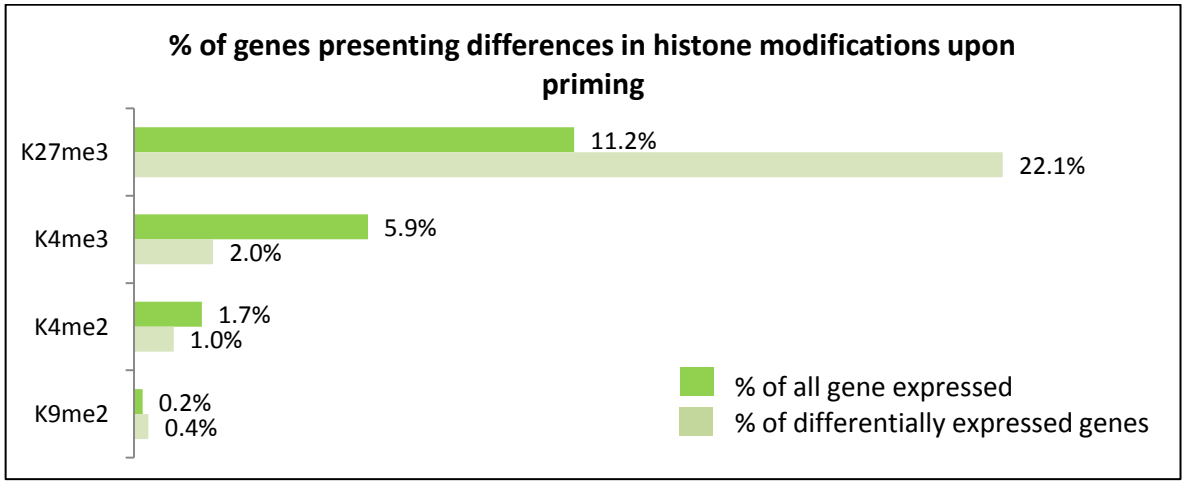


Fig. 6.3 Percentage of genes presenting differences in histone modifications upon priming.
Dark green: differences in the percentage of all expressed genes detected by RNA-Seq.
Light green: differences in the percentage of all differentially expressed genes between primed and non-primed roots.

6.2.3 Relationship between gene transcription and epigenetic modification in primed and non-primed plants

In order to investigate the quantitative relationship between the level of a specific epigenetic modification and the level of the transcripts obtained by RNA-Seq were compared against the methylation islands mapped to genes.

Each individual gene was assigned two values: one indicating the gene expression level (obtained from the RNA-Seq) and the other one the histone modification level (ChIP-Seq cumulative number of reads over the entire gene normalized to gene length). Genes with zero value for either histone modification or RNA level were excluded from the analysis. Genes were ranked by increasing levels of gene transcription then the average of the histone modification level over the neighbouring ~200 genes was assigned along with the average of the RNA level (± 100 windows size= 10% of total number of genes). Scatter-plots were generated by plotting the ranked genes against average histone methylation (Fig.6.4). The underlying mRNA profiles were also included. As shown in Fig.6.4, the data confirmed the previously reported correlation between histone modification level and gene expression: high level of H3K4me3 correlated with high transcripts level, while high levels of H3K27me3 corresponded to a low gene expression level. No correlation was observed for H3K4me2 and H3K9me2 (data not shown). The results show that even though priming impacts on histone modifications, differences in individual genes do not affect the general correlation between specific modifications and transcription.

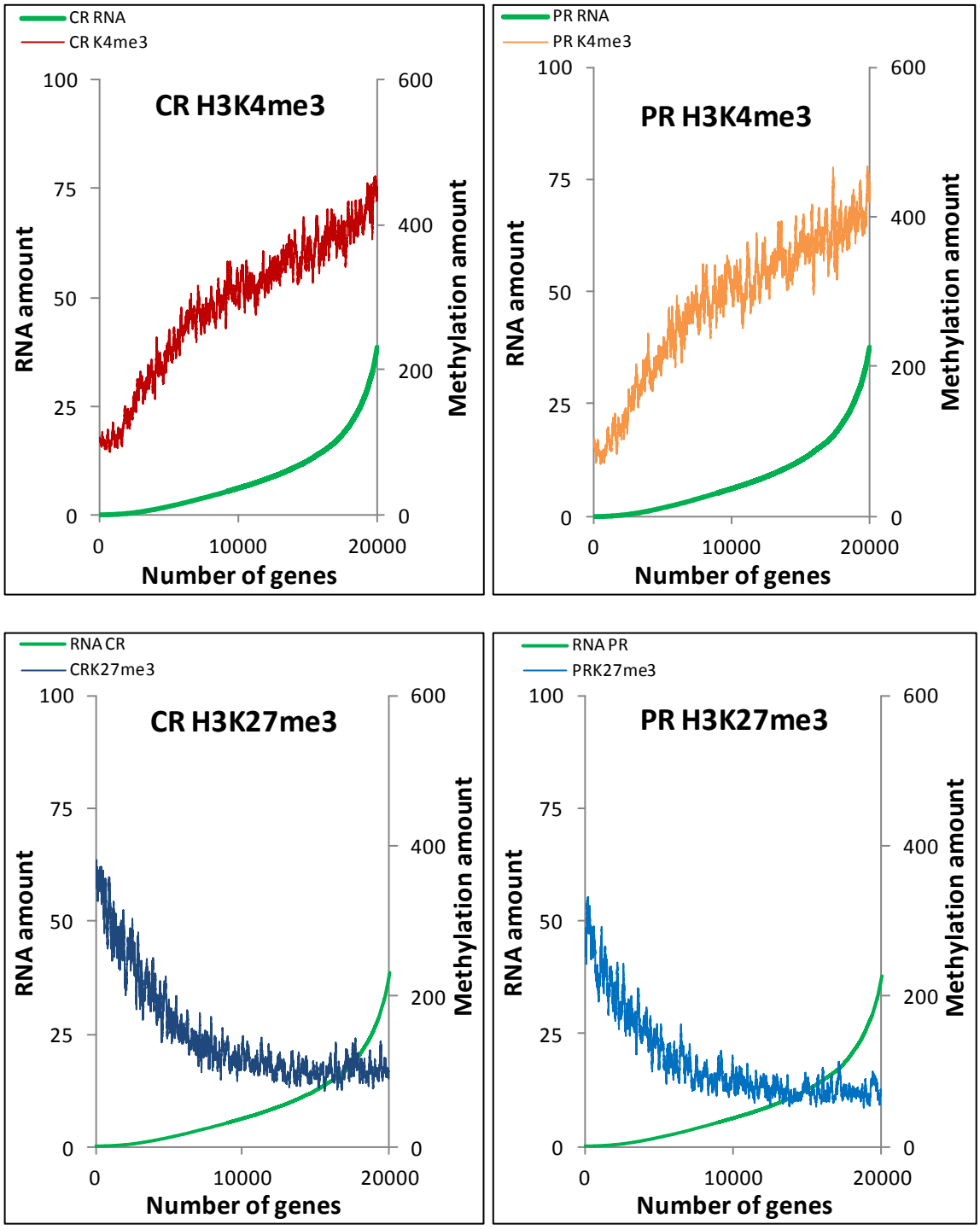


Fig. 6.4 Correlation between transcription level and methylation level.
On the x axis: Genes ranked by mRNA level.
Green curves are mRNA levels averaged over windows of 200 genes (left y axis). Other colours: histone modification levels over windows of 200 genes (right y axis).
Top: H3K4me3 level in non-primed (red) and primed (orange) roots.
Bottom: H3K27me3 level in non-primed (blue) and primed (light blue) roots.

6.2.4 Changes at the transcriptional level do not simply reflect changes in histone methylation

The overall correlation between transcript levels and H3K4me3 (positive) and H3K27me3 (negative) has been shown previously. Nevertheless, going through the exercise of establishing these correlations from my data, revealed two important facts. Firstly if the values were plotted individually for each gene the data was much too noisy to reveal any correlation. Secondly the correlation is much stronger for low mRNA levels than for high mRNA levels (Fig.6.4). These observations imply that at the level of individual genes changes in mRNA levels cannot be predicted from changes in histone modification levels and vice-versa. Indeed when mRNA levels and histone modifications levels were compared for individual genes the results did not reflect the established correlation.

Out of 1689 genes transcriptionally up-regulated upon priming only 372 also showed differences in methylation in H3K27me3, (83 up and 289 down), 42 genes in H3K4me3 (7 down, 35 up), 17 for H3K4me2 (2 down, 15 up) and 7 for H3K9me2 (1 down, 6 up). Out of the 1298 genes down-regulated upon priming, only 288 also showed changes in H3K27me3 (58 up, 230 down), 18 for H3K4me3 (0 down, 18 up), 13 for H3K4me2 (2 down, 11 up), and finally only 4 for H3K9me2 (1 down, 3 up) (Fig. 6.5). These findings indicate that at single genes level priming-induced changes in histone modifications were either largely independent of priming-induced changes in transcription or that the dynamics of histone modifications and mRNA are quite different and can therefore not be compared at one single time point only.

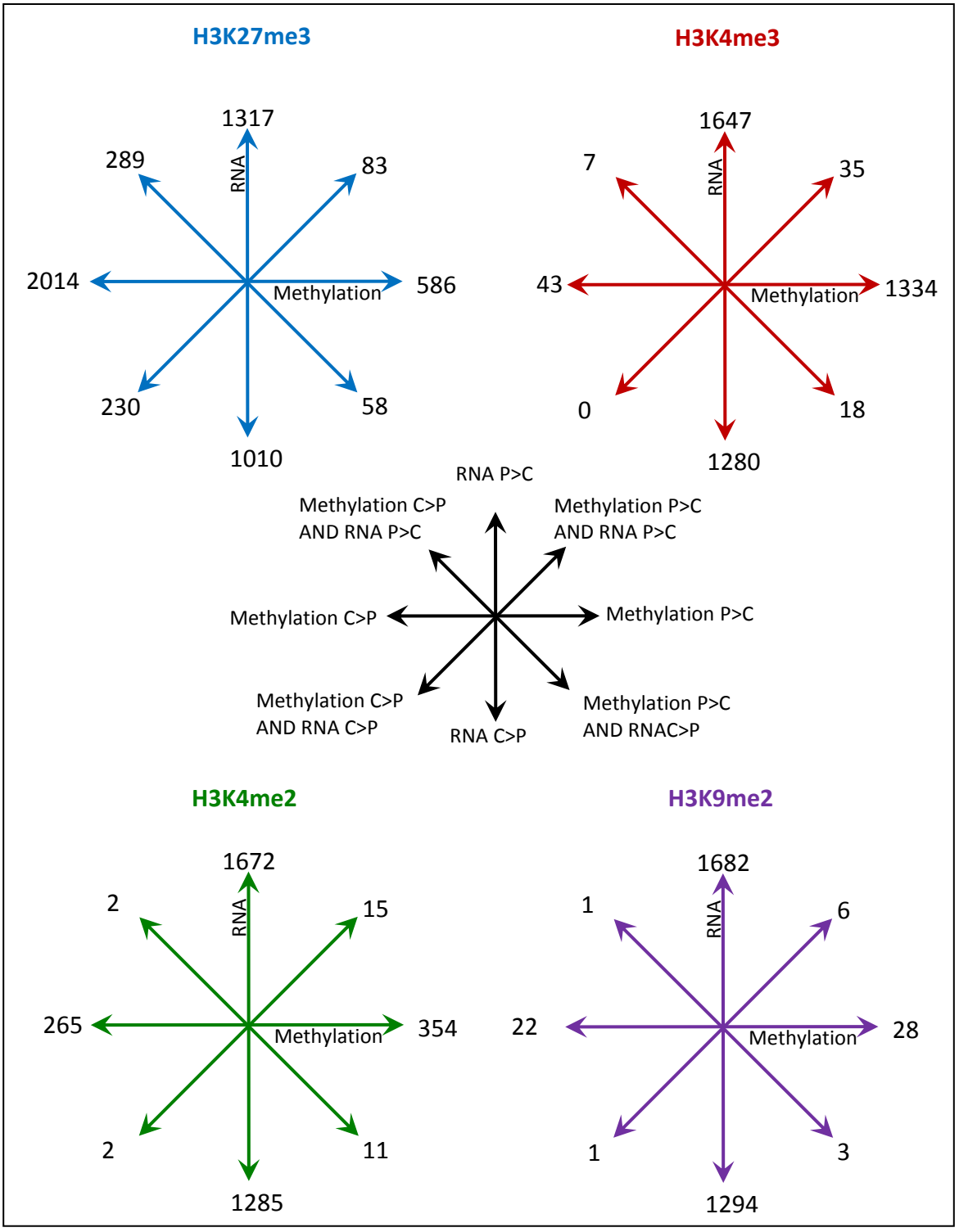


Fig. 6.5 Number and direction of priming induced changes of mRNA and of H3K27me3 (blue), H3K4me3 (red), H3K4me2 (green), H3K9me2 (purple).
Arrows indicate the direction of differences between primed (P) and non-primed (control, C) root samples as indicated in the central diagram.

6.2.5 Dynamics of histone methylation H3K27me3 and transcriptional changes

In order to investigate the dynamics between histone modification and transcriptional changes, selected genes indicated by the ChIP-Seq or RNA-Seq as being changed in H3K27me3 or mRNA after priming were analysed at additional time points using ChIP-RT qPCR. Roots from 3 weeks old plants (4L stage) were subjected, as before, to 50 mM NaCl and samples harvested after 0, 1, 4 and 8 hours. The results are reported in Fig.6.6.

In summary changes in methylation upon stress exposure can be extremely dynamic. This preliminary kinetic analysis, made from one experiment, show how little we understand about the exact relationship between histone modifications and gene expression in a non-steady state. The data indicate that H3K27me3 is indeed negatively correlated with mRNA for some genes but this correlation is reversed or absent for other genes.

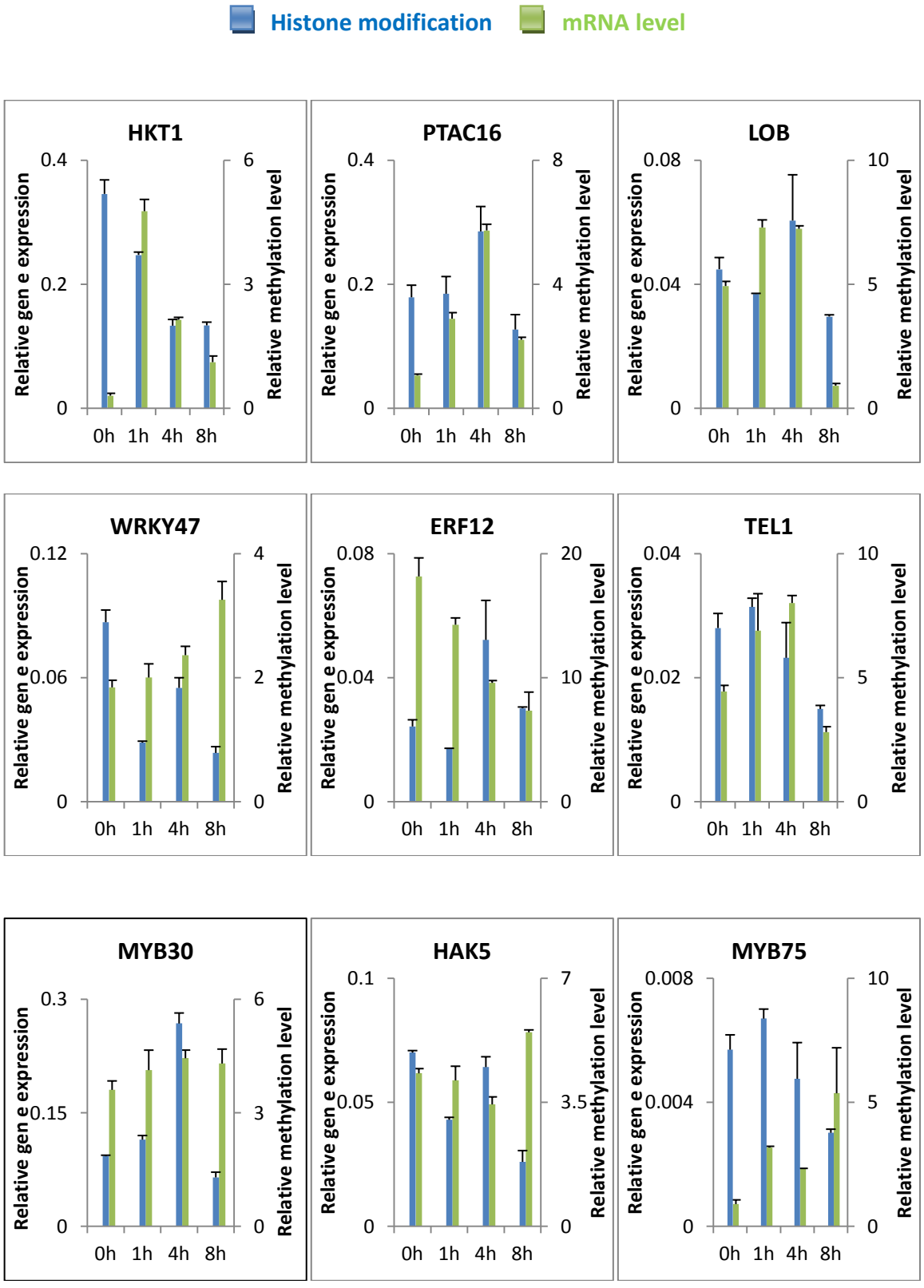


Fig. 6.6 Time course showing changes in H3K27me3 and mRNA during priming treatment.

qPCR were performed on ChIP samples to obtain the fold of enrichment relative to input for each time point. qPCR were performed on cDNA samples in order to detect gene expression relative to reference gene RpII. Errors bars are standard errors of technical duplicates of qPCR.

6.2.6 H3K27me3 differences and transcriptional responses after priming

Finally, I compared the differences in H3K27me3 levels apparent at 10 days after priming (Chapter 5) with the genes that showed differences in transcriptional responses to the second salt treatment applied 10 days after priming as detected by the microarray analysis (see Chapter 4).

After 10 days, 34 genes showed a difference in H3K27me3 between primed and non-primed samples and also showed a differential response to the second salt treatment. 21 of these responded less to salt in primed plants, while 13 genes responded more strongly. The complete gene lists are reported in Tab.6.2.

The list includes transcriptional factors such as *MYB92*, *MYB112*, *WRKY8*, electron carriers (*CYP714A1*, *CYP708A3*), oxidoreductase, peroxidase, transporter *HAK5*, *ATCHX17*, calcium ion binding *TSA1* (*TSK-ASSOCIATING PROTEIN 1*); *WAK1* (*CELL WALL-ASSOCIATED KINASE*); kinase, as well as several unknown proteins. Only four genes showed a decrease in H3K27me3 and an increase in transcriptional response. Other genes combined higher H3K27me3 with a lower transcriptional response (9 genes) or had parallel changes (12 genes combined lower H3K27me3 with lower transcriptional response, while 8 genes combined higher H3K27me3 with a high transcriptional response.

TAIR ID	H3K27me3 day 10 (P/C)	Description	Response (P+/P-)/(C+/C-)
AT1G52060	down	Function unknown	UP
AT1G52070	down	jacalin lectin family protein	UP
AT1G62510	down	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	UP
AT1G33840	down	unknown protein	UP
AT5G24910	down	CYP714A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	UP
AT5G10280	up	ATMYB92 (MYB DOMAIN PROTEIN 92); DNA binding / transcription factor	UP
AT1G63560	up	Function unknown	UP
AT4G13420	up	HAK5 (HIGH AFFINITY K+ TRANSPORTER 5); potassium ion transmembrane transporter/ potassium:sodium symporter	UP
AT2G36690	up	oxidoreductase, 2OG-Fe(II) oxygenase family protein	UP
AT2G45220	up	pectinesterase family protein	UP
AT3G49960	up	peroxidase, putative	UP
AT1G12080	up	unknown protein	UP
AT1G56660	up	unknown protein	UP
AT1G62710	up	BETA-VPE (BETA VACUOLAR PROCESSING ENZYME); cysteine-type endopeptidase	DOWN
AT4G23700	up	ATCHX17 (CATION/H+ EXCHANGER 17); monovalent cation:proton antiporter/ sodium:hydrogen antiporter	DOWN
AT3G29970	up	germination protein-related	DOWN
AT2G14960	up	GH3.1	DOWN
AT2G23170	up	GH3.3; indole-3-acetic acid amido synthetase	DOWN
AT5G59070	up	glycosyl transferase family 1 protein	DOWN
AT5G60250	up	zinc finger (C3HC4-type RING finger) family protein	DOWN
AT1G21250	up	WAK1 (CELL WALL-ASSOCIATED KINASE); kinase	DOWN
AT5G13880	up	unknown protein	DOWN
AT5G53020	down	unknown protein	DOWN
AT1G78490	down	CYP708A3; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	DOWN
AT4G11170	down	disease resistance protein (TIR-NBS-LRR class)	DOWN
AT2G36080	down	DNA-binding protein, putative	DOWN
AT3G29970	down	germination protein-related	DOWN
AT5G48010	down	THAS1 (THALIANOL SYNTHASE 1); catalytic/ thalianol synthase	DOWN
AT1G52410	down	TSA1 (TSK-ASSOCIATING PROTEIN 1); calcium ion binding /	DOWN
AT5G46350	down	WRKY8; transcription factor	DOWN
AT4G04840	down	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	DOWN
AT1G48000	down	MYB112 (myb domain protein 112); transcription factor	DOWN
AT2G22760	down	basic helix-loop-helix (bHLH) family protein	DOWN
AT5G10180	down	AST68; sulfate transmembrane transporter	DOWN

Tab. 6.2 List of genes maintained epigenetically different in H3K27me3 10 days after priming which were also differentially responding to salt stress on day 10 after priming.

6.3 Discussion

6.3.1 Priming induced changes in histone modifications observed immediately after priming are not a direct reflection of transcriptional changes induced by the treatment

The general correlation between particular histone modifications and transcription occurring in the steady state is well known: H3K4me3 is positively correlated while H3K27me3 is negative (Roudier et al., 2009). However, if and how this correlation changes in case a stress occurs has never been addressed. In order to investigate this aspect the transcriptional data obtained by RNA-Seq were compared with the islands of histone methylation occurring within genes (Fig.6.4). Results confirmed the previous reported overall correlation between H3K4me3 and H3K27me3 levels and gene expression and showed that the priming treatment does not affect this general correlation.
However two important results were found:

- A strong correlation (i.e. the slope of the curve is not 1) is only observed in constitutively low expressed genes (approx. a third of total) meaning that only within these genes changes in the transcription can predict changes in the histone methylation and *vice versa*.
- By simply plotting the histone methylation level against the transcript level for each gene no correlation is visible. In order to obtain the shown curves both values have to be averaged over many ranked genes. This showed that at single gene level the correlation is not always followed.

The latter point was further exemplified by plotting the direction of changes in H3K4me3 and H3K27me3 against the direction of changes in RNA levels for the same genes (Fig.6.5). The majority of genes did not lie on the expected lines with a positive or negative slope respectively. These findings could suggest that at a single gene level the correlations between histone methylation and transcript level are interrupted by the salt treatment, i.e. changes at epigenetic and transcriptional level occur independently from each other. Alternatively, it could be that the dynamics of epigenetic and transcriptional

processes differ and hence monitoring them at the same time does not account for these differences, i.e methylation differences take place after transcriptional changes have occurred.

6.3.1.1 Changes in histone methylation upon stress are dynamic at single gene level

For selected genes ChIP assays coupled with qRT-PCR were performed at different time points to investigate the dynamic behaviour of gene expression and histone methylation after application of the priming treatment. It has to be noticed that this method does not distinguish between transcriptional and post-transcriptional effects on mRNA levels making it impossible to determine the exact timing of gene activation. In future RNA-Pol II binding assay should be carried out at loci of interest. The results showed that changes in histone methylation upon stress are extremely dynamic displaying different temporal behaviours (Fig.6.6). Clearly, it is not possible to generalize the kinetic of histone methylation and gene transcription because they are differently correlated depending on the gene considered. Three possible cases were distinguished. I) Direct correlation at the same time (i.e. high level of H3K27me3 and low level of transcription at the same time point). II) No correlation. A lack of correlation does not necessarily exclude the possibility that histone modifications have a different turnover from the transcripts and the modification might be placed at a later time point not sampled here. III) Partial correlation (e.g. high H3K27me3 and low transcript levels at one or more but not all time points). This indicates that one of the changes may be only transient or follow the other one in time. Interestingly, in some cases histone methylation preceded transcriptional changes, while in others the opposite was the case (Fig.6.6).
These results explained, for selected genes, why correlation analysis at one time point in a non-steady state does not necessarily reveal the expected correlations.

6.3.1.2 Salt responsive genes were preferentially marked with changes in H3K27me3

It was found that priming induced differences in H3K4me3 mostly occurred in expressed genes (Fig.6.2), however, this value was reduced to half if differentially expressed genes were considered (Fig.6.3). On the contrary, the percentage of priming induced differences

in H3K27me3 doubled when the salt differentially expressed genes were considered (Fig.6.3). This suggests that differentially expressed genes are more likely to have changes in H3K27me3 suggesting that this modification modulates the transcriptional response to environmental changes.

6.3.2 Priming-induced changes in H3K27me3 that were maintained for 10 days underlie the different transcriptional responses of primed plants to a second stress

Initially it was thought that changes in histone modification after 10 days were reflecting the differential gene expression shown after salt stress in the microarray (Chapter 4). However, only 34 genes showed differential methylation level of H3K27 between primed and non-primed plants as well as a differential salt response upon priming (Tab.6.2). Thus only few modifications are dynamically responding to salt stress, maintained during plant growth, and perhaps having a direct influence on gene transcription. It is possible that most of the H3K27me3 marks established by priming are transient and are lost if the stress does not re-occur within a shorter time period, and the few detected differences only reflected the end of the memory process. Further experiments should be performed either waiting fewer days between priming and stress, or applying repeated priming treatments, to assess whether this could improve the “memorization” process.

Chapter 7: Discussion

7.1 Original hypothesis and experimental observation

At the beginning of this project it was hypothesized that a priming treatment causes epigenetic changes that modify the chromatin structure (I). These epigenetic changes are reflected by changes in specific histone residues and make the stress responsive genes more accessible to transcription factors and to the transcriptional machinery (II). When a second stress is applied primed plants respond with a greater and faster induction of stress protective genes (III) and consequently become more salt tolerant (IV) (Fig.7.1).

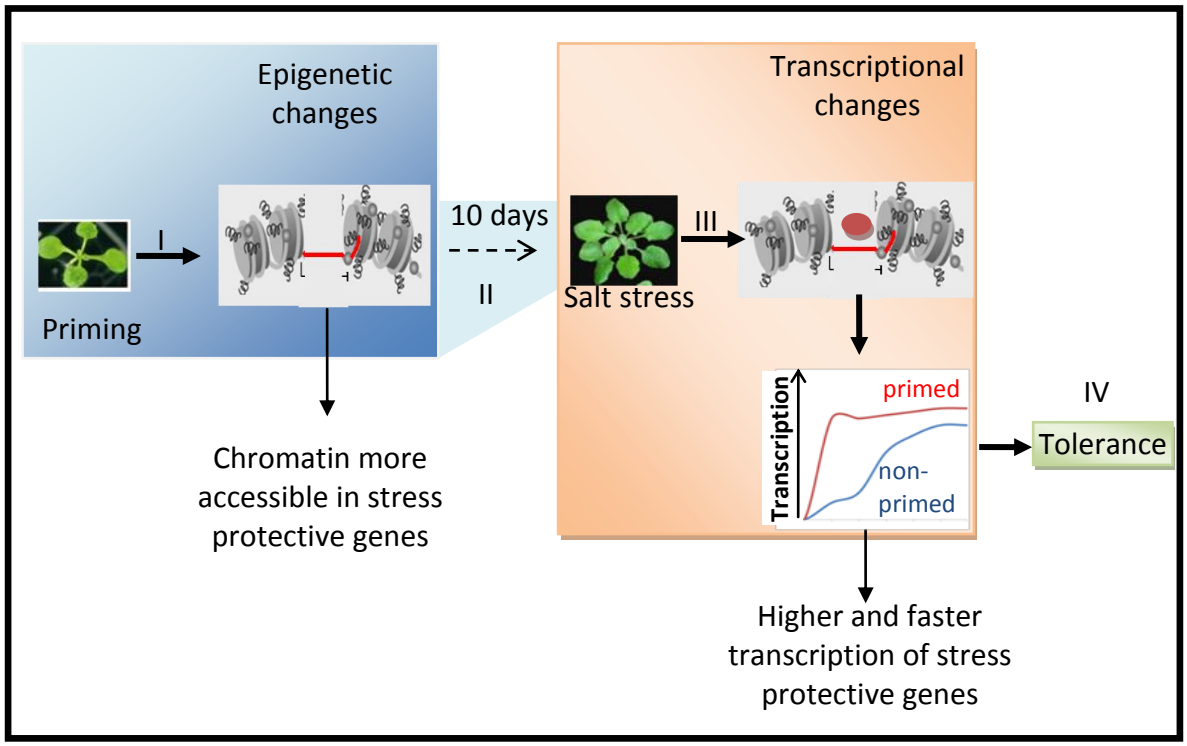


Fig. 7.1 Schematic representation of the original hypothesis.

Whether and how the experimental evidence obtained in this work supported the original hypothesis will be discussed in the following paragraphs and summarized in Tab.7.1.

Does the application of priming lead to increased salt tolerance?

The main aim of this study was to investigate differences in genome wide epigenetic profiles between primed and non-primed plants. Considering that these profiles represent the overlay of specific profiles from many different cell types, it was important to develop a mild priming treatment that did not lead to visible morphological differences. In addition, roots and shoots were harvested separately and plants were grown under short day condition in order to delay the transition from vegetative to reproductive phase during which many epigenetic differences occur. The use of this optimal experimental procedure allowed us to compare adult plants of the same size, with similar cell type content but with the priming/stress applications as the only source of variability. This was very important considering that previous studies demonstrated that changes in the photoperiod (long day vs short day) or light intensity strongly influenced the plant stress response (Becker et al., 2006).

During the preliminary assessment of the priming treatments it was found that, when the second stress was applied, the transcriptional response of two known salt responsive genes was different depending on which developmental stage the priming treatment was applied.

Nevertheless, primed plants did not show signs of long term acquired salt tolerance although in the short term they accumulated less sodium in the shoots. To understand if priming treatment leads only to transient alleviation of salt stress or can lead to long term adaptation more accurate physiological analyses are required. These were not the main focus of the thesis but should be carried out in the future.

It was observed, however, that primed plants coped better with drought stress suggesting an overlap between the two stress tolerance pathways as previously observed for the combination of drought and heat stress (Mittler et al., 2004). Therefore, it seems that the short and mild salt priming prepared plants to perform better upon osmotic stress than for salt toxicity. Unfortunately our laboratory is not equipped for controlled drought experiments and it was therefore not possible to carry out quantitative transcriptional studies on the drying plants. In general, it is difficult to assess fast responses to drought stress because of the gradual onset of the stress. The transcriptional response was therefore assessed immediately after a second salt application, representing the ‘osmotic’ phase of salt stress. In the future, transcriptional responses of primed/non-primed plants

exposed to salt and drought should be carried out over extended time courses. Future experiments should also compare the effect of priming in *Arabidopsis* mutants of specific salt genes to elucidate which signalling pathways are influenced by priming.

Do primed plants have a stronger response to a second stress?

A 10-day period of growth resulting in a 10 times increase of fresh weight was deemed long enough to revert any transcriptional changes induced by the priming treatment, as shown by the microarray data, where no changes were detected between control and primed plants not exposed to the second salt stress. Indeed, microarray analysis showed differences in transcript levels between primed and non-primed plants only when the second stress was applied. Against the original expectation, primed plants generally responded less (less genes and lower transcriptional response) than non-primed plants. Furthermore, it was found that primed plants accumulated less sodium in the shoots but not in the roots. It was therefore surprising that less transcriptional activity was detected in the roots of primed plants as they were less stressed. Nevertheless, a few genes did actually respond more strongly. These genes belong to diverse functional classes but may act all together to relieve the stress. This brings about the possibility that primed plants are channelling their stress response to a specific set of genes that collectively have the potential to enhance stress tolerance. To investigate this possibility a selection of salt/drought tolerance *Arabidopsis* mutants for the genes responding more strongly after priming should be further investigated.

Does priming lead to epigenetic differences?

Differences at the epigenetic level were detected after priming as originally hypothesized. Differences were small but robust. The observed differences have to be seen in the context that epigenetic profiles shape the landscape of a cell/s differentiation. Thus plants cannot 'risk' changing the entire epigenetic profile in response to a mild environmental perturbation because this leads to severe cell defects. Therefore it is more likely that either subtle changes occur in a wide range of genes or bigger changes occur in a precise gene subset in specific cell types. To explore these possibilities further experiments should be conducted in order to enhance either the spatial resolution using

cell sorting or micro-dissection, or enhance the time resolution by using different time points. Another interesting finding was that the epigenetic differences in primed plants were mainly detected as a reduction of H3K27me3 (where the most changes in number and amplitude were found) together with an increase in H3K4me3. This suggests that upon priming there is a pronounced release of silencing in the genes marked by H3K27me3 and a small increase of genes associated with active marker such as H3K4me3.

Does the epigenetic difference induced by priming lead to differences in gene transcription?

Very few epigenetic differences that occurred after priming matched the differentially responding genes after the second stress application. Furthermore, when the differences detected by ChIP-Seq were compared with the differences detected by RNA-Seq, after 24h of priming, not much correlation was found either. This could indicate that in non-steady state the changes at the epigenetic level are not necessarily reflected by changes at the transcriptional level, despite the fact that the overall general correlation between specific histone modification and transcription was still maintained.

Plants respond to salt stress with very dramatic transcriptional changes, in particular, in the first phase of the stress when a huge variety of genes have been reported to be differentially regulated (Killian et al., 2007). However, the genes encoding enzymes involved in chromatin modification were not found to be differentially regulated upon stress, indicating that chromatin modification enzymes are likely to be regulated at the post-transcriptional level.

It could be that the epigenetic response is the first step in preparing a reorganization of the transcriptional response to a stress. Or, alternatively, the differences in the transcriptional profile created by a perturbation leads to changes at the epigenetic level. Analysing both epigenetic and transcriptional changes over a time course as carried out here, is the first step towards answering these questions and should be carried out more extensively in the future. However the lack of a relationship between the epigenetic and the transcriptional changes raises the question if and how the observed epigenetic differences do actually cause the altered stress response of primed plants.

Do plants have memory?

The lack of correlation between differences found at the epigenetic level and differences in transcriptional response to the second stress could indicate that either the two responses are independent from each other or the epigenetic changes are 'forgotten' 10 days after the priming. The second possibility is supported by the fact that very few epigenetic changes were still detected 10 days after priming both with respect to the genes that were targeted and to the position within these genes. However, the overall decrease in H3K27me3 island length produced by the priming treatment was still apparent after 10 days indicating that the overall effect of the priming treatment on the epigenetic landscape was maintained. From these results it looks like that the priming treatment generates initial localised interruptions of silenced islands that are maintained but are eventually lost over time. It would be interesting to investigate whether repeated priming treatments enlarge these interruptions. Further experiments using several administrations of priming before the stress could answer this question.

Future outlook

- The identified candidate genes shown to be differentially responsive upon priming can be now followed by qPCR: further experiments using plants harvested at different time points after the priming treatment can elucidate for how long the priming-induced-changes are maintained.
- To investigate if the epigenetic changes detected upon priming take place before the changes in the transcriptional level or *vice versa,* more extended time course following candidate genes by coupling ChIP-qPCR and RT-qPCR are needed.
- The specific genes responding more strongly to the salt application upon priming can be further investigated in specific localization and types of cells, e.g. root epidermis, xylem parenchyma.
- Functional mutant analysis of genes responding differentially to stress upon priming could elucidate the effect of the priming treatment on the plant. In particular, the use of *HKT1* mutants will clarify the role of this gene in the acquisition of salt tolerance.

- It is not clear if the priming response is lost after 10 days. Further experiments with repeated priming treatments might further stimulate the “stress memory” using a selection of specific genes to be followed by ChIP-qPCR.
- The specific genes responding more strongly to the salt application upon priming can be tested using ChIP-qPCR if associated with other histone modifications eg. acetylation.

Tab. 7.1 Summary of the hypothesis and experimental observation

	Initial Hypothesis	Experimental observation	Accepted/rejected	Dataset evidence	Future directions
I	Priming induces changes at the epigenetic level	Epigenetic changes are induced upon priming	Accepted	ChIP-Seq after 24h of priming	
	Epigenetic-priming-induced-changes are reflected in gene transcription	Priming induced changes at epigenetic level are independent of gene expression	Rejected- (Epigenetic changes and transcriptional changes in different genes)	ChIP-Seq coupled with RNA-Seq	Extended time course
	Priming induced changes are tissue specific	Roots and shoots show transcriptional differential responses Shoots show few differences at H3K4me2 and H3K4me3	ND	ChIP-Seq coupled with RNA-Seq	Increase spatial resolution. Alternative way of administration of the stress
II	Priming induced changes are maintained until the next stress occurs	Transcriptional changes are lost if second stress is not recurring while epigenetic changes are lasting	Partially Accepted	ChIP-Seq after 24h of priming and microarray dataset after 2nd stress	Transient response at the transcriptional level
III	Priming induces changes at the transcriptional level	Transcriptional changes were detected upon priming	Accepted	Microarray dataset	Functional mutants
	Priming leads to higher and faster transcriptional response after 2nd stress	Overall no higher and faster response detected BUT few specific genes are responding more strongly	Partially Rejected	Microarray dataset/DAVID analysis	Increase resolution by investigating specific types of cells, e.g. root epidermis, xylem parenchyma
	Changes in gene transcription are caused by epigenetic differences induced by priming	Few genes differentially expressed upon priming have also epigenetic changes in methylation	Partially Rejected	Microarray dataset and ChIP-Seq	Repeated priming treatments might further stimulate the “stress memory”
IV	Primed plants are more tolerant to stress	Increased tolerance to drought but no salt tolerance was observed. But a transiently decreased accumulation of Na+ in the shoots of primed plants was seen which is typical of the *HKT1* mutant.	Partially Rejected	Physiological analysis	Functional mutant analysis i.e *HKT1* mutants

References

Abmayr, S. M., and Workman, J. L. (2012). Holding on through DNA Replication: Histone Modification or Modifier? Cell *150*, 875–877.

Alvarez-Venegas, R., Pien, S., Sadder, M., Witmer, X., Grossniklaus, U., and Avramova, Z. (2003). ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. Current Biology *13*, 627–637.

Angel, A., Song, J., Dean, C., and Howard, M. (2011). A Polycomb-based switch underlying quantitative epigenetic memory. Nature *476*, 105–108.

Apse, M. P. (1999). Salt Tolerance Conferred by Overexpression of a Vacuolar Na+/H+ Antiport in Arabidopsis. Science *285*, 1256–1258.

Apse, M. P., and Blumwald, E. (2007). Na+ transport in plants. FEBS letters *581*, 2247–2254.

Apse, M. P., Sottosanto, J. B., and Blumwald, E. (2003). Vacuolar cation/H + exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1 , the Arabidopsis vacuolar Na + /H + antiporter. The Plant Journal *36*, 229–239.

Bari, R., and Jones, J. D. G. (2009). Role of plant hormones in plant defence responses. Plant Molecular Biology *69*, 473–488.

Baroux, C., Gagliardini, V., Page, D. R., and Grossniklaus, U. (2006). Dynamic regulatory interactions of Polycomb group genes: MEDEA autoregulation is required for imprinted gene expression in Arabidopsis. Genes & development *20*, 1081–1086.

Barrett, T., Troup, D. B., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., Marshall, K. a, Phillippy, K. H., Sherman, P. M., et al. (2011). NCBI GEO: archive for functional genomics data sets--10 years on. Nucleic acids research *39*, D1005–10.

Becker, B., Holtgrefe, S., Jung, S., Wunrau, C., Kandlbinder, A., Baier, M., Dietz, K.-J., Backhausen, J. E., and Scheibe, R. (2006). Influence of the photoperiod on redox regulation and stress responses in Arabidopsis thaliana L. (Heynh.) plants under long- and short-day conditions. Planta *224*, 380–393.

Beemster, G. T. S., De Veylder, L., Vercruysse, S., West, G., Rombaut, D., Van Hummelen, P., Galichet, A., Gruissem, W., Inzé, D., and Vuylsteke, M. (2005). Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant physiology *138*, 734–743.

Berger, S. L. (2007). The complex language of chromatin regulation during transcription. Nature *447*, 407–412.

Bernatavichute, Y. V, Zhang, X., Cokus, S., Pellegrini, M., and Jacobsen, S. E. (2008). Genome-wide association of histone H3 lysine nine methylation with CHG DNA methylation in Arabidopsis thaliana. PloS one *3*, e3156.

Berthomieu, P., Conéjéro, G., Nublat, A., Brackenbury, W. J., Lambert, C., Savio, C., Uozumi, N., Oiki, S., Yamada, K., Cellier, F., et al. (2003). Functional analysis of AtHKT1 in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance. the The European Molecular Biology Organization Journal *22*, 2004–2014.

Boursiac, Y., Chen, S., Luu, D. T., Sorieul, M., Van Den Dries, N., and Maurel, C. (2005). Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. Plant Physiology *139*, 790–805.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A. V, Tariq, M., and Paszkowski, J. (2004). Chromatin techniques for plant cells. The Plant journal : for cell and molecular biology *39*, 776–789.

Breitling, R., Armengaud, P., and Amtmann, A. (2005). Vector analysis as a fast and easy method to compare gene expression responses between different experimental backgrounds. BMC bioinformatics *6*, 181.

Bruce, T. J. A., Matthes, M. C., Napier, J. A., and Pickett, J. A. (2007). Stressful “memories” of plants: Evidence and possible mechanisms. Plant Science *173*, 603–608.

Brusslan, J. a, Rus Alvarez-Canterbury, A. M., Nair, N. U., Rice, J. C., Hitchler, M. J., and Pellegrini, M. (2012). Genome-wide evaluation of histone methylation changes associated with leaf senescence in Arabidopsis. PloS one *7*, e33151.

Cantero, a, Barthakur, S., Bushart, T. J., Chou, S., Morgan, R. O., Fernandez, M. P., Clark, G. B., and Roux, S. J. (2006). Expression profiling of the Arabidopsis annexin gene family during germination, de-etiolation and abiotic stress. Plant physiology and biochemistry : PPB / Société française de physiologie végétale *44*, 13–24.

Catusse, J., Job, C., and Job, D. (2008). Transcriptome- and proteome-wide analyses of seed germination. Comptes rendus biologies *331*, 815–822.

Cayuela, E., Parra, M., and Caro, M. (2001). NaCl pre-treatment at the seedling stage enhances fruit yield of tomato plants irrigated with salt water. Plant and Soil, 231–238.

Cayuela, E., Perez-Alfocea, F., Caro, M., and Bolarin, M. C. (1996). Priming of seeds with NaCl induces physiological changes in tomato plants grown under salt stress. Physiologia Plantarum *96*, 231–236.

Cellier, F., Conéjéro, G., Ricaud, L., Luu, D. T., Lepetit, M., Gosti, F., and Casse, F. (2004). Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K+ homeostasis. The Plant journal : for cell and molecular biology *39*, 834–846.

Chanroj, S., Lu, Y., Padmanaban, S., Nanatani, K., Uozumi, N., Rao, R., and Sze, H. (2011). Plant-specific cation/H+ exchanger 17 and its homologs are endomembrane K+ transporters with roles in protein sorting. The Journal of biological chemistry *286*, 33931–33941.

Charron, J.-B. F., He, H., Elling, A. a, and Deng, X. W. (2009). Dynamic landscapes of four histone modifications during deetiolation in Arabidopsis. The Plant cell *21*, 3732–3748.

Chen, L.-T., Luo, M., Wang, Y.-Y., and Wu, K. (2010). Involvement of Arabidopsis histone deacetylase HDA6 in ABA and salt stress response. Journal of Experimental Botany *61*, 3345–3353.

Chen, L.-T., and Wu, K. (2010). Role of histone deacetylases HDA6 and HDA19 in ABA and abiotic stress response. Plant signaling & behavior *5*, 1318–1320.

Chen, Z. J., and Tian, L. (2007). Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochimica et biophysica acta *1769*, 295–307.

Chigri, F., Flosdorff, S., Pilz, S., Kölle, E., Dolze, E., Gietl, C., and Vothknecht, U. C. (2012). The Arabidopsis calmodulin-like proteins AtCML30 and AtCML3 are targeted to mitochondria and peroxisomes, respectively. Plant molecular biology *78*, 211–222.

Chinnusamy, V., Schumaker, K., and Zhu, J.-K. (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. Journal of experimental botany *55*, 225–236.

Chinnusamy, V., and Zhu, J.-K. (2009). Epigenetic regulation of stress responses in plants. Current opinion in plant biology *12*, 133–139.

Chua, Y. L., Brown, A. P. C., and Gray, J. C. (2001). Targeted Histone Acetylation and Altered Nuclease Accessibility over Short Regions of the Pea Plastocyanin Gene. The Plant Cell *13*, 599–612.

Chua, Y. L., Watson, L. A., and Gray, J. C. (2003). The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. The Plant Cell *15*, 1468–1479.

Conrath, U. (2011). Molecular aspects of defence priming. Trends in plant science *16*, 524–531.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., et al. (2006). Priming: getting ready for battle. Molecular plant-microbe interactions : MPMI *19*, 1062–1071.

Conrath, U., Thulke, O., Katz, V., Schwindling, S., and Kohler, A. (2001). Priming as a mechanism in induced systemic resistance of plants. European Journal of Plant Pathology *107*, 113–119.

Corpet, A., and Almouzni, G. (2009). Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information. Trends in cell biology *19*, 29–41.

Cuartero, J., Bolarín, M. C., Asíns, M. J., and Moreno, V. (2006). Increasing salt tolerance in the tomato. Journal of experimental botany *57*, 1045–1058.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J. G. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. The Plant Journal *32*, 457–466.

Van Dijk, K., Ding, Y., Malkaram, S., Riethoven, J.-J. M., Liu, R., Yang, J., Laczko, P., Chen, H., Xia, Y., Ladunga, I., et al. (2010). Dynamic changes in genome-wide histone H3 lysine 4 methylation patterns in response to dehydration stress in Arabidopsis thaliana. BMC plant biology *10*, 238.

Ding, Y., Avramova, Z., and Fromm, M. (2011). The Arabidopsis trithorax-like factor ATX1 functions in dehydration stress responses via ABA-dependent and ABA-independent pathways. The Plant journal : for cell and molecular biology *66*, 735–744.

Earley, K. W., Shook, M. S., Brower-Toland, B., Hicks, L., and Pikaard, C. S. (2007). In vitro specificities of Arabidopsis co-activator histone acetyltransferases: implications for histone hyperacetylation in gene activation. The Plant Journal *52*, 615–626.

Enami, K., Ichikawa, M., Uemura, T., Kutsuna, N., Hasezawa, S., Nakagawa, T., Nakano, A., and Sato, M. H. (2009). Differential expression control and polarized distribution of plasma membrane-resident SYP1 SNAREs in Arabidopsis thaliana. Plant & cell physiology *50*, 280–289.

Farooq, M., Aziz, T., Basra, S. M. A., Cheema, M. A., and Rehman, H. (2008). Chilling tolerance in hybrid maize induced by seed priming with salicylic acid. Journal of Agronomy and Crop Science *194*, 161–168.

Von der Fecht-Bartenbach, J., Bogner, M., Dynowski, M., and Ludewig, U. (2010). CLC-b-mediated NO-3/H+ exchange across the tonoplast of Arabidopsis vacuoles. Plant & cell physiology *51*, 960–968.

Finch-Savage, W. E., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. New Phytologist *171*, 501–523.

Gális, I., Gaquerel, E., Pandey, S. P., and Baldwin, I. T. (2009). Molecular mechanisms underlying plant memory in JA-mediated defence responses. Plant cell environment *32*, 617–627.

Gattolin, S., Sorieul, M., Hunter, P. R., Khonsari, R. H., and Frigerio, L. (2009). In vivo imaging of the tonoplast intrinsic protein family in Arabidopsis roots. BMC plant biology *9*, 133.

Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L., and Fink, G. R. (1999). The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proceedings of the National Academy of Sciences of the United States of America *96*, 1480–1485.

Gendrel, A.-V., and Colot, V. (2005). Arabidopsis epigenetics: when RNA meets chromatin. Current opinion in plant biology *8*, 142–147.

Golldack, D., Lüking, I., and Yang, O. (2011). Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant cell reports.

Gong, Q., Li, P., Ma, S., Indu Rupassara, S., and Bohnert, H. J. (2005). Salinity stress adaptation competence in the extremophile Thellungiella halophila in comparison with its relative Arabidopsis thaliana. The Plant journal : for cell and molecular biology *44*, 826–839.

Guo, L., Yu, Y., Law, J. a, and Zhang, X. (2010). SET DOMAIN GROUP2 is the major histone H3 lysine [corrected] 4 trimethyltransferase in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America *107*, 18557–18562.

Ha, M., Ng, D. W.-K., Li, W.-H., and Chen, Z. J. (2011). Coordinated histone modifications are associated with gene expression variation within and between species. Genome research *21*, 590–598.

Halfter, U., Ishitani, M., and Zhu, J. K. (2000). The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proceedings of the National Academy of Sciences of the United States of America *97*, 3735–3740.

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. Frontiers in plant science *3*, 1–5.

Heil, M., and Kost, C. (2006). Priming of indirect defences. Ecology letters *9*, 813–817.

Van Hengel, A. J., and Roberts, K. (2003). AtAGP30, an arabinogalactan-protein in the cell walls of the primary root, plays a role in root regeneration and seed germination. The Plant Journal *36*, 256–270.

Ho, J. W. K., Bishop, E., Karchenko, P. V, Nègre, N., White, K. P., and Park, P. J. (2011). ChIP-chip versus ChIP-seq: lessons for experimental design and data analysis. BMC genomics *12*, 134.

Hossain, Z., Nouri, M.-Z., and Komatsu, S. (2012). Plant cell organelle proteomics in response to abiotic stress. Journal of proteome research *11*, 37–48.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008). Genevestigator V3: A Reference Expression Database for the Meta-Analysis of Transcriptomes. Advances in bioinformatics *2008*, 420747.

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols *4*, 44–57.

Van Hulten, M., Pelser, M., Van Loon, L. C., Pieterse, C. M. J., and Ton, J. (2006). Costs and benefits of priming for defense in Arabidopsis. Proccedings of the National Academy of Sciences *103*, 5602–5607.

Hussain, K., Nisar, M. F., Majeed, A., Nawaz, K., Bhatti, K. H., Afghan, S., Shahazad, A., and Zia-ul-Hussnian, S. (2010). What molecular mechanism is adapted by plants during salt stress tolerance. Afr. J. Biotechnol *9*, 416–422.

Ishitani, M., Liu, J., Halfter, U., Kim, C.-S., Shi, W., and Zhu, J.-K. (2000). SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. The Plant Cell *12*, 1667–1678.

Jacob, Y., Feng, S., LeBlanc, C. a, Bernatavichute, Y. V, Stroud, H., Cokus, S., Johnson, L. M., Pellegrini, M., Jacobsen, S. E., and Michaels, S. D. (2009). ATXR5 and ATXR6 are H3K27 monomethyltransferases required for chromatin structure and gene silencing. Nature structural & molecular biology *16*, 763–768.

Jacobs, J., and Roe, J. L. (2005). SKS6, a multicopper oxidase-like gene, participates in cotyledon vascular patterning during Arabidopsis thaliana development. Planta *222*, 652–666.

Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J.-P., and Mauch-Mani, B. (2005). Enhancing Arabidopsis Salt and Drought Stress Tolerance by Chemical Priming for Its Abscisic Acid Responses1. Plant Physiology *139*, 267–274.

Jang, I.-C., Pahk, Y.-M., Song, S. I., Kwon, H. J., Nahm, B. H., and Kim, J.-K. (2003). Structure and expression of the rice class-I type histone deacetylase genes OsHDAC1-3: OsHDAC1 overexpression in transgenic plants leads to increased growth rate and altered architecture. The Plant journal : for cell and molecular biology *33*, 531–541.

Jasencakova, Z., Meister, A., and Schubert, I. (2001). Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. Chromosoma *110*, 83–92.

Jaskiewicz, M., Conrath, U., and Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. EMBO Reports *12*, 50–55.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science (New York, N.Y.) *293*, 1074–1080.

Jiang, Y., and Deyholos, M. K. (2006). Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. BMC plant biology *6*, 25.

Johnson, L., Mollah, S., Garcia, B. a, Muratore, T. L., Shabanowitz, J., Hunt, D. F., and Jacobsen, S. E. (2004). Mass spectrometry analysis of Arabidopsis histone H3 reveals distinct combinations of post-translational modifications. Nucleic acids research *32*, 6511–6518.

Kagale, S., Divi, U. K., Krochko, J. E., Keller, W. a, and Krishna, P. (2007). Brassinosteroid confers tolerance in Arabidopsis thaliana and Brassica napus to a range of abiotic stresses. Planta *225*, 353–364.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. The Plant Journal *50*, 347–363.

Kim, J.-M., To, T. K., Ishida, J., Morosawa, T., Kawashima, M., Matsui, A., Toyoda, T., Kimura, H., Shinozaki, K., and Seki, M. (2008). Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. Plant & cell physiology *49*, 1580–1588.

Knight, H., Trewavas, A. J., and Knight, M. R. (1997). Calcium signalling in Arabidopsis thaliana responding to drought and salinity. The Plant journal : for cell and molecular biology *12*, 1067–1078.

Kravchuk, Z., Vicedo, B., Flors, V., Camañes, G., González-Bosch, C., and García-Agustín, P. (2011). Priming for JA-dependent defenses using hexanoic acid is an effective

mechanism to protect Arabidopsis against B. cinerea. Journal of Plant Physiology *168*, 359–366.

Kreps, J. A., Wu, Y., Chang, H. S., Zhu, T., Wang, X., and Harper, J. F. (2002). Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology *130*, 2129–2141.

Kugler, A., Köhler, B., Palme, K., Wolff, P., and Dietrich, P. (2009). Salt-dependent regulation of a CNG channel subfamily in Arabidopsis. BMC plant biology *9*, 140.

Lafos, M., Kroll, P., Hohenstatt, M. L., Thorpe, F. L., Clarenz, O., and Schubert, D. (2011). Dynamic regulation of H3K27 trimethylation during Arabidopsis differentiation. PLoS genetics *7*, e1002040.

Lallous, Nada; Ramón-Maiques, S. (2011). Chromatin Recognition Protein Modules : The PHD. Encyclopedia of Life Sciences (ELS)J ohn Wiley & Sons, Ltd: Chichester, 1–14.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology *10*, R25.

Laohavisit, A., and Davies, J. M. (2011). Annexins. The New phytologist *189*, 40–53.

Laohavisit, A., Shang, Z., Rubio, L., Cuin, T. a, Véry, A.-A., Wang, A., Mortimer, J. C., Macpherson, N., Coxon, K. M., Battey, N. H., et al. (2012). Arabidopsis annexin1 mediates the radical-activated plasma membrane Ca^{2}+- and K+-permeable conductance in root cells. The Plant cell *24*, 1522–1533.

Li, B., Carey, M., and Workman, J. L. (2007a). The role of chromatin during transcription. Cell *128*, 707–719.

Li, F., Asami, T., Wu, X., Tsang, E. W. T., and Cutler, A. J. (2007b). A putative hydroxysteroid dehydrogenase involved in regulating plant growth and development. Plant physiology *145*, 87–97.

Lin, W.-D., Liao, Y.-Y., Yang, T. J. W., Pan, C.-Y., Buckhout, T. J., and Schmidt, W. (2011). Coexpression-based clustering of Arabidopsis root genes predicts functional modules in early phosphate deficiency signaling. Plant physiology *155*, 1383–1402.

Liu, C., Lu, F., Cui, X., and Cao, X. (2010). Histone methylation in higher plants. Annual review of plant biology *61*, 395–420.

Liu, J., Ishitani, M., Halfter, U., Kim, C. S., and Zhu, J. K. (2000). The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. Proceedings of the National Academy of Sciences of the United States of America *97*, 3730–3734.

Luo, M., Liu, X., Singh, P., Cui, Y., Zimmerli, L., and Wu, K. (2012a). Chromatin modifications and remodeling in plant abiotic stress responses. Biochimica et biophysica acta *1819*, 129–136.

Luo, M., Wang, Y.-Y., Liu, X., Yang, S., Lu, Q., Cui, Y., and Wu, K. (2012b). HD2C interacts with HDA6 and is involved in ABA and salt stress response in Arabidopsis. Journal of experimental botany *3*, 1–10.

Ma, S., Gong, Q., and Bohnert, H. J. (2006). Dissecting salt stress pathways. Journal of experimental botany *57*, 1097–1107.

Maathuis, F. (2001). Sodium uptake in Arabidopsis roots is regulated by cyclic nucleotides. Plant Physiology *127*, 1617–1625.

Mahajan, S., and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. Archives of biochemistry and biophysics *444*, 139–158.

Mao, X., Jia, D., Li, A., Zhang, H., Tian, S., Zhang, X., Jia, J., and Jing, R. (2011). Transgenic expression of TaMYB2A confers enhanced tolerance to multiple abiotic stresses in Arabidopsis. Functional & integrative genomics *11*, 445–465.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. Trends in plant science *11*, 15–19.

Morant, M., Jørgensen, K., Schaller, H., Pinot, F., Møller, B. L., Werck-Reichhart, D., and Bak, S. (2007). CYP703 is an ancient cytochrome P450 in land plants catalyzing in-chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. The Plant cell *19*, 1473–1487.

Mosher, R. A., Durrant, W. E., Wang, D., Song, J., and Dong, X. (2006). A Comprehensive Structure – Function Analysis of Arabidopsis SNI1 Defines Essential Regions and Transcriptional Repressor Activity. *18*, 1750–1765.

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. Annual review of plant biology *59*, 651–681. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18444910 [Accessed June 13, 2011].

Nakajima, K., Furutani, I., Tachimoto, H., Matsubara, H., and Hashimoto, T. (2004). SPIRAL1 Encodes a Plant-Specific Microtubule-Localized Protein Required for Directional Control of Rapidly Expanding Arabidopsis Cells. *16*, 1178–1190.

Nawaz, A., Amjad, M., Pervez, M. A., and Afzal, I. (2011). Effect of halopriming on germination and seedling vigor of tomato. African Journal of Agricultural Research *6*, 3551–3559.

Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M., and Iba, K. (2008). CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature *452*, 483–486.

Ng, D., Wang, T., and Chandrasekharan, M. (2007). Plant SET domain-containing proteins: structure, function and regulation. (BBA)-Gene Structure *1769*, 316–329.

Nicol, J. W., Helt, G. A., Blanchard, S. G., Raja, A., and Loraine, A. E. (2009). The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics (Oxford, England) *25*, 2730–2731.

Nolan, T., Hands, R. E., and Bustin, S. a (2006). Quantification of mRNA using real-time RT-PCR. Nature protocols *1*, 1559–1582.

Pandey, R., Müller, A., Napoli, C. A., Selinger, D. A., Pikaard, C. S., Richards, E. J., Bender, J., Mount, D. W., and Jorgensen, R. A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Research *30*, 5036–5055.

Patade, V. Y., Bhargava, S., and Suprasanna, P. (2009). Halopriming imparts tolerance to salt and PEG induced drought stress in sugarcane. Agriculture, Ecosystems & Environment *134*, 24–28.

Petruk, S., Sedkov, Y., Johnston, D. M., Hodgson, J. W., Black, K. L., Kovermann, S. K., Beck, S., Canaani, E., Brock, H. W., and Mazo, A. (2012). TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. Cell *150*, 922–933.

Pfluger, J., and Wagner, D. (2007). Histone modifications and dynamic regulation of genome accessibility in plants. Current opinion in plant biology *10*, 645–652.

Pien, S., Fleury, D., Mylne, J. S., Crevillen, P., Inzé, D., Avramova, Z., Dean, C., and Grossniklaus, U. (2008). ARABIDOPSIS TRITHORAX1 dynamically regulates FLOWERING LOCUS C activation via histone 3 lysine 4 trimethylation. The Plant cell *20*, 580–588.

Pontvianne, F., Blevins, T., and Pikaard, C. S. (2010). Arabidopsis Histone Lysine Methyltransferases. Advances in botanical research *53*, 1–22.

Qiu, Q.-S., Barkla, B. J., Vera-Estrella, R., Zhu, J.-K., and Schumaker, K. S. (2003). Na+/H+ Exchange Activity in the Plasma Membrane of Arabidopsis1. Plant Physiology *132*, 1041–1052.

Qiu, Q.-S., Guo, Y., Dietrich, M. A., Schumaker, K. S., and Zhu, J.-K. (2002). Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proceedings of the National Academy of Sciences of the United States of America *99*, 8436–8441.

Quintero, F. J., Blatt, M. R., and Pardo, J. M. (2000). Functional conservation between yeast and plant endosomal Na(+)/H(+) antiporters. FEBS letters *471*, 224–228.

Ringnér, M. (2008). What is principal component analysis ? Nature Biotechnology *26*, 303–304.

Rodríguez-Rosales, M. P., Gálvez, F. J., Huertas, R., Aranda, M. N., Baghour, M., Cagnac, O., and Venema, K. (2009). Plant NHX cation/proton antiporters. Plant signaling & behavior *4*, 265–276.

Rossi, V., Locatelli, S., Varotto, S., Donn, G., Pirona, R., Henderson, D. a, Hartings, H., and Motto, M. (2007). Maize histone deacetylase hda101 is involved in plant development, gene transcription, and sequence-specific modulation of histone modification of genes and repeats. The Plant cell *19*, 1145–1162.

Roudier, F., Teixeira, F. K., and Colot, V. (2009). Chromatin indexing in Arabidopsis: an epigenomic tale of tails and more. Trends in genetics : TIG *25*, 511–517.

Rubio, F., Gassmann, W., and Schroeder, J. I. (1995). Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science (New York, N.Y.) *270*, 1660–1663.

Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B., Matsumoto, T. K., Koiwa, H., Zhu, J.-K., Bressan, R. A., and Hasegawa, P. M. (2001). AtHKT1 is a salt tolerance determinant that controls Na(+) entry into plant roots. Proceedings of the National Academy of Sciences of the United States of America *98*, 14150–14155.

Saleh, A., Alvarez-Venegas, R., Yilmaz, M., Le, O., Hou, G., Sadder, M., Al-Abdallat, A., Xia, Y., Lu, G., Ladunga, I., et al. (2008). The highly similar Arabidopsis homologs of trithorax ATX1 and ATX2 encode proteins with divergent biochemical functions. The Plant cell *20*, 568–579.

Schachtman, D. P., and Schroeder, J. I. (1994). Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature *370*, 655–658.

Schwember, A. R., and Bradford, K. J. (2010). A genetic locus and gene expression patterns associated with the priming effect on lettuce seed germination at elevated temperatures. Plant molecular biology *73*, 105–118.

Sedbrook, J. C., Carroll, K. L., Hung, K. F., Masson, P. H., and Somerville, C. R. (2002). The Arabidopsis SKU5 gene encodes an extracellular glycosyl phosphatidylinositol–anchored glycoprotein involved in directional root growth. The Plant Cell Online *14*, 1635–1648.

Sedghi, M., Nemati, A., and Esmaielpour, B. (2010). Effect of seed priming on germination and seedling growth of two medicinal plants under salinity. Emirates Journal of Food and Agriculture *22*, 130–139.

Servet, C., Conde e Silva, N., and Zhou, D.-X. (2010). Histone acetyltransferase AtGCN5/HAG1 is a versatile regulator of developmental and inducible gene expression in Arabidopsis. Molecular plant *3*, 670–677.

Sheldon, C. C., Finnegan, E. J., Dennis, E. S., and Peacock, W. J. (2006). Quantitative effects of vernalization on FLC and SOC1 expression. The Plant Journal *45*, 871–883.

Shi, H., Ishitani, M., Kim, C., and Zhu, J. K. (2000). The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proceedings of the National Academy of Sciences of the United States of America *97*, 6896–6901.

Shi, H., Quintero, F. J., Pardo, J. M., and Zhu, J.-K. (2002). The putative plasma membrane Na(+)/H(+) antiporter SOS1 controls long-distance Na(+) transport in plants. The Plant Cell *14*, 465–477.

Shi, H., and Zhu, J.-K. (2002). Regulation of expression of the vacuolar Na+/H+ antiporter gene AtNHX1 by salt stress and abscisic acid. Plant molecular biology *50*, 543–550.

Simon, S. a, and Meyers, B. C. (2011). Small RNA-mediated epigenetic modifications in plants. Current opinion in plant biology *14*, 148–155.

Skirycz, A., Claeys, H., De Bodt, S., Oikawa, A., Shinoda, S., Andriankaja, M., Maleux, K., Eloy, N. B., Coppens, F., Yoo, S.-D., et al. (2011). Pause-and-stop: the effects of

osmotic stress on cell proliferation during early leaf development in Arabidopsis and a role for ethylene signaling in cell cycle arrest. The Plant cell *23*, 1876–1888.

Sokol, A., Kwiatkowska, A., Jerzmanowski, A., and Prymakowska-Bosak, M. (2007). Up-regulation of stress-inducible genes in tobacco and Arabidopsis cells in response to abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications. Planta *227*, 245–254.

Steppuhn, H., and Raney, J. (2005). Emergence, height, and yield of canola and barley grown in saline root zones. Canadian journal of plant science *85*, 815–827.

Sunarpi, Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., Chan, W.-Y., Leung, H.-Y., Hattori, K., et al. (2005). Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na unloading from xylem vessels to xylem parenchyma cells. The Plant journal : for cell and molecular biology *44*, 928–938.

Tamada, Y., Yun, J.-Y., Woo, S. C., and Amasino, R. M. (2009). ARABIDOPSIS TRITHORAX-RELATED7 Is Required for Methylation of Lysine 4 of Histone H3 and for Transcriptional Activation of FLOWERING LOCUS C[C][W]. The Plant Cell *21*, 3257–3269.

Tanaka, M., Kikuchi, A., and Kamada, H. (2008). The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. Plant physiology *146*, 149–161.

Tian, L., and Chen, Z. J. (2001). Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. Proceedings of the National Academy of Sciences of the United States of America *98*, 200–205.

To, T. K., Nakaminami, K., Kim, J.-M., Morosawa, T., Ishida, J., Tanaka, M., Yokoyama, S., Shinozaki, K., and Seki, M. (2011). Arabidopsis HDA6 is required for freezing tolerance. Biochemical and biophysical research communications *406*, 414–419.

Ton, J., Ent, S. Van Der, Hulten, M. Van, and Pozo, M. (2009). Priming as a mechanism behind induced resistance against pathogens, insects and abiotic stress. IOBC/wprs Bull *44*, 3–13.

Tracy, F. E., Gilliham, M., Dodd, A. N., Webb, A. A. R., and Tester, M. (2008). NaCl-induced changes in cytosolic free Ca2+ in Arabidopsis thaliana are heterogeneous and modified by external ionic composition. Plant, cell & environment *31*, 1063–1073.

Tsai, C.-H., Singh, P., Chen, C.-W., Thomas, J., Weber, J., Mauch-Mani, B., and Zimmerli, L. (2011). Priming for enhanced defence responses by specific inhibition of the Arabidopsis response to coronatine. The Plant Journal *65*, 469–479.

Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, a, Bakker, E. P., Nakamura, T., and Schroeder, J. I. (2000). The Arabidopsis HKT1 gene homolog mediates inward Na(+) currents in xenopus laevis oocytes and Na(+) uptake in Saccharomyces cerevisiae. Plant physiology *122*, 1249–1259.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology *3*, research0034.1–research0034.11.

Veiseth, S. V, Rahman, M. a, Yap, K. L., Fischer, A., Egge-Jacobsen, W., Reuter, G., Zhou, M.-M., Aalen, R. B., and Thorstensen, T. (2011). The SUVR4 histone lysine methyltransferase binds ubiquitin and converts H3K9me1 to H3K9me3 on transposon chromatin in Arabidopsis. PLoS genetics *7*, e1001325.

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M. Lou, Briat, J.-F., and Curie, C. (2002). IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. The Plant cell *14*, 1223–1233.

Vicedo, B., Flors, V., and Leyva, M. de la O. (2009). Hexanoic acid-induced resistance against Botrytis cinerea in tomato plants. Molecular plant- *22*, 1455–1465.

Volkov, V., and Amtmann, A. (2006). Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana, has specific root ion-channel features supporting K+/Na+ homeostasis under salinity stress. The Plant journal : for cell and molecular biology *48*, 342–353.

Wang, B., Davenport, R. J., Volkov, V., and Amtmann, A. (2006). Low unidirectional sodium influx into root cells restricts net sodium accumulation in Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana. Journal of experimental botany *57*, 1161–1170.

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta *218*, 1–14.

Wierzbicki, A. T., Cocklin, R., Mayampurath, A., Lister, R., Rowley, M. J., Gregory, B. D., Ecker, J. R., Tang, H., and Pikaard, C. S. (2012). Spatial and functional relationships among Pol V-associated loci, Pol IV-dependent siRNAs, and cytosine methylation in the Arabidopsis epigenome. Genes & development *26*, 1825–1836.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V, and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PloS one *2*, e718.

Wong, C. E., Li, Y., Labbe, A., Guevara, D., Nuin, P., Whitty, B., Diaz, C., Golding, G. B., Gray, G. R., Weretilnyk, E. A., et al. (2006). Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in Thellungiella, a close relative of Arabidopsis. Plant physiology *140*, 1437–1450.

Worrall, D., Holroyd, G. H., Moore, J. P., Glowacz, M., Croft, P., Taylor, J. E., Paul, N. D., and Roberts, M. R. (2012). Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. The New phytologist *193*, 770–778.

Xu, H., Wei, C.-L., Lin, F., and Sung, W.-K. (2008a). An HMM approach to genome-wide identification of differential histone modification sites from ChIP-seq data. Bioinformatics (Oxford, England) *24*, 2344–2349.

Xu, L., Zhao, Z., Dong, A., Soubigou-Taconnat, L., Renou, J.-P., Steinmetz, A., and Shen, W.-H. (2008b). Di- and tri- but not monomethylation on histone H3 lysine 36 marks

active transcription of genes involved in flowering time regulation and other processes in Arabidopsis thaliana. Molecular and cellular biology *28*, 1348–1360.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants. Molecular general genetics MGG *236*, 331–340.

Yokoi, S., Bressan, R. A., and Hasegawa, P. M. (2002a). Salt Stress Tolerance of Plants. Plant Cell, 25–33.

Yokoi, S., Quintero, F. J., Cubero, B., Ruiz, M. T., Bressan, R. a, Hasegawa, P. M., and Pardo, J. M. (2002b). Differential expression and function of Arabidopsis thaliana NHX Na+/H+ antiporters in the salt stress response. The Plant journal : for cell and molecular biology *30*, 529–539.

Yoshiba, Y., Nanjo, T., Miura, S., Yamaguchi-shinozaki, K., and Shinozaki, K. (1999). Stress-Responsive and Developmental Regulation Gene Expression in Arabidopsis thaliana. Biochemical and Biophysical Research Communications *772*, 766–772.

Yu, C.-W., Liu, X., Luo, M., Chen, C., Lin, X., Tian, G., Lu, Q., Cui, Y., and Wu, K. (2011). HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in Arabidopsis. Plant physiology *156*, 173–184.

Yuan, L., Loqué, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., Takahashi, H., and Von Wirén, N. (2007). The organization of high-affinity ammonium uptake in Arabidopsis roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. The Plant cell *19*, 2636–2652.

Zang, C., Schones, D. E., Zeng, C., Cui, K., Zhao, K., and Peng, W. (2009). A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics (Oxford, England) *25*, 1952–1958.

Zar, J. H. (1999). Biostatistical Analysis 4th ed. Prentice-Hall, ed. (Upper Saddle River, NJ.).

Zeng, L., and Zhou, M. M. (2002). Bromodomain: an acetyl-lysine binding domain. FEBS Letters *513*, 124–128.

Zhang, K., Sridhar, V. V, Zhu, J., Kapoor, A., and Zhu, J.-K. (2007a). Distinctive Core Histone Post-Translational Modification Patterns in Arabidopsis thaliana. PLoS ONE *2*, 11.

Zhang, X., Bernatavichute, Y. V, Cokus, S., Pellegrini, M., and Jacobsen, S. E. (2009). Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in Arabidopsis thaliana. Genome biology *10*, R62.

Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y. V, Pellegrini, M., Goodrich, J., and Jacobsen, S. E. (2007b). Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis. PLoS biology *5*, e129.

Zhao, J., Barkla, B. J., Marshall, J., Pittman, J. K., and Hirschi, K. D. (2008). The Arabidopsis cax3 mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H+-ATPase activity. Planta *227*, 659–669.

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. The Plant cell *17*, 1196–1204.

Zhou, J., Wang, X., He, K., Charron, J.-B. F., Elling, A. a, and Deng, X. W. (2010). Genome-wide profiling of histone H3 lysine 9 acetylation and dimethylation in Arabidopsis reveals correlation between multiple histone marks and gene expression. Plant molecular biology *72*, 585–595.

Zhu, J., Jeong, J. C., Zhu, Y., Sokolchik, I., Miyazaki, S., Zhu, J.-K., Hasegawa, P. M., Bohnert, H. J., Shi, H., Yun, D.-J., et al. (2008). Involvement of Arabidopsis HOS15 in histone deacetylation and cold tolerance. Proceedings of the National Academy of Sciences of the United States of America *105*, 4945–4950.

Zhu, J.-K. (2002). Salt and drought stress signal transduction in plants. Annual review of plant biology *53*, 247–273.

Zimmerli, L., Métraux, J. P., and Mauch-Mani, B. (2001). beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus Botrytis cinerea. Plant physiology *126*, 517–523.

Appendix I: Primers sequence

Sequence	Primer name	AGI	Feature	
GCGTTACTCGTATGCGAGTTG	WRKY40-QPCR-F	AT1G80840	Downregulated upon priming	Microarray
AGGGCTGATTTGATCCCTCT	WRKY40-QPCR-R			
CCGCATCTCGTCGCTCAGAC	NAC19-QPCR-F	AT1G52890	Downregulated upon priming	
CCGTGACTGCTCTCGACTTC	NAC19-QPCR-R			
GATGGTTGTTCTCCGGTTGT	ERF1-QPCR-F	AT3G23240	Downregulated upon priming	
CTCAAGGTACTGTTCTCCCA	ERF1-QPCR-R			
CCTCGGTTGTGGTGGATAAT	CYP715A1 -QPCR-F	AT5G52400	Downregulated upon priming	
CCTTCGACCATAACCAAACC	CYP715A1 -QPCR-R			
GCTGGACGATGGATTCAGTT	WRKY30-QPCR-F	AT5G24110	Downregulated upon priming	
CGGCATTGTTGTACCGACA	WRKY30-QPCR-R			
GACGACGTTCTCGAGTCGTTG	NAC55-QPCR-F	AT3G15500	Downregulated upon priming	
CCGTCACCGTAACGAATACTC	NAC55-QPCR-R			
CCTCGAACTTCATTCCCGTTG	MYB108-QPCR-F	AT3G06490	Downregulated upon priming	
GGTTGTGATGCAAGATGACG	MYB108-QPCR-R			
GGCTCTATTGATGATGGTCACT	WRKY46-QPCR-F	AT2G46400	Downregulated upon priming	
GTGAATCGATGCGTGCATCTG	WRKY46-QPCR-R			
TGAAGGTCGCTAATGAAGCA	JAZ10-QPCR-F	AT5G13220	Downregulated upon priming	
CTCGAGAAAACGTTGCAGTG	JAZ10-QPCR-R			
TCCAGTTCATAAGGGGATGC	RNA-HSD4-F	AT5G50590	Upregulated upon priming	
GACTGAGACATCACCGCGTA	RNA-HSD4-R			
GTTTGGCATTGGAGGTGACT	RNA-PHT3-F	AT5G43360	Upregulated upon priming	
GCTTGAGGAGGCGTTGATAG	RNA-PHT3-R			
CTGACGTTGTCGCTACTGGA	RNA-GMD1-F	AT5G66280	Upregulated upon priming	
ACCGTAGGCCTCTCGGTAAT	RNA-GMD1-R			
GTACGTTGAAGATCAAAGGC	RNA-TOM7,2-F	AT1G64220	Upregulated upon priming	
AGGATCTGAGTTCATGCCGAT	RNA-TOM7,2-R			
TTGGCACATGGACGGTACTA	RNA-SKS15-F	AT4G37160	Upregulated upon priming	
TCTCATCGTTCCAAACACGA	RNA-SKS15-R			
CTCCGTCCAATGGTTTCATC	RNA-AMT1;1-F	AT4G13510	Upregulated upon priming	
TCAACTGACCAGAACCAGTGAG	RNA-AMT1;1-R			
TGTGAACCACTGGCCATAAA	RNA-SLAH1-F	AT1G62280	Upregulated upon priming	
CCATCTATGGCGTTCCATCT	RNA-SLAH1-R			
TTCACCGTGTTCTGTTGGAA	RNA-CHX16-F	AT1G64170	Upregulated upon priming	
GTGATGTTTCTTGCGCTTCA	RNA-CHX16-R			
TGGCAACTGTTGGTTATGGA	RNA-SKOR-F	AT3G02850	Upregulated upon priming	
CACGGATGTTCCTACCGAGT	RNA-SKOR-R			
ATAGCTAGCTGATGGGTCAAGTC	At4g35800-QPCR-F	AT4G35800	Reference gene	qPCR refernces genes RNA
GTTTTGAGTGTTTTGAAAAGGATT	At4g35800-QPCR-R			
TCGTAGTAGAGAGAAATACCAGTGGA	At1g56070-QPCR-F	AT1G56070	Reference gene	
AAGTTTACAGCTGATGAGCTTCG	At1g56070-QPCR-R			
TCCAGCTAAGGGTGCC	EF1-QPCR-F	AT5G60390	Reference gene	
GGTGGGTACTCGGAGA	EF1-QPCR-R			
ACTGACCTCTTAGCTCG	eIF4A-QPCR-F	AT3G13920	Reference gene	
CAGATCGGCCACGTTC	eIF4A-QPCR-R			
AGTGTCCCTGAGCTAAC	TUB9-QPCR-F	AT4G20890	Reference gene	

AGTGGGAGCTATATCGC	TUB9-QPCR-R			
AGGAAGGGATTCCACC	UBQ4-QPCR-F	AT5G20620	Reference gene	
AGTCCGACCATCCTCA	UBQ4-QPCR-R			
ATCACCCTTGAAGTGGA	UBQ10-QPCR-F	AT4G05320	Reference gene	
GAAACCACCACGAAGAC	UBQ10-QPCR-R			
GGAGCTAATAGCGGAGCTTG	ChipCont1-1F	AT1G24560	Positive Control H3K4me3	qPCR References genes ChIP
TCCTTCAATGCTTCATCACG	ChipCont1-1R			
GCACAGAGTTTGGTGGAAGAG	ChipCont1-2F	AT1G24560	Positive Control H3K4me2	
CACCATCCCTCAAACCATTC	ChipCont1-2R			
ACCAAGTCTAGCATGGGAGAGA	ChipCont1-3F	AT1G24560	Negative Control me2-3	
TCGAGGGTTTGATTTACATGG	ChipCont1-3R			
TGGCTCTGTTTCACATCTCG	ChipCont2-1F	AT1G24560	Positive Control H3K4me3	
AGATCATGCGAAGGAATGCT	ChipCont2-1R			
TGAGGCTGGGTAATGCTTCT	ChipCont2-2F	AT1G24560	Positive Control H3K4me2	
AATCGGAATCATCTCCATCG	ChipCont2-2R			
TGTGAATCCTGGTGTGGAAA	ChipCont2-3F	AT1G24560	Negative Control me2-3	
TGCAGAAGAACACCGCATTA	ChipCont2-3R			
CATCATCCGCGTCAATCAC	H3K9me2 3-1F	AT2G05920	Negative Control H3K9me2	
GAAACTCAGGAGTACGCG	H3K9me2 3-1R			
AAGAGAGCTGGCAGAAGCAGTTGA	H3K9me2 1-1F	AT1G37110	Positive Control K9me2	
ACGCCCTTTACCTTGACCTCCTTT	H3K9me2 1-1R			
TGTGTGGAAGGGTCTTGTGGACTT	H3K9me2 2-2F	AT4G03745	Positive Control K9me2	
AACTTACATGTTTGCGGGCACGAG	H3K9me2 2-2R			
GGAATCAGAAATTCCAACAGG	H3K27me3-1-1F	AT5G56900	Negative Control H3K27me3	
TTGATGCTCTTCGTGCACTT	H3K27me3-1-1R			
AAGCAATGCCGTATTGATCC	H3K27me3-1-2F	AT5G56920	Positive Control H3K27me3	
GCTGCACTTCAACAGCTTCA	H3K27me3-1-2R			
GAGAAGCTAGCAATACGCAACG	MYB75-F	AT1G56650	24h H3K27me3	qPCR ChIP
TGCTATTAAAGGTTGTGCATGA	MYB75-R			
GAATTGTCTACTTATATTGTAC	CYP71B14 -F	AT5G25180	24h/10d H3K27me3	
CCATCATAAATTGTCAACGA	CYP71B14 -R			
ACTGCTGGCCACCTGGTACT	RLP43-F	AT3G28890	24h/10d H3K27me3	
CTTCATCAATGGGGAAGCTG	RLP43-R			
ATTCTGCACATCACCAGTGC	GA1-F	AT4G02780	24h/10d H3K27me3	
CTATTCGTTAGGGTACTGTTCG	GA1-R			
GATGAGTTGTCACTAGGGATCC	SHP1 -F	AT3G58780	24h/10d H3K27me3	
CAATGTTAGACAAAGTCATCCG	SHP1 -R			
CGATCTTCTAAGCTAGATTTGA	AG8-F	AT4G18960	24h/10d H3K27me3	
CAAATATTCCCACTAATGTTAGTG	AG8-R			
CCATTTCTTCAGTGGCTAATG	SOS5 RH3K4me3-F	AT3G46550	ChIP-Seq H3K4me3 me2	
TCCAGTCATTACCGGCGGAG	SOS5 RH3K4me3-R			
CGCCTGATTTCCACAGTCTTG	LRP1 RH3K4me3-F	AT5G12330	ChIP-Seq H3K4me3 me2	
CCACGAAACGCAGTATCTTCAC	LRP1 RH3K4me3-R			
CCGACCCGTTTCAAGTTACC	SCARECROW-LIKE RH3K4me3-F	AT5G66770	ChIP-Seq H3K4me3 me2	
TCCACCGCCGAAATCAGATA	SCARECROW-LIKERH3K4me3-R			
GAGAGACCCAAGAGTCCAAGAC	MYB30-F	AT3G28910	Time-course ChIP	qPCR Time course ChIP
CTACTTTAGGTGGTAATGAGTC	MYB30-R			
AGGCTTCACGAGGAGAATCA	WRKY47-F	AT4G01720	Time-course ChIP	
AGCTAATCTCGGTGCCACAT	WRKY47-R			

GCAGGATATGCGACACATGA	HAK5-F	AT4G13420	Time-course ChIP	
TCGGAGGTGTTTTCCTCTGC	HAK5-R			
CACGGAAACTTGAAATATTTCAT	MYB75-F	AT1G56650	Time-course ChIP	
GACGTTGATCAACTTTGGAGTC	MYB75-R			
CGGTTCTTGATCGTGTTCATT	LOB-F	AT2G42440	Time-course ChIP	
ACTCTGAATTGGATCAGCACTTG	LOB-R			
GCTGAGATACGAGATCCTTGG	ERF12-F	AT1G28360	Time-course ChIP	
GGTGGTTGAGGTCGAGAGAG	ERF12-R			
GTTTCGACCTCAGACGCATT	PTAC16-F	AT3G46780	Time-course ChIP	
AGAGATAGTCAATGGCTGAGA	PTAC16-R			
GGAAGATCCGAGTTGCAGAG	TEL1-F	AT3G26120	Time-course ChIP	
TATAGCACACGAATCCATGTG	TEL1-R			
CACTCCCATGGATTGAGGAA	HKT1-K27me3-F	AT4G10310	Time-course ChIP	
TTGTGGAAAGTTTGACAAGCA	HKT1-K27me3-R			
CCAATACTGGGCTGCTTAGA	RNA-MYB30-F	AT3G28910	Time-course RNA	qPCR Time course RNA
GATGAGAATCTTGATTGACT	RNA-MYB30-R			
CAACATGAGGATGTACCTCA	RNA-WRKY47-F	AT4G01720	Time-course RNA	
GCTCTAACGGAAACCCTAGC	RNA-WRKY47-R			
CTCCGGGATATAGACTTGTCCCT	AtHAK5-F	AT4G13420	Time-course RNA	
GCAATGTTTGCTGATCTAGGTCA	AtHAK5-R			
GTCCAAGGCATGGAGGATTAACG	MYB75-F	AT1G56650	Time-course RNA	
GGTCGGACCGCAAATGACGTC	MYB75-R			
CTGAGATACGAGATCCTTGGA	ERF12-F	AT1G28360	Time-course RNA	
CGCAGCGAACCAGAGCTGTTG	ERF12-R			
GGAGTCACAGTGGACGGTTT	PTAC16-F	AT3G46780	Time-course RNA	
CTTCGGACGTTCTTCTTTCG	PTAC16-R			
ACCATGGAAGATCCGAGTTG	TEL1-F	AT3G26120	Time-course RNA	
ATCCATGGGGAGATACACGA	TEL1-R			
TTGGTTGGATCGTTGTTTCA	AtHKT1-F	AT4G10310	Time-course RNA	
CTTTCGGTGATTGAAATGAG	AtHKT1-R			

Appendix II :**Top 300 genes list rank where P+/P- > C+/C-**

Rank	Affymetrix ID	TAIR ID	Description	Response C+/C-	Response P+/P-	Fold change
1	254550_AT	AT4G19690	IRT1 (iron-regulATed transporter 1);	0.2	3.0	16.0
2	253502_AT	AT4G31940	CYP82C4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	0.2	1.9	8.4
3	246880_s_AT	AT5G26000 ;AT5G2598 0	[AT5G26000, TGG1 (THIOGLUCOSIDE GLUCOHYDROLASE 1); hydrolase, hydrolyzing O-glycosyl compounds / thioglucosidase];[AT5G25980, TGG2 (GLUCOSIDE GLUCOHYDROLASE 2); hydrolase, hydrolyzing O-glycosyl compounds / thioglucosidase]	1.0	6.0	6.3
4	253763_AT	AT4G28850	xyloglucanxyloglucosyl transferase, putATive / xyloglucan endotransglycosylase, putATive / endo-xyloglucan transferase, putATive	0.6	2.9	4.9
5	248519_AT	AT5G50590	[AT5G50590, AtHSD4 (hydroxysteroid dehydrogenase 4); binding / cATalytic/ oxidoreductase];[AT5G50690, AtHSD7 (hydroxysteroid dehydrogenase 7); binding / cATalytic/ oxidoreductase]	1.9	9.2	4.9
6	261542_AT	AT1G63560	unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; CONTAINS InterPro DOMAIN/s Protein of unknown function DUF26 (InterProIPR002902); BEST *Arabidopsis* thaliana protein mATch is protein kinase-relATed (TAIRAT1G63600.1); Has 899 Blast hits to 869 proteins in 13 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 899; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	1.2	5.5	4.5
7	263376_AT	AT2G20520	FLA6 (FASCICLIN-LIKE ARABINOGALACTAN 6)	0.3	1.1	3.9
8	251755_AT	AT3G55790	unknown protein	17.7	61.4	3.5
9	256593_AT	AT3G28510	AAA-type ATPase family protein	0.9	3.0	3.4
10	252302_AT	AT3G49190	condensATion domain-containing protein	0.5	1.8	3.4
11	248406_AT	AT5G51490	pectinesterase family protein	0.1	0.4	3.3
12	248571_AT	AT5G49790	transposable element gene	0.7	2.3	3.3
13	250651_AT	AT5G06900	CYP93D1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	6.3	20.3	3.2
14	262427_s_AT	AT1G47600	[AT1G47600, BGLU34 (BETA GLUCOSIDASE 34); hydrolase, hydrolyzing O-glycosyl compounds / thioglucosidase];[AT1G51470, BGLU35 (BETA GLUCOSIDASE 35); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds]	0.6	1.7	3.1
15	246138_AT	AT5G19870	unknown protein	0.6	1.9	3.0
16	252510_AT	AT3G46270	receptor protein kinase-relATed	0.8	2.4	3.0
17	259328_AT	AT3G16440	ATMLP-300B (MYROSINASE-BINDING PROTEIN-LIKE PROTEIN-300B)	0.6	1.7	3.0
18	246652_AT	AT5G35190	proline-rich extensin-like family protein	0.7	2.1	3.0
19	266649_AT	AT2G25810	TIP4;1 (tonoplast intrinsic protein 4;1); wATer channel	0.4	1.1	2.8
20	258912_AT	AT3G06460	GNS1/SUR4 membrane family protein	0.4	1.1	2.8
21	254056_AT	AT4G25250	invertase/pectin methylesterase inhibitor family protein	0.7	1.9	2.8
22	265246_AT	AT2G43050	ATPMEPCRD; enzyme inhibitor/ pectinesterase	0.3	0.8	2.8
23	256099_AT	AT1G13710	CYP78A5; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	0.5	1.3	2.7
24	264682_AT	AT1G65570	polygalacturonase, putATive / pectinase, putATive	0.4	1.1	2.7
25	251545_AT	AT3G58810	MTPA2 (METAL TOLERANCE PROTEIN A2); efflux transmembrane transporter/ inorganic anion transmembrane transporter/ zinc ion transmembrane transporter	0.8	2.2	2.7
26	263689_AT	AT1G26820	RNS3 (RIBONUCLEASE 3); RNA binding / endoribonuclease/ ribonuclease T2	0.7	1.9	2.7

27	251116_AT	AT3G63470	scpl40 (serine carboxypeptidase-like 40); serine-type carboxypeptidase	0.2	0.5	2.6
28	254539_s_AT	AT4G19750	[AT4G19750, glycosyl hydrolase family 18 protein];[AT4G19760, cATalytic/ cATion binding / chitinase/ hydrolase, hydrolyzing O-glycosyl compounds]	0.4	1.2	2.6
29	252238_AT	AT3G49960	peroxidase, putATive	0.8	2.2	2.6
30	260091_AT	AT1G73290	scpl5 (serine carboxypeptidase-like 5); serine-type carboxypeptidase	1.5	3.8	2.6
31	249474_s_AT	AT5G39160	[AT5G39160, germin-like protein (GLP2a) (GLP5a)];[AT5G39130, germin-like protein, putATive];[AT5G39190, GER2 (GERMIN-LIKE PROTEIN 2); oxalATe oxidase]	1.2	3.1	2.6
32	245697_AT	AT5G04200	AtMC9 (metacaspase 9); cysteine-type peptidase	0.8	2.1	2.6
33	265049_AT	AT1G52060	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN endomembrane system; EXPRESSED IN root; CONTAINS InterPro DOMAIN/s Mannose-binding lectin (InterProIPR001229); BEST *Arabidopsis* thaliana protein mATch is jacalin lectin family protein (TAIRAT1G52070.1); Has 1057 Blast hits to 529 proteins in 40 species Archae - 0; Bacteria - 2; Metazoa - 4; Fungi - 0; Plants - 1048; Viruses - 0; Other Eukaryotes - 3 (source NCBI BLink).	0.5	1.2	2.6
34	267626_AT	AT2G42250	CYP712A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	2.3	5.8	2.5
35	248048_AT	AT5G56080	NAS2 (NICOTIANAMINE SYNTHASE 2); nicotianamine synthase	0.4	1.0	2.5
36	256929_AT	no_mATch	no_mATch	1.7	4.2	2.5
37	263229_s_AT	AT1G05660	[AT1G05660, polygalacturonase, putATive / pectinase, putATive];[AT1G05650, polygalacturonase, putATive / pectinase, putATive]	0.9	2.3	2.5
38	258008_AT	AT3G19430	lATe embryogenesis abundant protein-relATed / LEA protein-relATed	0.8	2.0	2.5
39	260950_s_AT	AT1G06120	[AT1G06120, fATty acid desATurase family protein];[AT1G06090, fATty acid desATurase family protein]	0.6	1.4	2.5
40	245966_AT	AT5G19790	RAP2.11 (relATed to AP2 11); DNA binding / transcription factor	0.4	1.0	2.5
41	255075_AT	AT4G09110	zinc finger (C3HC4-type RING finger) family protein	0.8	1.8	2.5
42	261550_AT	AT1G63450	cATalytic	0.2	0.5	2.5
43	249151_AT	AT5G43360	PHT3 (PHOSPHATE TRANSPORTER 3); carbohydrATe transmembrane transporter/ inorganic phosphATe transmembrane transporter/ phosphATe transmembrane transporter/ sugarhydrogen symporter	1.0	2.6	2.5
44	265048_AT	AT1G52050	jacalin lectin family protein	0.2	0.6	2.4
45	263941_AT	no_mATch	no_mATch	0.7	1.8	2.4
46	261562_AT	AT1G01750	ADF11 (ACTIN DEPOLYMERIZING FACTOR 11); actin binding	0.4	0.9	2.4
47	257323_AT	ATMG01200	hypothetical protein	0.7	1.6	2.4
48	248844_s_AT	AT5G46900	[AT5G46900, protease inhibitor/seed storage/lipid transfer protein (LTP) family protein];[AT5G46890, protease inhibitor/seed storage/lipid transfer protein (LTP) family protein]	0.2	0.4	2.4
49	255632_AT	AT4G00680	ADF8 (ACTIN DEPOLYMERIZING FACTOR 8); actin binding	0.4	1.0	2.4
50	265050_AT	AT1G52070	jacalin lectin family protein	0.6	1.4	2.4
51	253968_AT	AT4G26560	CBL7 (CALCINEURIN B-LIKE 7); calcium ion binding	0.9	2.1	2.4
52	250801_AT	AT5G04960	pectinesterase family protein	0.4	1.0	2.3
53	247657_AT	AT5G59845	gibberellin-regulATed family protein	0.6	1.5	2.3
54	248178_AT	AT5G54370	lATe embryogenesis abundant protein-relATed / LEA protein-relATed	1.1	2.6	2.3
55	260758_AT	AT1G48930	AtGH9C1 (*Arabidopsis* thaliana glycosyl hydrolase	0.6	1.4	2.3

			9C1); cATalytic/ hydrolase, hydrolyzing O-glycosyl compounds			
56	245967_AT	AT5G19800	hydroxyproline-rich glycoprotein family protein	0.3	0.6	2.3
57	253527_AT	AT4G31470	pAThogenesis-relATed protein, putATive	0.5	1.1	2.3
58	250778_AT	AT5G05500	pollen Ole e 1 allergen and extensin family protein	0.5	1.3	2.3
59	246912_AT	AT5G25820	exostosin family protein	0.8	1.9	2.3
60	260970_AT	AT1G53640	unknown protein	0.4	0.9	2.3
61	259581_AT	AT1G28040	protein binding / zinc ion binding	0.5	1.1	2.3
62	260148_AT	AT1G52800	oxidoreductase, 2OG-Fe(II) oxygenase family protein	0.6	1.3	2.3
63	257041_AT	no_mATch	no_mATch	0.5	1.0	2.3
64	258013_AT	AT3G19320	leucine-rich repeAT family protein	1.9	4.3	2.3
65	266978_AT	AT2G39430	disease resistance-responsive protein-relATed / dirigent protein-relATed	0.5	1.2	2.3
66	247094_AT	AT5G66280	GMD1 (GDP-D-MANNOSE 4,6-DEHYDRATASE 1); GDP-mannose 4,6-dehydrATase/ binding / cATalytic/ coenzyme binding	0.5	1.2	2.3
67	267457_AT	AT2G33790	AGP30 (ARABINOGALACTAN PROTEIN30)	0.9	1.9	2.3
68	260867_AT	AT1G43790	TED6 (TRACHEARY ELEMENT DIFFERENTIATION-RELATED 6)	0.7	1.5	2.3
69	248904_AT	AT5G46295	unknown protein	1.6	3.7	2.3
70	261691_AT	AT1G50060	pAThogenesis-relATed protein, putATive	0.8	1.9	2.2
71	248652_AT	AT5G49270	SHV2 (SHAVEN 2)	0.3	0.8	2.2
72	255138_AT	AT4G08380	proline-rich extensin-like family protein	0.6	1.4	2.2
73	259462_AT	AT1G18940	nodulin family protein	0.3	0.6	2.2
74	249934_AT	AT5G22410	peroxidase, putATive	0.4	1.0	2.2
75	260957_AT	AT1G06080	ADS1 (DELTA 9 DESATURASE 1); oxidoreductase	1.0	2.1	2.2
76	264404_AT	AT2G25160	CYP82F1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	0.3	0.7	2.2
77	246067_AT	AT5G19410	ABC transporter family protein	3.3	7.3	2.2
78	257607_AT	AT3G13880	pentATricopeptide (PPR) repeAT-containing protein	0.5	1.2	2.2
79	267024_s_AT	AT2G34390	[AT2G34390, NIP2;1 (NOD26-LIKE INTRINSIC PROTEIN 2;1); lactATe transmembrane transporter/ wATer channel];[AT2G29870, major intrinsic family protein / MIP family protein]	1.1	2.4	2.2
80	258145_AT	AT3G18200	nodulin MtN21 family protein	0.8	1.8	2.2
81	260067_AT	AT1G73780	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0.5	1.0	2.2
82	245677_AT	AT1G56660	unknown protein	0.8	1.8	2.2
83	245172_AT	AT2G47540	pollen Ole e 1 allergen and extensin family protein	0.6	1.3	2.2
84	263850_AT	AT2G04480	unknown protein	0.3	0.7	2.2
85	253998_AT	AT4G26010	peroxidase, putATive	0.5	1.1	2.2
86	256684_AT	AT3G32040	geranylgeranyl pyrophosphATe synthase, putATive / GGPP synthetase, putATive / farnesyltranstransferase, putATive	0.6	1.4	2.2
87	259525_AT	AT1G12560	ATEXPA7 (*ARABIDOPSIS* THALIANA EXPANSIN A7)	0.7	1.5	2.2
88	261543_AT	AT1G63550	LOCATED IN anchored to membrane; EXPRESSED IN flower, root, carpel, leaf; EXPRESSED DURING 4 leaf senescence stage, petal differentiATion and expansion stage; CONTAINS InterPro DOMAIN/s Protein of unknown function DUF26 (InterProIPR002902); BEST *Arabidopsis* thaliana protein mATch is receptor-like protein kinase-relATed (TAIRAT1G63570.1); Has 4939 Blast hits to 2168 proteins in 204 species Archae - 8; Bacteria - 183; Metazoa - 580; Fungi - 134; Plants - 2707; Viruses - 661; Other Eukaryotes - 666 (source NCBI BLink).	0.6	1.3	2.1

89	250832_AT	AT5G04950	NAS1 (NICOTIANAMINE SYNTHASE 1); nicotianamine synthase	0.5	1.0	2.1
90	267287_AT	AT2G23630	sks16 (SKU5 Similar 16); copper ion binding / pectinesterase	0.5	1.1	2.1
91	266191_AT	AT2G39040	peroxidase, putATive	1.1	2.3	2.1
92	255432_AT	AT4G03330	SYP123 (SYNTAXIN OF PLANTS 123); SNAP receptor	0.3	0.7	2.1
93	245412_AT	AT4G17280	INVOLVED IN multicellular organismal development; LOCATED IN membrane; EXPRESSED IN 11 plant structures; EXPRESSED DURING 6 growth stages; CONTAINS InterPro DOMAIN/s Uncharacterised conserved protein UCP037471 (InterProIPR017214), Protein of unknown function DUF568, DOMON-like (InterProIPR007613), DOMON relATed (InterProIPR005018), Cytochrome b561/ferric reductase transmembrane (InterProIPR006593); BEST *Arabidopsis* thaliana protein mATch is auxin-responsive protein, putATive (TAIRAT5G47530.1); Has 203 Blast hits to 203 proteins in 14 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 203; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	1.6	3.4	2.1
94	244960_AT	ATCG01020	encodes a chloroplast ribosomal protein L32, a constituent of the large subunit of the ribosomal complex	0.6	1.2	2.1
95	248324_AT	no_mATch	no_mATch	0.3	0.7	2.1
96	250802_AT	AT5G04970	pectinesterase, putATive	0.9	1.9	2.1
97	256970_AT	AT3G21090	ABC transporter family protein	0.7	1.5	2.1
98	253696_AT	AT4G29740	CKX4 (CYTOKININ OXIDASE 4); amine oxidase/ cytokinin dehydrogenase	0.8	1.7	2.1
99	265102_AT	AT1G30870	cATionic peroxidase, putATive	0.7	1.4	2.1
100	264391_AT	AT1G11920	pectATe lyase family protein	0.9	1.9	2.1
101	259291_AT	AT3G11550	integral membrane family protein	0.5	1.0	2.1
102	251953_AT	AT3G53668	[AT3G53668, CPuORF51 (Conserved peptide upstream open reading frame 51)];[AT3G53670, unknown protein]	0.3	0.6	2.1
103	256141_AT	AT1G48640	lysine and histidine specific transporter, putATive	0.4	0.8	2.1
104	246302_AT	AT3G51860	CAX3 (CATION EXCHANGER 3); calciumcATion antiporter/ calciumhydrogen antiporter/ cATioncATion antiporter	4.5	9.4	2.1
105	253870_AT	AT4G27530	unknown protein	2.0	4.2	2.1
106	259222_AT	AT3G03680	C2 domain-containing protein	0.4	0.9	2.1
107	261099_AT	AT1G62980	ATEXPA18 (*ARABIDOPSIS* THALIANA EXPANSIN A18)	0.6	1.1	2.1
108	258080_AT	AT3G25930	universal stress protein (USP) family protein	0.4	0.9	2.1
109	246403_AT	AT1G57590	carboxylesterase	0.5	1.1	2.1
110	265111_AT	AT1G62510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0.2	0.5	2.0
111	250717_AT	AT5G06200	integral membrane family protein	0.6	1.1	2.0
112	251315_AT	AT3G61410	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; BEST *Arabidopsis* thaliana protein mATch is protein kinase family protein / U-box domain-containing protein (TAIRAT2G45910.1); Has 146 Blast hits to 144 proteins in 9 species Archae - 0; Bacteria - 0; Metazoa - 13; Fungi - 0; Plants - 132; Viruses - 0; Other Eukaryotes - 1 (source NCBI BLink).	0.8	1.5	2.0
113	264998_AT	AT1G67330	unknown protein	0.4	0.7	2.0
114	249779_AT	AT5G24230	LOCATED IN cellular_component unknown; EXPRESSED IN root; BEST *Arabidopsis* thaliana protein mATch is triacylglycerol lipase (TAIRAT5G24200.1); Has 125 Blast hits to 125 proteins in 14 species Archae - 0; Bacteria - 0;	0.7	1.3	2.0

			Metazoa - 3; Fungi - 7; Plants - 115; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).			
115	250205_AT	AT5G14020	INVOLVED IN N-terminal protein myristoylATion; LOCATED IN vacuole; EXPRESSED IN root; CONTAINS InterPro DOMAIN/s BRO1 (InterProIPR004328); BEST *Arabidopsis* thaliana protein mATch is unknown protein (TAIRAT1G73390.3); Has 53 Blast hits to 53 proteins in 7 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 53; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	0.1	0.3	2.0
116	248941_s_AT	AT5G45460	[AT5G45460, unknown protein];[AT5G45470, unknown protein]	0.5	1.0	2.0
117	250958_AT	AT5G03260	LAC11 (laccase 11); laccase	0.9	1.8	2.0
118	245262_AT	AT4G16563	aspartyl protease family protein	0.2	0.4	2.0
119	262017_AT	AT1G35550	elongATion factor Tu C-terminal domain-containing protein	0.6	1.3	2.0
120	255105_AT	AT4G08620	SULTR1;1 (SULPHATE TRANSPORTER 1;1); sulfATe transmembrane transporter	0.3	0.5	2.0
121	259720_AT	AT1G61080	proline-rich family protein	0.6	1.1	2.0
122	246530_AT	AT5G15725	unknown protein	0.3	0.5	2.0
123	264287_AT	AT1G61930	unknown protein	2.0	4.0	2.0
124	255005_AT	AT4G09990	unknown protein	0.5	1.0	2.0
125	254404_AT	AT4G21340	B70; transcription factor	1.3	2.5	2.0
126	249037_AT	AT5G44130	FLA13 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 PRECURSOR)	0.0	0.1	2.0
127	245875_AT	AT1G26240	proline-rich extensin-like family protein	1.6	3.2	2.0
128	254324_AT	AT4G22640	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN anchored to membrane; EXPRESSED IN male gametophyte, root; CONTAINS InterPro DOMAIN/s Bifunctional inhibitor/plant lipid transfer protein/seed storage (InterProIPR016140); BEST *Arabidopsis* thaliana protein mATch is unknown protein (TAIRAT4G22666.2); Has 19 Blast hits to 19 proteins in 1 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 19; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	1.6	3.1	2.0
129	259120_AT	AT3G02240	unknown protein	0.8	1.5	2.0
130	255814_AT	AT1G19900	glyoxal oxidase-relATed	0.6	1.2	2.0
131	248898_AT	AT5G46370	KCO2 (CA2+ ACTIVATED OUTWARD RECTIFYING K+ CHANNEL 2); calcium ion binding / outward rectifier potassium channel	0.6	1.2	2.0
132	253914_AT	AT4G27400	lATe embryogenesis abundant protein-relATed / LEA protein-relATed	0.8	1.5	2.0
133	265764_AT	AT2G48060	unknown protein	0.9	1.7	2.0
134	257473_AT	AT1G33840	unknown protein	0.4	0.8	2.0
135	248636_AT	no_mATch	no_mATch	0.3	0.7	2.0
136	254977_s_AT	AT4G10520	[AT4G10520, subtilase family protein];[AT4G10530, subtilase family protein]	1.5	3.0	2.0
137	256097_AT	AT1G13670	unknown protein	0.5	1.0	2.0
138	247871_AT	AT5G57530	xyloglucanxyloglucosyl transferase, putATive / xyloglucan endotransglycosylase, putATive / endo-xyloglucan transferase, putATive	1.1	2.1	2.0
139	267343_AT	AT2G44260	unknown protein	0.6	1.3	1.9
140	254338_s_AT	AT4G22080	[AT4G22080, pectATe lyase family protein];[AT4G22090, pectATe lyase family protein]	0.4	0.9	1.9
141	260558_AT	AT2G43600	glycoside hydrolase family 19 protein	1.1	2.1	1.9
142	250855_AT	AT5G04730	unknown protein	0.3	0.6	1.9
143	247091_AT	AT5G66390	peroxidase 72 (PER72) (P72) (PRXR8)	2.6	5.0	1.9

144	266941_AT	AT2G18980	peroxidase, putATive	0.4	0.7	1.9
145	259345_s_AT	AT3G03700	[AT3G03700, unknown protein];[AT3G04440, unknown protein]	1.0	1.9	1.9
146	248577_AT	AT5G49870	jacalin lectin family protein	0.4	0.8	1.9
147	252152_AT	no_mATch	no_mATch	1.1	2.1	1.9
148	260754_AT	AT1G49000	unknown protein	3.3	6.4	1.9
149	246969_AT	AT5G24880	INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; EXPRESSED IN 8 plant structures; EXPRESSED DURING L mATure pollen stage, M germinATed pollen stage, 4 anthesis, petal differentiATion and expansion stage; BEST *Arabidopsis* thaliana protein mATch is calmodulin-binding protein-relATed (TAIRAT5G10660.1); Has 195834 Blast hits to 92446 proteins in 2480 species Archae - 1007; Bacteria - 19200; Metazoa - 85171; Fungi - 18359; Plants - 7291; Viruses - 1072; Other Eukaryotes - 63734 (source NCBI BLink).	0.5	1.0	1.9
150	254587_AT	no_mATch	no_mATch	0.5	1.0	1.9
151	255528_AT	AT4G02090	unknown protein	0.9	1.7	1.9
152	254013_AT	AT4G26050	leucine-rich repeAT family protein	0.8	1.6	1.9
153	265645_AT	AT2G27370	integral membrane family protein	0.6	1.1	1.9
154	251434_AT	AT3G59850	polygalacturonase, putATive / pectinase, putATive	1.2	2.3	1.9
155	260122_AT	AT1G33900	avirulence-responsive protein, putATive / avirulence induced gene protein, putATive / AIG protein, putATive	0.3	0.5	1.9
156	254093_AT	AT4G25110	AtMC2 (metacaspase 2); cysteine-type endopeptidase	0.6	1.2	1.9
157	246229_AT	AT4G37160	sks15 (SKU5 Similar 15); copper ion binding / oxidoreductase	1.2	2.4	1.9
158	252046_AT	AT3G52460	hydroxyproline-rich glycoprotein family protein	0.3	0.6	1.9
159	255377_AT	AT4G03500	ankyrin repeAT family protein	1.0	1.8	1.9
160	263316_s_AT	AT2G24710	[AT2G24710, ATGLR2.3; intracellular ligand-gATed ion channel];[AT2G24720, ATGLR2.2; intracellular ligand-gATed ion channel]	0.3	0.5	1.9
161	254978_AT	AT4G10540	subtilase family protein	2.3	4.4	1.9
162	254718_AT	AT4G13580	disease resistance-responsive family protein	0.6	1.2	1.9
163	245553_AT	AT4G15370	BARS1 (BARUOL SYNTHASE 1); baruol synthase/ cATalytic	0.5	0.9	1.9
164	262978_AT	AT1G75780	TUB1; GTP binding / GTPase/ structural molecule	0.4	0.8	1.9
165	251677_AT	AT3G56980	BHLH039; DNA binding / transcription factor	0.8	1.4	1.9
166	248408_AT	AT5G51520	invertase/pectin methylesterase inhibitor family protein	1.7	3.3	1.9
167	253004_AT	AT4G38280	[AT4G38280, unknown protein];[AT4G38330, FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN integral to membrane; EXPRESSED IN male gametophyte, pollen tube; EXPRESSED DURING L mATure pollen stage, M germinATed pollen stage; CONTAINS InterPro DOMAIN/s Hly-III relATed (InterProIPR004254); BEST *Arabidopsis* thaliana protein mATch is unknown protein (TAIRAT4G38290.1); Has 8 Blast hits to 8 proteins in 1 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 8; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).];[AT2G45250, unknown protein]	0.6	1.1	1.9
168	263560_s_AT	AT2G15350	[AT2G15350, FUT10 (FUCOSYLTRANSFERASE 10); fucosyltransferase/ transferase, transferring glycosyl groups];[AT2G15370, FUT5; fucosyltransferase/ transferase, transferring glycosyl groups]	0.6	1.1	1.9
169	247895_AT	AT5G58010	basic helix-loop-helix (bHLH) family protein	0.9	1.7	1.9

170	252098_AT	AT3G51330	aspartyl protease family protein	0.7	1.4	1.9
171	250576_AT	AT5G08250	cytochrome P450 family protein	4.6	8.6	1.9
172	253309_AT	AT4G33790	CER4 (ECERIFERUM 4); fATty acyl-CoA reductase (alcohol-forming)/ oxidoreductase, acting on the CH-CH group of donors	0.5	1.0	1.9
173	254025_AT	AT4G25790	allergen V5/Tpx-1-relATed family protein	0.3	0.6	1.9
174	264842_AT	AT1G03700	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN endomembrane system; EXPRESSED IN hypocotyl, flower; EXPRESSED DURING petal differentiATion and expansion stage; CONTAINS InterPro DOMAIN/s Uncharacterised protein family UPF0497, trans-membrane plant (InterProIPR006702), Uncharacterised protein family UPF0497, trans-membrane plant subgroup (InterProIPR006459); BEST *Arabidopsis* thaliana protein mATch is integral membrane family protein (TAIRAT4G03540.1); Has 260 Blast hits to 260 proteins in 13 species Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 260; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	3.9	7.3	1.9
175	257184_AT	AT3G13090	ATMRP8; ATPase, coupled to transmembrane movement of substances	1.0	1.9	1.9
176	251143_AT	AT5G01220	SQD2 (sulfoquinovosyldiacylglycerol 2); UDP-glycosyltransferase/ UDP-sulfoquinovoseDAG sulfoquinovosyltransferase/ transferase, transferring glycosyl groups	0.5	0.9	1.9
177	254092_AT	AT4G25090	respirATory burst oxidase, putATive / NADPH oxidase, putATive	0.6	1.0	1.9
178	254743_AT	AT4G13420	HAK5 (HIGH AFFINITY K+ TRANSPORTER 5); potassium ion transmembrane transporter/ potassiumsodium symporter	7.3	13.7	1.9
179	265852_AT	AT2G42350	zinc finger (C3HC4-type RING finger) family protein	1.0	1.8	1.9
180	250683_x_AT	AT5G06640	proline-rich extensin-like family protein	0.3	0.6	1.9
181	262168_AT	AT1G74730	unknown protein	0.9	1.6	1.9
182	263338_AT	AT2G05000	transposable element gene	1.1	2.1	1.9
183	249847_AT	AT5G23210	SCPL34; serine-type carboxypeptidase	0.3	0.6	1.9
184	255127_AT	AT4G08300	nodulin MtN21 family protein	0.1	0.2	1.9
185	258359_s_AT	AT3G14415	[AT3G14415, (S)-2-hydroxy-acid oxidase, peroxisomal, putATive / glycolATe oxidase, putATive / short chain alpha-hydroxy acid oxidase, putATive];[AT3G14420, (S)-2-hydroxy-acid oxidase, peroxisomal, putATive / glycolATe oxidase, putATive / short chain alpha-hydroxy acid oxidase, putATive]	0.1	0.3	1.9
186	245036_AT	AT2G26410	Iqd4 (IQ-domain 4); calmodulin binding	0.9	1.7	1.8
187	257720_AT	AT3G18450	unknown protein	0.2	0.3	1.8
188	259351_AT	AT3G05150	sugar transporter family protein	0.2	0.5	1.8
189	257405_AT	AT1G24620	polcalcin, putATive / calcium-binding pollen allergen, putATive	0.5	1.0	1.8
190	245318_AT	AT4G16980	arabinogalactan-protein family	0.7	1.4	1.8
191	261203_AT	AT1G12845	unknown protein	1.7	3.0	1.8
192	255140_x_AT	AT4G08410	proline-rich extensin-like family protein	0.4	0.8	1.8
193	263571_AT	AT2G17050	disease resistance protein (TIR-NBS-LRR class), putATive	1.0	1.8	1.8
194	264988_AT	AT1G27140	ATGSTU14 (*ARABIDOPSIS* THALIANA GLUTATHIONE S-TRANSFERASE TAU 14); glutAThione transferase	0.4	0.7	1.8
195	255659_AT	AT4G00895	ATP synthase delta chain-relATed	0.8	1.5	1.8
196	246798_AT	AT5G26930	zinc finger (GATA type) family protein	0.9	1.6	1.8
197	251857_AT	AT3G54770	RNA recognition motif (RRM)-containing protein	0.5	0.8	1.8
198	263227_AT	AT1G30750	unknown protein	0.7	1.3	1.8
199	257679_AT	AT3G20470	GRP5 (GLYCINE-RICH PROTEIN 5); structural	0.5	0.9	1.8

			constituent of cell wall			
200	262217_AT	AT1G74770	protein binding / zinc ion binding	0.6	1.0	1.8
201	257143_AT	AT3G20110	CYP705A20; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	0.3	0.6	1.8
202	252222_AT	AT3G49845	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; EXPRESSED IN root; CONTAINS InterPro DOMAIN/s XYPPX repeAT (InterProIPR006031); Has 14038 Blast hits to 7746 proteins in 541 species Archae - 8; Bacteria - 1083; Metazoa - 5225; Fungi - 1793; Plants - 3748; Viruses - 424; Other Eukaryotes - 1757 (source NCBI BLink).	0.8	1.4	1.8
203	252183_AT	AT3G50740	UGT72E1 (UDP-glucosyl transferase 72E1); UDP-glycosyltransferase/ coniferyl-alcohol glucosyltransferase/ transferase, transferring glycosyl groups	1.1	1.9	1.8
204	245574_AT	AT4G14750	IQD19 (IQ-domain 19); calmodulin binding	0.4	0.8	1.8
205	254774_AT	AT4G13440	calcium-binding EF hand family protein	0.7	1.3	1.8
206	262212_AT	AT1G74890	ARR15 (RESPONSE REGULATOR 15); transcription regulATor/ two-component response regulATor	0.6	1.1	1.8
207	245510_AT	AT4G15740	C2 domain-containing protein	0.5	0.9	1.8
208	264884_AT	AT1G61170	unknown protein	0.5	1.0	1.8
209	258955_s_AT	AT3G18530	[AT3G18530, binding];[AT3G01450, binding]	0.7	1.3	1.8
210	255548_AT	AT4G01930	DC1 domain-containing protein	0.7	1.2	1.8
211	245546_AT	AT4G15290	ATCSLB05; cellulose synthase/ transferase/ transferase, transferring glycosyl groups	0.4	0.7	1.8
212	245012_AT	ATCG00440	Encodes NADH dehydrogenase D3 subunit of the chloroplast NAD(P)H dehydrogenase complex	0.5	0.9	1.8
213	258920_AT	AT3G10520	AHB2 (*ARABIDOPSIS* HAEMOGLOBIN 2); oxygen transporter	0.6	1.0	1.8
214	262097_AT	AT1G55990	glycine-rich protein	1.8	3.2	1.8
215	260567_AT	AT2G43820	UGT74F2 (UDP-GLUCOSYLTRANSFERASE 74F2); UDP-glucose4-aminobenzoATe acylglucosyltransferase/ UDP-glucosyltransferase/ UDP-glycosyltransferase/ transferase, transferring glycosyl groups / transferase, transferring hexosyl groups	0.7	1.2	1.8
216	260623_AT	AT1G08090	ATNRT21 (NITRATE TRANSPORTER 21); nitrATe transmembrane transporter	1.0	1.7	1.8
217	244969_AT	ATCG00650	chloroplast-encoded ribosomal protein S18	0.7	1.3	1.8
218	260298_AT	AT1G80320	oxidoreductase, 2OG-Fe(II) oxygenase family protein	7.5	13.3	1.8
219	254313_AT	AT4G22460	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0.1	0.1	1.8
220	263437_AT	AT2G28670	disease resistance-responsive family protein / fibroin-relATed	0.8	1.4	1.8
221	250173_AT	AT5G14340	AtMYB40 (myb domain protein 40); DNA binding / transcription factor	0.3	0.5	1.8
222	264567_s_AT	AT1G05250	[AT1G05250, peroxidase, putATive];[AT1G05240, peroxidase, putATive]	0.3	0.6	1.8
223	265031_AT	AT1G61590	protein kinase, putATive	0.9	1.7	1.8
224	254648_AT	AT4G18550	lipase class 3 family protein	0.6	1.1	1.8
225	259481_AT	AT1G18970	GLP4 (GERMIN-LIKE PROTEIN 4); manganese ion binding / nutrient reservoir	2.7	4.8	1.8
226	254820_s_AT	AT4G12510	[AT4G12510, protease inhibitor/seed storage/lipid transfer protein (LTP) family protein];[AT4G12520, protease inhibitor/seed storage/lipid transfer protein (LTP) family protein]	0.3	0.5	1.8
227	260733_AT	AT1G17640	RNA recognition motif (RRM)-containing protein	0.7	1.2	1.8
228	253613_AT	AT4G30320	allergen V5/Tpx-1-relATed family protein	0.3	0.6	1.8
229	255533_AT	AT4G02180	DC1 domain-containing protein	0.3	0.6	1.8
230	262198_AT	AT1G53830	ATPME2; pectinesterase	0.9	1.6	1.8

231	257668_AT	AT3G20460	sugar transporter, putATive	0.6	1.0	1.8
232	248971_AT	AT5G45000	transmembrane receptor	1.0	1.7	1.8
233	249756_AT	AT5G24313	unknown protein	0.5	0.9	1.8
234	260643_AT	AT1G53270	ABC transporter family protein	2.6	4.5	1.8
235	255550_AT	AT4G01970	AtSTS (*Arabidopsis* thaliana stachyose synthase); galactinol-raffinose galactosyltransferase/ hydrolase, hydrolyzing O-glycosyl compounds	0.1	0.2	1.8
236	251349_s_AT	AT3G61020	[AT3G61020, pseudogene, similar to P0034A04.28, several hypothetical proteins - *Arabidopsis* thaliana; blastp mATch of 43% identity and 2.2e-46 P-value to GP|29837187|dbj|BAC75569.1||AP004333 P0034A04.28 {Oryza sATiva (japonica cultivar-group)}];[AT3G11030, unknown protein]	0.7	1.3	1.8
237	256738_AT	AT3G29430	geranylgeranyl pyrophosphATe synthase, putATive / GGPP synthetase, putATive / farnesyltranstransferase, putATive	0.3	0.6	1.8
238	260941_AT	AT1G44970	peroxidase, putATive	0.5	0.9	1.8
239	266356_AT	AT2G32300	UCC1 (UCLACYANIN 1); copper ion binding / electron carrier	0.6	1.0	1.8
240	254125_AT	AT4G24670	TAR2 (TRYPTOPHAN AMINOTRANSFERASE RELATED 2); L-tryptophan2-oxoglutarATe aminotransferase/ L-tryptophanpyruvATe aminotransferase/ carbon-sulfur lyase	0.4	0.7	1.8
241	250682_x_AT	AT5G06630	proline-rich extensin-like family protein	0.4	0.7	1.8
242	264342_AT	AT1G12080	unknown protein	0.4	0.6	1.7
243	263709_AT	AT1G09310	unknown protein	3.1	5.4	1.7
244	254836_AT	AT4G12330	CYP706A7; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	0.8	1.4	1.7
245	264014_AT	AT2G21210	auxin-responsive protein, putATive	0.2	0.4	1.7
246	266571_AT	AT2G23830	vesicle-associATed membrane protein, putATive / VAMP, putATive	0.9	1.5	1.7
247	262530_AT	AT1G17240	AtRLP2 (Receptor Like Protein 2); protein binding / protein kinase	0.7	1.2	1.7
248	267037_AT	AT2G38320	unknown protein	0.5	0.9	1.7
249	248980_AT	AT5G45090	AtPP2-A7 (Phloem protein 2-A7); carbohydrATe binding	1.2	2.1	1.7
250	263284_AT	AT2G36100	integral membrane family protein	0.6	1.0	1.7
251	264794_AT	AT1G08670	epsin N-terminal homology (ENTH) domain-containing protein / clAThrin assembly protein-relATed	0.9	1.6	1.7
252	259493_AT	AT1G15840	unknown protein	0.7	1.2	1.7
253	256352_AT	AT1G54970	ATPRP1 (PROLINE-RICH PROTEIN 1); structural constituent of cell wall	9.2	15.9	1.7
254	265443_AT	AT2G20750	ATEXPB1 (*ARABIDOPSIS* THALIANA EXPANSIN B1)	0.9	1.5	1.7
255	246839_AT	AT5G26720	unknown protein	0.7	1.2	1.7
256	251231_AT	AT3G62760	ATGSTF13; glutAThione transferase	0.2	0.4	1.7
257	252605_s_AT	AT3G45070	[AT3G45070, sulfotransferase family protein];[AT3G45080, sulfotransferase family protein]	0.4	0.7	1.7
258	246807_AT	AT5G27100	ATGLR2.1; intracellular ligand-gATed ion channel	0.8	1.4	1.7
259	259433_AT	AT1G01570	fringe-relATed protein	3.1	5.3	1.7
260	255564_s_AT	AT4G01770	[AT4G01770, RGXT1 (rhamnogalacturonan xylosyltransferase 1); UDP-xylosyltransferase];[AT4G01750, RGXT2 (rhamnogalacturonan xylosyltransferase 2); UDP-xylosyltransferase]	0.7	1.3	1.7
261	253416_AT	AT4G33070	pyruvATe decarboxylase, putATive	1.3	2.2	1.7
262	254912_AT	AT4G11230	respirATory burst oxidase, putATive / NADPH oxidase, putATive	0.8	1.4	1.7
263	249451_s_AT	AT5G39490	[AT5G39490, F-box family protein];[AT5G39480, F-box family protein]	0.7	1.2	1.7

264	267385_AT	AT2G44380	DC1 domain-containing protein	0.6	1.0	1.7
265	251196_AT	AT3G62950	glutaredoxin family protein	0.1	0.2	1.7
266	260139_AT	AT1G66380	MYB114 (myb domain protein 114); DNA binding / transcription factor	0.8	1.3	1.7
267	266100_AT	AT2G37980	unknown protein	0.9	1.5	1.7
268	265897_AT	AT2G25680	MOT1 (molybdATe transporter 1); molybdATe ion transmembrane transporter/ sulfATe transmembrane transporter	0.7	1.1	1.7
269	250664_AT	AT5G07080	transferase family protein	0.6	1.1	1.7
270	246540_AT	AT5G15600	SP1L4 (SPIRAL1-LIKE4)	0.6	1.1	1.7
271	259064_AT	AT3G07490	AGD11 (ARF-GAP domain 11); calcium ion binding	0.4	0.7	1.7
272	260495_AT	AT2G41810	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN endomembrane system; EXPRESSED IN root; CONTAINS InterPro DOMAIN/s Protein of unknown function DUF642 (InterProIPR006946), Galactose-binding like (InterProIPR008979); BEST *Arabidopsis* thaliana protein mATch is unknown protein (TAIRAT2G41800.1); Has 161 Blast hits to 157 proteins in 12 species Archae - 0; Bacteria - 2; Metazoa - 0; Fungi - 0; Plants - 159; Viruses - 0; Other Eukaryotes - 0 (source NCBI BLink).	3.2	5.5	1.7
273	264144_AT	AT1G79320	AtMC6 (metacaspase 6); cysteine-type endopeptidase	1.3	2.2	1.7
274	250059_AT	AT5G17820	peroxidase 57 (PER57) (P57) (PRXR10)	0.5	0.8	1.7
275	249477_s_AT	AT5G38940	[AT5G38940, manganese ion binding / nutrient reservoir];[AT5G38930, germin-like protein, putATive]	0.5	0.9	1.7
276	248750_AT	AT5G47530	auxin-responsive protein, putATive	2.2	3.8	1.7
277	257500_s_AT	AT5G36180	[AT5G36180, scpl1 (serine carboxypeptidase-like 1); serine-type carboxypeptidase];[AT1G73300, scpl2 (serine carboxypeptidase-like 2); serine-type carboxypeptidase]	0.5	0.9	1.7
278	253347_AT	AT4G33610	glycine-rich protein	1.2	2.1	1.7
279	265588_AT	AT2G19970	pAThogenesis-relATed protein, putATive	0.5	0.8	1.7
280	246855_AT	AT5G26280	meprin and TRAF homology domain-containing protein / MATH domain-containing protein	0.6	1.1	1.7
281	246854_AT	AT5G26200	mitochondrial substrATe carrier family protein	0.4	0.6	1.7
282	266162_AT	no_mATch	no_mATch	1.1	1.9	1.7
283	247831_AT	AT5G58540	protein kinase family protein	0.4	0.7	1.7
284	261464_AT	AT1G07740	[AT1G07740, pentATricopeptide (PPR) repeAT-containing protein];[AT1G07730, disease resistance-responsive family protein]	0.3	0.5	1.7
285	263876_AT	AT2G21880	ATRAB7A; GTP binding	0.4	0.7	1.7
286	260123_AT	AT1G33890	avirulence-responsive protein, putATive / avirulence induced gene protein, putATive / AIG protein, putATive	0.7	1.1	1.7
287	260035_AT	AT1G68850	peroxidase, putATive	2.5	4.2	1.7
288	259842_AT	AT1G73602	[AT1G73602, CPuORF32 (Conserved peptide upstream open reading frame 32)];[AT1G73600, methyltransferase/ phosphoethanolamine N-methyltransferase]	0.7	1.1	1.7
289	249718_AT	AT5G35740	glycosyl hydrolase family protein 17	0.4	0.6	1.7
290	263290_AT	AT2G10930	unknown protein	0.6	1.1	1.7
291	253244_AT	AT4G34580	COW1 (CAN OF WORMS1); phosphATidylinositol transporter/ transporter	0.7	1.1	1.7
292	249279_AT	AT5G41920	scarecrow transcription factor family protein	0.7	1.2	1.7
293	259413_AT	AT1G02320	unknown protein	0.8	1.3	1.7
294	247991_AT	AT5G56320	ATEXPA14 (*ARABIDOPSIS* THALIANA EXPANSIN A14)	0.8	1.3	1.7
295	262935_AT	AT1G79410	AtOCT5 (*Arabidopsis* thaliana ORGANIC CATION/CARNITINE TRANSPORTER5);	0.9	1.6	1.7

			carbohydrATe transmembrane transporter/ sugarhydrogen symporter			
296	258143_AT	AT3G18170	transferase, transferring glycosyl groups	0.5	0.9	1.7
297	265483_AT	AT2G15790	SQN (SQUINT); peptidyl-prolyl cis-trans isomerase	1.0	1.7	1.7
298	255903_AT	AT1G17950	MYB52 (MYB DOMAIN PROTEIN 52); DNA binding / transcription factor	1.3	2.1	1.7
299	253432_AT	AT4G32450	pentATricopeptide (PPR) repeAT-containing protein	0.6	1.0	1.7
300	250640_AT	AT5G07150	leucine-rich repeAT family protein	0.5	0.8	1.7

Appendix III: **Top 300 genes list rank where C+/C- > P+/P-**

Rank	Affymetrix ID	TAIR ID	Description	Response C+/C-	Response P+/P-	Fold change
1	250207_AT	AT5G13930	TT4 (TRANSPARENT TESTA 4); naringenin-chalcone synthase	158.4	8.4	18.9
2	260706_AT	AT1G32350	AOX1D (alternATive oxidase 1D); alternATive oxidase	25.2	1.5	17.3
3	250296_AT	AT5G12020	HSP17.6II (17.6 KDA CLASS II HEAT SHOCK PROTEIN)	35.9	2.3	15.3
4	248062_AT	AT5G55450	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	11.7	1.0	12.2
5	247492_AT	AT5G61890	AP2 domain-containing transcription factor family protein	20.3	1.7	11.7
6	260978_AT	AT1G53540	17.6 kDa class I small heAT shock protein (HSP17.6C-CI) (AA 1-156)	12.5	1.1	11.1
7	257917_AT	AT3G23220	DNA binding / transcription factor	46.4	4.6	10.2
8	254283_s_AT	AT4G22870	[AT4G22870, leucoanthocyanidin dioxygenase, putATive / anthocyanidin synthase, putATive	50.0	5.4	9.3
9	252739_AT	AT3G43250	cell cycle control protein-relATed	10.1	1.1	9.1
10	251625_AT	AT3G57260	BGL2 (BETA-1,3-GLUCANASE 2); cellulase/ glucan 1,3-beta-glucosidase/ hydrolase, hydrolyzing O-glycosyl	12.8	1.6	7.8
11	248676_AT	AT5G48850	ATSDI1 (SULPHUR DEFICIENCY-INDUCED 1); binding	12.2	1.7	7.4
12	266455_AT	AT2G22760	basic helix-loop-helix (bHLH) family protein	40.2	5.5	7.4
13	260581_AT	AT2G47190	MYB2 (MYB DOMAIN PROTEIN 2); DNA binding / calmodulin binding / transcription activATor/	28.1	3.8	7.3
14	264718_AT	AT1G70130	lectin protein kinase, putATive	78.5	11.0	7.1
15	260551_AT	AT2G43510	ATTI1; serine-type endopeptidase inhibitor	6.0	0.9	7.0
16	245531_AT	AT4G15100	scpl30 (serine carboxypeptidase-like 30); serine-type carboxypeptidase	77.3	11.1	7.0
17	247026_AT	AT5G67080	MAPKKK19; ATP binding / kinase/ protein kinase/ protein serine/threonine kinase	15.0	2.2	6.8
18	261394_AT	AT1G79680	wall-associATed kinase, putATive	8.7	1.3	6.8
19	262911_s_AT	AT1G59860	[AT1G59860, 17.6 kDa class I heAT shock protein (HSP17.6A-CI)];[AT1G07400, 17.8 kDa class I heAT shock protein (HSP17.8-CI)]	12.6	1.9	6.7
20	259161_AT	AT3G01500	CA1 (CARBONIC ANHYDRASE 1); carbonATe dehydrATase/ zinc ion binding	13.0	2.0	6.7
21	259743_AT	AT1G71140	MATE efflux family protein	7.7	1.2	6.6
22	250083_AT	AT5G17220	ATGSTF12 (*ARABIDOPSIS* THALIANA GLUTATHIONE S-TRANSFERASE PHI 12); glutAThione transferase	26.9	4.1	6.5
23	248896_AT	AT5G46350	WRKY8; transcription factor	12.8	2.0	6.5
24	250445_AT	AT5G10760	aspartyl protease family protein	9.9	1.6	6.3
25	252265_AT	AT3G49620	DIN11 (DARK INDUCIBLE 11); iron ion binding / oxidoreductase	10.5	1.7	6.3
26	249312_AT	AT5G41550	disease resistance protein (TIR-NBS-LRR class), putATive	18.1	2.9	6.2
27	249743_AT	AT5G24540	BGLU31 (BETA GLUCOSIDASE 31); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	13.1	2.2	6.0
28	261763_AT	AT1G15520	PDR12 (PLEIOTROPIC DRUG RESISTANCE 12); ATPase, coupled to transmembrane movement of substances	4.6	0.8	5.9
29	254255_AT	AT4G23220	kinase	12.5	2.1	5.9
30	258063_AT	AT3G14620	CYP72A8; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	12.6	2.1	5.9
31	246293_AT	AT3G56710	SIB1 (SIGMA FACTOR BINDING PROTEIN 1); binding / protein binding	19.6	3.4	5.8
32	260203_AT	AT1G52890	ANAC019 (*Arabidopsis* NAC domain containing protein 19); transcription factor	66.9	11.9	5.6
33	251770_AT	AT3G55970	oxidoreductase, 2OG-Fe(II) oxygenase family protein	45.2	8.1	5.6
34	259925_AT	AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	5.0	0.9	5.5
35	252515_AT	AT3G46230	ATHSP17.4	17.1	3.1	5.5
36	262092_AT	AT1G56150	auxin-responsive family protein	7.2	1.4	5.3
37	254042_AT	AT4G25810	XTR6 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6);	3.5	0.7	5.2

			hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucanxyloglucosyl transferase			
38	249333_AT	AT5G40990	GLIP1 (GDSL LIPASE1); carboxylesterase/ lipase	9.0	1.8	5.1
39	260015_AT	AT1G67980	CCoAMT; caffeoyl-CoA O-methyltransferase	35.7	7.0	5.1
40	261037_AT	AT1G17420	LOX3; electron carrier/ iron ion binding / lipoxygenase/ metal ion binding / oxidoreductase, acting on single donors with incorporATion of molecular oxygen, incorporATion of two AToms of oxygen	40.8	8.0	5.1
41	252123_AT	AT3G51240	F3H (FLAVANONE 3-HYDROXYLASE); naringenin 3-dioxygenase	74.7	15.2	4.9
42	249940_AT	AT5G22380	anac090 (*Arabidopsis* NAC domain containing protein 90); transcription factor	5.3	1.1	4.9
43	251428_AT	AT3G60140	DIN2 (DARK INDUCIBLE 2); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	1.6	0.3	4.9
44	248317_AT	AT5G52680	heavy-metal-associATed domain-containing protein	21.2	4.3	4.9
45	261033_AT	AT1G17380	JAZ5 (JASMONATE-ZIM-DOMAIN PROTEIN 5)	21.3	4.5	4.7
46	266299_AT	AT2G29450	ATGSTU5 (*ARABIDOPSIS* THALIANA GLUTATHIONE S-TRANSFERASE TAU 5); glutAThione binding / glutAThione transferase	28.2	6.0	4.7
47	263539_AT	AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3); L-tyrosine2-oxoglutarATe aminotransferase/ transaminase	525.5	111.8	4.7
48	251166_AT	AT3G63350	AT-HSFA7B; DNA binding / transcription factor	12.8	2.8	4.5
49	264646_AT	AT1G08860	BON3 (BONZAI 3); calcium-dependent phospholipid binding	11.1	2.5	4.5
50	255937_AT	AT1G12610	DDF1 (DWARF AND DELAYED FLOWERING 1); DNA binding / sequence-specific DNA binding / transcription factor	17.7	4.0	4.5
51	260278_AT	AT1G80590	WRKY66; transcription factor	4.5	1.0	4.4
52	262211_AT	AT1G74930	ORA47; DNA binding / transcription factor	45.9	10.4	4.4
53	247290_AT	AT5G64450	unknown protein	7.2	1.6	4.4
54	249744_AT	AT5G24550	BGLU32 (BETA GLUCOSIDASE 32); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	37.3	8.7	4.3
55	246620_AT	AT5G36220	CYP81D1 (CYTOCHROME P450 81D1); electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	15.8	3.7	4.3
56	262126_AT	AT1G59620	CW9; ATP binding	6.2	1.5	4.2
57	261922_AT	AT1G65890	AAE12 (ACYL ACTIVATING ENZYME 12); cATalytic	4.5	1.1	4.2
58	266821_AT	AT2G44840	ERF13 (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13); DNA binding / transcription factor	287.0	70.5	4.1
59	260848_AT	AT1G21850	sks8 (SKU5 Similar 8); copper ion binding / oxidoreductase	6.1	1.5	4.1
60	250558_AT	AT5G07990	TT7 (TRANSPARENT TESTA 7); flavonoid 3'-monooxygenase/ oxygen binding	8.2	2.1	4.0
61	255298_AT	AT4G04840	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	5.1	1.3	4.0
62	251482_s_AT	AT3G59750	[AT3G59750, receptor lectin kinase, putATive];[AT3G59740, receptor lectin kinase 3 (lecRK3)]	8.9	2.2	4.0
63	254869_AT	AT4G11890	protein kinase family protein	12.2	3.1	4.0
64	254062_AT	AT4G25380	zinc finger (AN1-like) family protein	9.8	2.5	3.9
65	257641_s_AT	AT3G25760	[AT3G25760, AOC1 (ALLENE OXIDE CYCLASE 1); allene-oxide cyclase];[AT3G25770, AOC2 (ALLENE OXIDE CYCLASE 2); allene-oxide cyclase]	22.7	5.8	3.9
66	253070_AT	AT4G37850	basic helix-loop-helix (bHLH) family protein	13.2	3.4	3.9
67	250199_AT	AT5G14180	MPL1 (MYZUS PERSICAE-INDUCED LIPASE 1); cATalytic	2.1	0.5	3.9
68	254158_AT	AT4G24380	unknown protein	50.3	13.1	3.9
69	245528_AT	AT4G15530	PPDK (pyruvATe orthophosphATe dikinase); kinase/ pyruvATe, phosphATe dikinase	2.6	0.7	3.8
70	245173_AT	AT2G47520	AP2 domain-containing transcription factor, putATive	10.9	2.9	3.8

71	248775_AT	AT5G47850	CCR4 (*ARABIDOPSIS* THALIANA CRINKLY4 RELATED 4); kinase	7.1	1.9	3.8
72	249197_AT	AT5G42380	CML37 (CALMODULIN LIKE 37); calcium ion binding	223.5	59.4	3.8
73	250351_AT	AT5G12030	AT-HSP17.6A (*ARABIDOPSIS* THALIANA HEAT SHOCK PROTEIN 17.6A); unfolded protein binding	16.8	4.5	3.7
74	252136_AT	AT3G50770	calmodulin-relATed protein, putATive	17.1	4.6	3.7
75	261215_AT	AT1G32970	subtilase family protein	118.8	32.1	3.7
76	264217_AT	AT1G60190	armadillo/beta-cATenin repeAT family protein / U-box domain-containing protein	42.3	11.5	3.7
77	261216_AT	AT1G33030	O-methyltransferase family 2 protein	6.2	1.7	3.6
78	257918_AT	AT3G23230	ethylene-responsive factor, putATive	99.7	27.7	3.6
79	248160_AT	AT5G54470	zinc finger (B-box type) family protein	16.6	4.6	3.6
80	261443_AT	AT1G28480	GRX480; electron carrier/ protein disulfide oxidoreductase	65.6	18.4	3.6
81	255599_AT	AT4G01010	ATCNGC13; calmodulin binding / cyclic nucleotide binding / ion channel	9.8	2.7	3.6
82	252269_AT	AT3G49580	LSU1 (RESPONSE TO LOW SULFUR 1)	4.2	1.2	3.6
83	256159_AT	AT1G30135	JAZ8 (JASMONATE-ZIM-DOMAIN PROTEIN 8)	37.1	10.4	3.6
84	257927_AT	AT3G23240	ERF1 (ETHYLENE RESPONSE FACTOR 1); DNA binding / transcription activATor/ transcription factor	93.4	26.3	3.5
85	267607_s_AT	AT2G26740	[AT2G26740, ATSEH (*Arabidopsis* thaliana soluble epoxide hydrolase); epoxide hydrolase];[AT2G26750, epoxide hydrolase, putATive]	4.1	1.2	3.5
86	266658_AT	AT2G25735	unknown protein	9.4	2.6	3.5
87	255879_AT	AT1G67000	ATP binding / kinase/ protein kinase/ protein serine/threonine kinase	5.6	1.6	3.5
88	248794_AT	AT5G47220	ERF2 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2); DNA binding / transcription activATor/ transcription factor	44.0	12.5	3.5
89	253181_AT	AT4G35180	LHT7 (Lys/His transporter 7); amino acid transmembrane transporter	8.0	2.3	3.5
90	266486_AT	AT2G47950	unknown protein	3.0	0.9	3.5
91	245613_AT	no_mATch	no_mATch	24.4	7.0	3.5
92	245041_AT	AT2G26530	AR781	43.0	12.5	3.4
93	265632_AT	AT2G14290	F-box family protein	22.3	6.5	3.4
94	247754_AT	AT5G59080	unknown protein	0.2	0.0	3.4
95	253259_AT	AT4G34410	RRTF1 ({REDOX RESPONSIVE TRANSCRIPTION FACTOR 1); DNA binding / transcription factor	103.2	30.1	3.4
96	257540_AT	AT3G21520	unknown protein	13.0	3.8	3.4
97	256009_AT	AT1G19210	AP2 domain-containing transcription factor, putATive	9.0	2.7	3.4
98	250798_AT	AT5G05340	peroxidase, putATive	7.6	2.3	3.4
99	251603_AT	AT3G57760	protein kinase family protein	5.0	1.5	3.4
100	261242_AT	AT1G32960	SBT3.3; identical protein binding / serine-type endopeptidase	3.3	1.0	3.4
101	247360_AT	AT5G63450	CYP94B1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	18.1	5.4	3.4
102	247925_AT	AT5G57560	TCH4 (Touch 4); hydrolase, acting on glycosyl bonds / xyloglucanxyloglucosyl transferase	15.0	4.5	3.4
103	250292_AT	AT5G13220	JAZ10 (JASMONATE-ZIM-DOMAIN PROTEIN 10)	112.5	33.7	3.3
104	247431_AT	AT5G62520	SRO5 (SIMILAR TO RCD ONE 5); NAD+ ADP-ribosyltransferase	5.0	1.5	3.3
105	259640_AT	AT1G52400	BGLU18 (BETA GLUCOSIDASE 18); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	6.1	1.8	3.3
106	266270_AT	AT2G29470	ATGSTU3 (*ARABIDOPSIS* THALIANA GLUTATHIONE S-TRANSFERASE TAU 3); glutAThione transferase	3.5	1.0	3.3
107	256991_AT	AT3G28600	ATP binding / ATPase/ nucleoside-triphosphATase/ nucleotide binding	20.8	6.3	3.3
108	247789_AT	AT5G58680	armadillo/beta-cATenin repeAT family protein	10.3	3.1	3.3
109	253060_AT	AT4G37710	VQ motif-containing protein	286.5	87.8	3.3

110	258975_AT	AT3G01970	WRKY45; transcription factor	2.9	0.9	3.3
111	265091_s_AT	AT1G03940	[AT1G03940, transferase family protein];[AT1G03495, transferase/ transferase, transferring acyl groups other than amino-acyl groups]	28.2	8.7	3.3
112	257919_AT	AT3G23250	MYB15 (MYB DOMAIN PROTEIN 15); DNA binding / transcription factor	19.2	5.9	3.3
113	264758_AT	AT1G61340	F-box family protein	27.2	8.4	3.2
114	248185_AT	AT5G54060	UF3GT (udp-glucoseflavonoid 3-O-glucosyltransferase); transferase, transferring glycosyl groups	19.2	6.0	3.2
115	251456_AT	AT3G60120	BGLU27 (BETA GLUCOSIDASE 27); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	34.6	10.8	3.2
116	262325_AT	AT1G64160	disease resistance-responsive family protein / dirigent family protein	10.5	3.3	3.2
117	252648_AT	AT3G44630	disease resistance protein RPP1-WsB-like (TIR-NBS-LRR class), putATive	14.5	4.6	3.2
118	256526_AT	AT1G66090	disease resistance protein (TIR-NBS class), putATive	56.5	17.8	3.2
119	247655_AT	AT5G59820	RHL41 (RESPONSIVE TO HIGH LIGHT 41); nucleic acid binding / transcription factor/ zinc ion binding	20.1	6.3	3.2
120	252679_AT	AT3G44260	CCR4-NOT transcription complex protein, putATive	15.5	4.9	3.2
121	247848_AT	AT5G58120	disease resistance protein (TIR-NBS-LRR class), putATive	8.2	2.6	3.2
122	246406_AT	AT1G57650	INVOLVED IN defense response; LOCATED IN cellular_component unknown; EXPRESSED IN stamen;	8.4	2.7	3.1
123	253859_AT	AT4G27657	unknown protein	4.6	1.5	3.1
124	249747_AT	AT5G24600	unknown protein	28.3	9.1	3.1
125	251774_AT	AT3G55840	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; EXPRESSED IN 10 plant structures	14.0	4.5	3.1
126	251827_AT	AT3G55120	TT5 (TRANSPARENT TESTA 5); chalcone isomerase	10.1	3.3	3.1
127	253066_AT	AT4G37770	ACS8; 1-aminocyclopropane-1-carboxylATe synthase	17.1	5.5	3.1
128	256430_AT	AT3G11020	DREB2B (DRE/CRT-BINDING PROTEIN 2B); DNA binding / transcription activATor/ transcription factor	5.5	1.8	3.1
129	249971_AT	AT5G19110	extracellular dermal glycoprotein-relATed / EDGP-relATed	14.3	4.6	3.1
130	245033_AT	AT2G26380	disease resistance protein-relATed / LRR protein-relATed	132.1	43.0	3.1
131	249889_AT	AT5G22540	unknown protein	6.3	2.1	3.1
132	263783_AT	AT2G46400	WRKY46; transcription factor	47.5	15.6	3.1
133	261558_AT	AT1G01770	unknown protein	1.0	0.3	3.0
134	253724_AT	AT4G29285	LCR24 (Low-molecular-weight cysteine-rich 24)	5.3	1.7	3.0
135	255900_AT	AT1G17830	unknown protein	9.0	3.0	3.0
136	259866_AT	AT1G76640	calmodulin-relATed protein, putATive	37.6	12.5	3.0
137	245624_AT	AT4G14090	UDP-glucoronosyl/UDP-glucosyl transferase family protein	24.5	8.2	3.0
138	267140_AT	AT2G38250	DNA-binding protein-relATed	3.7	1.2	3.0
139	265030_AT	AT1G61610	S-locus lectin protein kinase family protein	3.9	1.3	3.0
140	267147_AT	AT2G38240	oxidoreductase, 2OG-Fe(II) oxygenase family protein	43.1	14.5	3.0
141	258606_AT	AT3G02840	immediATe-early fungal elicitor family protein	45.0	15.2	3.0
142	262229_AT	AT1G68620	hydrolase	16.4	5.5	3.0
143	245953_AT	AT5G28520	unknown	13.8	4.7	2.9
144	257386_AT	AT2G42440	unknown; BEST *Arabidopsis* thaliana protein mATch is LBD29 (LATERAL ORGAN BOUNDARIES-DOMAIN 29) (TAIRAT3G58190.1);	4.2	1.4	2.9
145	250415_AT	AT5G11210	ATGLR2.5; intracellular ligand-gATed ion channel	10.6	3.6	2.9
146	257840_AT	AT3G25250	AGC2-1 (OXIDATIVE SIGNAL-INDUCIBLE1); kinase	76.9	26.2	2.9

147	260541_AT	AT2G43530	trypsin inhibitor, putATive	2.7	0.9	2.9
148	257206_AT	AT3G16530	legume lectin family protein	3.4	1.2	2.9
149	266878_AT	no_mATch	no_mATch	15.2	5.2	2.9
150	253643_AT	AT4G29780	unknown protein	34.5	11.9	2.9
151	252989_AT	AT4G38420	sks9 (SKU5 Similar 9); copper ion binding / oxidoreductase	10.8	3.7	2.9
152	265569_AT	AT2G05620	PGR5 (proton gradient regulATion 5); electron carrier	3.0	1.0	2.9
153	266267_AT	AT2G29460	ATGSTU4 (*ARABIDOPSIS* THALIANA GLUTATHIONE S-TRANSFERASE TAU 4); glutAThione transferase	24.0	8.3	2.9
154	261892_AT	AT1G80840	WRKY40; transcription factor	52.1	18.1	2.9
155	265327_AT	AT2G18210	unknown protein	11.2	3.9	2.9
156	254265_s_AT	AT4G23140	[AT4G23140, CRK6 (CYSTEINE-RICH RLK 6); kinase];[AT4G23160, protein kinase family protein]	3.0	1.0	2.9
157	248618_AT	AT5G49620	AtMYB78 (myb domain protein 78); DNA binding / transcription factor	2.6	0.9	2.9
158	253477_AT	AT4G32320	APX6; L-ascorbATe peroxidase/ heme binding / peroxidase	2.3	0.8	2.9
159	247543_AT	AT5G61600	ethylene-responsive element-binding family protein	2.7	0.9	2.9
160	263378_AT	AT2G40180	ATHPP2C5; cATalytic/ protein serine/threonine phosphATase	9.7	3.4	2.8
161	250455_AT	AT5G09980	PROPEP4 (Elicitor peptide 4 precursor)	13.2	4.7	2.8
162	261957_AT	AT1G64660	ATMGL (*ARABIDOPSIS* THALIANA METHIONINE GAMMA-LYASE); cATalytic/ methionine gamma-lyase	1.6	0.6	2.8
163	250781_AT	AT5G05410	DREB2A; DNA binding / transcription activATor/ transcription factor	7.4	2.6	2.8
164	248789_AT	AT5G47440	phosphoinositide binding	16.3	5.8	2.8
165	266971_AT	AT2G39580	unknown protein	1.3	0.5	2.8
166	247691_AT	AT5G59720	HSP18.2 (heAT shock protein 18.2)	2.4	0.9	2.8
167	265674_AT	no_mATch	no_mATch	40.4	14.5	2.8
168	252070_AT	AT3G51680	short-chain dehydrogenase/reductase (SDR) family protein	17.5	6.3	2.8
169	250794_AT	AT5G05270	chalcone-flavanone isomerase family protein	4.2	1.5	2.8
170	262047_AT	AT1G80160	lactoylglutAThione lyase family protein / glyoxalase I family protein	1.3	0.5	2.8
171	247070_AT	AT5G66815	unknown protein	2.2	0.8	2.7
172	246099_AT	AT5G20230	ATBCB (*ARABIDOPSIS* BLUE-COPPER-BINDING PROTEIN); copper ion binding / electron carrier	37.4	13.6	2.7
173	266294_AT	AT2G29500	17.6 kDa class I small heAT shock protein (HSP17.6B-CI)	10.2	3.7	2.7
174	253044_AT	AT4G37290	unknown protein	18.4	6.7	2.7
175	261785_AT	AT1G08230	amino acid transporter family protein	2.4	0.9	2.7
176	258377_AT	AT3G17690	ATCNGC19; calmodulin binding / cyclic nucleotide binding / ion channel	36.0	13.2	2.7
177	266097_AT	AT2G37970	SOUL-1; binding	3.4	1.3	2.7
178	245755_AT	AT1G35210	unknown protein	45.8	16.8	2.7
179	262213_AT	AT1G74870	protein binding / zinc ion binding	9.4	3.4	2.7
180	264659_AT	AT1G09930	ATOPT2; oligopeptide transporter	21.1	7.8	2.7
181	262580_AT	AT1G15330	CBS domain-containing protein	1.7	0.6	2.7
182	260856_AT	AT1G21910	AP2 domain-containing transcription factor family protein	9.6	3.6	2.7
183	258516_AT	AT3G06490	MYB108 (myb domain protein 108); DNA binding / transcription factor	82.7	30.5	2.7
184	248625_AT	AT5G48880	PKT2 (PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 2); acetyl-CoA C-acyltransferase/ cATalytic	4.7	1.7	2.7
185	248606_AT	AT5G49450	[AT5G49450, AtbZIP1 (*Arabidopsis* thaliana basic leucine-zipper 1); DNA binding / protein heterodimerizATion/ transcription factor];[AT5G49448, CPuORF4 (Conserved peptide upstream open reading frame 4)]	3.6	1.3	2.7

186	259694_AT	AT1G63180	UGE3 (UDP-D-glucose/UDP-D-galactose 4-epimerase 3); UDP-glucose 4-epimerase/ protein dimerizATion	1.4	0.5	2.7
187	247327_AT	AT5G64120	peroxidase, putATive	8.2	3.1	2.7
188	246349_AT	AT1G51915	cryptdin protein-relATed	6.8	2.5	2.7
189	265482_AT	AT2G15780	glycine-rich protein	19.7	7.4	2.7
190	252131_AT	AT3G50930	BCS1 (CYTOCHROME BC1 SYNTHESIS); ATP binding / ATPase/ nucleoside-triphosphATase/ nucleotide binding	37.8	14.2	2.7
191	255884_AT	AT1G20310	unknown protein	53.5	20.1	2.7
192	253316_s_AT	AT4G34300	[AT4G34300, glycine-rich protein];[AT4G33930, glycine-rich protein]	4.6	1.7	2.7
193	256589_AT	AT3G28740	CYP81D1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	13.6	5.2	2.6
194	257763_s_AT	AT3G23120	[AT3G23120, AtRLP38 (Receptor Like Protein 38); kinase/ protein binding];[AT3G23110, AtRLP37 (Receptor Like Protein 37); kinase/ protein binding]	4.2	1.6	2.6
195	254074_AT	AT4G25490	CBF1 (C-REPEAT/DRE BINDING FACTOR 1); DNA binding / transcription activATor/ transcription factor	10.3	3.9	2.6
196	266800_AT	AT2G22880	VQ motif-containing protein	27.8	10.5	2.6
197	250187_AT	AT5G14370	LOCATED IN chloroplast; EXPRESSED IN 21 plant structures; EXPRESSED DURING 13 growth stages; CONTAINS InterPro DOMAIN/s CCT domain (InterProIPR010402); BEST *Arabidopsis* thaliana protein mATch is CIL (TAIRAT4G25990.1);	2.2	0.8	2.6
198	261053_AT	AT1G01320	tetrATricopeptide repeAT (TPR)-containing protein	1.3	0.5	2.6
199	248358_AT	AT5G52400	CYP715A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	110.2	42.0	2.6
200	248448_AT	AT5G51190	AP2 domain-containing transcription factor, putATive	9.1	3.5	2.6
201	256093_AT	AT1G20823	zinc finger (C3HC4-type RING finger) family protein	9.3	3.5	2.6
202	264202_AT	AT1G22810	AP2 domain-containing transcription factor, putATive	100.8	38.4	2.6
203	265983_AT	AT2G18550	HB-2 (HOMEOBOX-2); DNA binding / transcription factor	6.4	2.5	2.6
204	266720_s_AT	AT2G46790	[AT2G46790, APRR9 (*ARABIDOPSIS* PSEUDO-RESPONSE REGULATOR 9); protein binding / transcription regulATor/ two-component response regulATor];[AT2G46670, pseudo-response regulATor, putATive / timing of CAB expression 1-like protein, putATive]	7.9	3.0	2.6
205	250670_AT	AT5G06860	PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding	18.4	7.0	2.6
206	256128_AT	AT1G18140	LAC1 (Laccase 1); laccase	7.5	2.9	2.6
207	254905_AT	AT4G11170	disease resistance protein (TIR-NBS-LRR class), putATive	20.3	7.8	2.6
208	262383_AT	AT1G72940	disease resistance protein (TIR-NBS class), putATive	10.2	3.9	2.6
209	264761_AT	AT1G61280	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; EXPRESSED IN 17 plant structures; EXPRESSED DURING 9 growth stages; CONTAINS InterPro DOMAIN/s PIG-P (InterProIPR013717), PhosphATidylinositol N-acetylglucosaminyltransferase, GPI19/PIG-P subunit (InterProIPR016542); BEST *Arabidopsis* thaliana protein mATch is unknown protein (TAIRAT2G39445.1);	3.1	1.2	2.6
210	247913_AT	AT5G57510	unknown protein	57.3	22.1	2.6
211	264929_AT	AT1G60730	aldo/keto reductase family protein	4.2	1.6	2.6
212	247052_AT	AT5G66700	HB53; DNA binding / transcription factor	6.1	2.4	2.6
213	249773_AT	AT5G24140	SQP2; FAD binding / oxidoreductase/ squalene monooxygenase	3.9	1.5	2.6
214	256994_s_AT	AT3G25830	[AT3G25830, ATTPS-CIN (terpene synthase-like sequence-1,8-cineole); (E)-beta-ocimene synthase/ myrcene synthase];[AT3G25820, ATTPS-CIN (terpene synthase-like sequence-1,8-cineole); (E)-beta-ocimene synthase/ myrcene synthase]	3.2	1.2	2.6

215	254926_AT	AT4G11280	ACS6 (1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6); 1-aminocyclopropane-1-carboxylATe synthase	17.7	6.9	2.6
216	251023_AT	AT5G02170	amino acid transporter family protein	13.2	5.1	2.6
217	264618_AT	AT2G17680	unknown protein	11.5	4.5	2.6
218	254014_AT	AT4G26120	ankyrin repeAT family protein / BTB/POZ domain-containing protein	6.1	2.4	2.6
219	248286_AT	AT5G52870	unknown protein	4.4	1.7	2.6
220	249215_AT	AT5G42800	DFR (DIHYDROFLAVONOL 4-REDUCTASE); dihydrokaempferol 4-reductase	18.8	7.3	2.6
221	245540_AT	AT4G15230	PDR2 (PLEIOTROPIC DRUG RESISTANCE 2); ATPase, coupled to transmembrane movement of substances	5.1	2.0	2.6
222	254396_AT	AT4G21680	proton-dependent oligopeptide transport (POT) family protein	3.5	1.4	2.5
223	250435_AT	AT5G10380	RING1; protein binding / ubiquitin-protein ligase/ zinc ion binding	7.1	2.8	2.5
224	249558_AT	AT5G38310	unknown protein	4.1	1.6	2.5
225	253666_AT	AT4G30270	MERI5B (meristem-5); hydrolase, acting on glycosyl bonds / xyloglucanxyloglucosyl transferase	0.6	0.3	2.5
226	259293_AT	AT3G11580	DNA-binding protein, putATive	6.7	2.6	2.5
227	245627_AT	AT1G56600	AtGolS2 (*Arabidopsis* thaliana galactinol synthase 2); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups	32.1	12.8	2.5
228	255340_AT	AT4G04490	protein kinase family protein	3.1	1.2	2.5
229	249752_AT	AT5G24660	LSU2 (RESPONSE TO LOW SULFUR 2)	5.8	2.3	2.5
230	265501_AT	AT2G15490	UGT73B4 (UDP-GLYCOSYLTRANSFERASE 73B4); UDP-glucosyltransferase/ UDP-glycosyltransferase/ quercetin 3-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase/ transferase, transferring glycosyl groups	6.1	2.4	2.5
231	263150_AT	AT1G54050	17.4 kDa class III heAT shock protein (HSP17.4-CIII)	3.3	1.3	2.5
232	265723_AT	AT2G32140	transmembrane receptor	64.4	25.8	2.5
233	265204_AT	AT2G36650	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; EXPRESSED IN sperm cell, hypocotyl, flower, root; EXPRESSED DURING petal differentiATion and expansion stage; BEST *Arabidopsis* thaliana protein mATch is CHUP1 (CHLOROPLAST UNUSUAL POSITIONING 1) (TAIRAT3G25690.1	6.3	2.5	2.5
234	260405_AT	AT1G69930	ATGSTU11 (GLUTATHIONE S-TRANSFERASE TAU 11); glutAThione transferase	33.0	13.3	2.5
235	260205_AT	AT1G70700	TIFY7	6.5	2.6	2.5
236	255250_AT	AT4G05100	AtMYB74 (myb domain protein 74); DNA binding / transcription factor	7.3	3.0	2.5
237	259410_AT	AT1G13340	unknown protein	3.8	1.5	2.5
238	252408_AT	AT3G47600	ATMYB94 (MYB DOMAIN PROTEIN 94); DNA binding / transcription factor	3.3	1.3	2.5
239	262259_s_AT	AT1G53890	[AT1G53890, unknown protein];[AT1G53870, unknown protein]	3.9	1.6	2.5
240	253046_AT	AT4G37370	CYP81D8; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	32.0	13.0	2.5
241	256356_s_AT	AT5G43620	[AT5G43620, S-locus protein-relATed];[AT1G66500, zinc finger (C2H2-type) family protein]	10.0	4.1	2.5
242	260408_AT	AT1G69880	ATH8 (thioredoxin H-type 8)	0.5	0.2	2.4
243	263584_AT	AT2G17040	anac036 (*Arabidopsis* NAC domain containing protein 36); transcription factor	19.5	8.0	2.4
244	262012_s_AT	AT1G35625	[AT1G35625, peptidase/ protein binding / zinc ion binding];[AT1G35630, protease-associATed zinc finger (C3HC4-type RING finger) family protein]	2.2	0.9	2.4
245	250622_AT	AT5G07310	AP2 domain-containing transcription factor, putATive	2.9	1.2	2.4
246	260399_AT	AT1G72520	lipoxygenase, putATive	184.1	75.7	2.4
247	255543_AT	AT4G01870	tolB protein-relATed	12.8	5.3	2.4
248	257536_AT	AT3G02800	phosphATase/ phosphoprotein phosphATase/ protein tyrosine phosphATase	11.4	4.7	2.4

249	251488_AT	AT3G59440	calcium-binding protein, putATive	2.2	0.9	2.4
250	259134_AT	AT3G05390	unknown protein	4.6	1.9	2.4
251	262148_AT	AT1G52560	26.5 kDa class I small heAT shock protein-like (HSP26.5-P)	11.1	4.6	2.4
252	252557_AT	AT3G45960	ATEXLA3 (*Arabidopsis* thaliana expansin-like a3)	3.9	1.6	2.4
253	246884_AT	AT5G26220	ChaC-like family protein	11.8	4.9	2.4
254	258277_AT	AT3G26830	PAD3 (PHYTOALEXIN DEFICIENT 3); dihydrocamalexic acid decarboxylase/ monooxygenase/ oxygen binding	5.0	2.1	2.4
255	249094_AT	AT5G43890	YUCCA5; monooxygenase	35.7	14.8	2.4
256	259561_AT	AT1G21250	WAK1 (CELL WALL-ASSOCIATED KINASE); kinase	2.0	0.8	2.4
257	266910_AT	AT2G45920	U-box domain-containing protein	3.2	1.3	2.4
258	251039_AT	AT5G02020	unknown protein	5.2	2.2	2.4
259	259473_AT	AT1G19025	DNA cross-link repair protein-relATed	4.5	1.9	2.4
260	260429_AT	AT1G72450	JAZ6 (JASMONATE-ZIM-DOMAIN PROTEIN 6)	4.5	1.9	2.4
261	252193_AT	AT3G50060	MYB77; DNA binding / transcription factor	10.8	4.5	2.4
262	261470_AT	AT1G28370	ERF11 (ERF DOMAIN PROTEIN 11); DNA binding / transcription factor/ transcription repressor	27.1	11.3	2.4
263	259550_AT	AT1G35230	AGP5 (ARABINOGALACTAN-PROTEIN 5)	2.8	1.2	2.4
264	254229_AT	AT4G23610	unknown protein	5.9	2.5	2.4
265	267573_AT	AT2G30670	tropinone reductase, putATive / tropine dehydrogenase, putATive	2.2	0.9	2.4
266	264415_AT	AT1G43160	RAP2.6 (relATed to AP2 6); DNA binding / transcription factor	12.6	5.3	2.4
267	252363_AT	AT3G48460	GDSL-motif lipase/hydrolase family protein	3.7	1.6	2.4
268	253503_AT	AT4G31950	CYP82C3; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	14.1	5.9	2.4
269	267262_AT	AT2G22990	SNG1 (SINAPOYLGLUCOSE 1); serine-type carboxypeptidase/ sinapoylglucose-malATe O-sinapoyltransferase	0.9	0.4	2.4
270	265992_AT	AT2G24130	leucine-rich repeAT transmembrane protein kinase, putATive	10.9	4.6	2.4
271	266281_AT	AT2G29250	lectin protein kinase, putATive	7.4	3.1	2.4
272	263837_AT	AT2G04500	DC1 domain-containing protein	6.2	2.6	2.4
273	251971_AT	AT3G53160	UGT73C7 (UDP-glucosyl transferase 73C7); UDP-glycosyltransferase/ transferase, transferring glycosyl groups	6.1	2.6	2.4
274	259879_AT	AT1G76650	calcium-binding EF hand family protein	4.8	2.1	2.3
275	260741_AT	AT1G15040	glutamine amidotransferase-relATed	3.2	1.4	2.3
276	258537_AT	AT3G04210	disease resistance protein (TIR-NBS class), putATive	2.9	1.2	2.3
277	250287_AT	AT5G13330	Rap2.6L (relATed to AP2 6L); DNA binding / transcription factor	5.3	2.3	2.3
278	261476_AT	AT1G14480	protein binding	5.2	2.2	2.3
279	246831_AT	AT5G26340	MSS1; carbohydrATe transmembrane transporter/ hexosehydrogen symporter/ high-affinity hydrogenglucose symporter/ sugarhydrogen symporter	9.7	4.2	2.3
280	260237_AT	AT1G74430	MYB95 (myb domain protein 95); DNA binding / transcription factor	3.4	1.5	2.3
281	265990_AT	AT2G24280	serine carboxypeptidase S28 family protein	0.7	0.3	2.3
282	267076_AT	AT2G41090	calmodulin-like calcium-binding protein, 22 kDa (CaBP-22)	5.7	2.4	2.3
283	261005_AT	AT1G26420	FAD-binding domain-containing protein	13.7	5.9	2.3
284	265468_AT	AT2G37210	Encodes a protein of unknown function. It has been crystallized and shown to be structurally almost identical to the protein encoded by At5g11950.	2.3	1.0	2.3
285	247213_AT	AT5G64900	PROPEP1	6.6	2.9	2.3
286	266052_AT	AT2G40740	WRKY55; transcription factor	4.9	2.1	2.3

287	261564_AT	AT1G01720	ATAF1; transcription activATor/ transcription factor	10.1	4.4	2.3
288	251705_AT	AT3G56400	WRKY70; transcription factor/ transcription repressor	12.8	5.6	2.3
289	254215_AT	AT4G23700	ATCHX17 (CATION/H+ EXCHANGER 17); monovalent cATionproton antiporter/ sodiumhydrogen antiporter	6.3	2.8	2.3
290	261648_AT	AT1G27730	STZ (salt tolerance zinc finger); nucleic acid binding / transcription factor/ transcription repressor/ zinc ion binding	15.3	6.7	2.3
291	261135_AT	AT1G19610	PDF1.4	1.2	0.5	2.3
292	264815_AT	AT1G03620	phagocytosis and cell motility protein ELMO1-relATed	1.0	0.4	2.3
293	249890_AT	AT5G22570	WRKY38; transcription factor	36.9	16.3	2.3
294	252888_AT	AT4G39210	APL3; glucose-1-phosphATe adenylyltransferase	3.7	1.6	2.3
295	259033_AT	AT3G09405	FUNCTIONS IN molecular_function unknown; INVOLVED IN biological_process unknown; LOCATED IN cellular_component unknown; CONTAINS InterPro DOMAIN/s Pectinacetylesterase (InterProIPR004963); BEST *Arabidopsis* thaliana protein mATch is pectinacetylesterase family protein (TAIRAT3G09410.1).	8.3	3.7	2.3
296	260239_AT	AT1G74360	leucine-rich repeAT transmembrane protein kinase, putATive	8.2	3.6	2.3
297	259439_AT	AT1G01480	ACS2; 1-aminocyclopropane-1-carboxylATe synthase	31.8	14.1	2.2
298	249021_AT	AT5G44820	unknown protein	3.7	1.6	2.2
299	267627_AT	AT2G42270	U5 small nuclear ribonucleoprotein helicase, putATive	1.6	0.7	2.2
300	263866_AT	AT2G36950	heavy-metal-associATed domain-containing protein	1.1	0.4	2.2

Appendix IV**: Enriched GO categories for genes showing higher response to salt in primed plants compared to control plants in the selected background (FRD≤0.05).**
The top 300 genes from the list Primed>Control are compared against A) genes salt specific background and B) roots specific background.

A) Primed> Control on salt specific background

Cluster1	Enrichment Score 4.4	Count	%	P-Value	Affymetrix ID	FDR
GOTERM_ MF_FAT	GO0005199~structural constituent of cell wall	9	3.1	9.48E-08	256352_AT, 255140_X_AT, 245875_AT, 257679_AT, 258008_AT, 257041_AT, 246652_AT, 250683_X_AT, 250682_X_AT	1.22E-04
GOTERM_ BP_FAT	GO0009664~plant-type cell wall organizATion	9	3.1	9.51E-06	259525_AT, 255140_X_AT, 245875_AT, 265443_AT, 261099_AT, 257041_AT, 246652_AT, 250683_X_AT, 250682_X_AT	1.32E-02
Cluster2	**Enrichment Score 3.8**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_ BP_FAT	GO0042744~hydrogen peroxide cATabolic process	11	3.7	8.29E-07	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.15E-03
GOTERM_ BP_FAT	GO0070301~cellular response to hydrogen peroxide	11	3.7	8.29E-07	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.15E-03
GOTERM_ BP_FAT	GO0042743~hydrogen peroxide metabolic process	11	3.7	1.32E-06	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.84E-03
GOTERM_ BP_FAT	GO0034614~cellular response to reactive oxygen species	11	3.7	2.04E-06	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	2.85E-03
GOTERM_ BP_FAT	GO0034599~cellular response to oxidATive stress	11	3.7	2.52E-06	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	3.51E-03
GOTERM_ MF_FAT	GO0005509~calcium ion binding	20	6.8	5.97E-06	254338_S_AT, 249934_AT, 247091_AT, 257405_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 253968_AT, 264567_S_AT, 264391_AT, 266191_AT, 254092_AT, 246302_AT, 248898_AT, 265102_AT, 259064_AT, 250059_AT, 254774_AT	7.70E-03
GOTERM_ MF_FAT	GO0020037~heme binding	19	6.4	7.73E-06	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 250576_AT, 253998_AT, 264567_S_AT, 264404_AT, 258920_AT, 266191_AT, 250651_AT, 267626_AT, 257143_AT, 253502_AT, 265102_AT, 256099_AT, 250059_AT	9.96E-03
GOTERM_ BP_FAT	GO0006800~oxygen and reactive oxygen species metabolic process	11	3.7	9.52E-06	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.33E-02
GOTERM_ MF_FAT	GO0004601~peroxidase activity	12	4.1	1.48E-05	249934_AT, 266191_AT, 254092_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.91E-02
GOTERM_ MF_FAT	GO0016684~oxidoreductase activity, acting on peroxide as acceptor	12	4.1	1.48E-05	249934_AT, 266191_AT, 254092_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	1.91E-02
GOTERM_ MF_FAT	GO0046906~tetrapyrrole binding	19	6.4	1.61E-05	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 250576_AT, 253998_AT, 264567_S_AT, 264404_AT, 258920_AT, 266191_AT,	2.07E-02

					250651_AT, 267626_AT, 257143_AT, 253502_AT, 265102_AT, 256099_AT, 250059_AT	
GOTERM_BP_FAT	GO0042542~response to hydrogen peroxide	11	3.7	2.93E-05	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	4.08E-02
Cluster3	**Enrichment Score 2.9**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_BP_FAT	GO0007047~cell wall organizATion	15	5.1	7.82E-06	259525_AT, 260758_AT, 265443_AT, 263229_S_AT, 261099_AT, 251434_AT, 253763_AT, 263560_S_AT, 247871_AT, 262198_AT, 250802_AT, 245546_AT, 265246_AT, 264682_AT, 248406_AT	1.09E-02
GOTERM_BP_FAT	GO0045229~external encapsulATing structure organizATion	15	5.1	9.81E-06	259525_AT, 260758_AT, 265443_AT, 263229_S_AT, 261099_AT, 251434_AT, 253763_AT, 263560_S_AT, 247871_AT, 262198_AT, 250802_AT, 245546_AT, 265246_AT, 264682_AT, 248406_AT	1.37E-02

B) Primed>Control on roots background

Cluster 1	Enrichment Score 5.1	Affymetrix ID	Count	%	P-Value	FDR
GOTERM_MF_FAT	GO0020037~heme binding	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 250576_AT, 264567_S_AT, 264404_AT, 258920_AT, 266191_AT, 250651_AT, 267626_AT, 254836_AT, 257143_AT, 253502_AT, 265102_AT, 256099_AT, 250059_AT	20	6.8	1.09E-08	1.40E-05
GOTERM_MF_FAT	GO0046906~tetrapyrrole binding	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 250576_AT, 264567_S_AT, 264404_AT, 258920_AT, 266191_AT, 250651_AT, 267626_AT, 254836_AT, 257143_AT, 253502_AT, 265102_AT, 256099_AT, 250059_AT	20	6.8	3.22E-08	4.16E-05
GOTERM_BP_FAT	GO0042744~hydrogen peroxide cATabolic process	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	5.65E-08	7.89E-05
GOTERM_BP_FAT	GO0070301~cellular response to hydrogen peroxide	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	5.65E-08	7.89E-05
GOTERM_MF_FAT	GO0004601~peroxidase activity	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 254912_AT, 264567_S_AT, 266191_AT, 254092_AT, 265102_AT, 250059_AT	13	4.4	6.07E-08	7.83E-05
GOTERM_MF_FAT	GO0016684~oxidoreductase activity, acting on peroxide as acceptor	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 254912_AT, 264567_S_AT, 266191_AT, 254092_AT, 265102_AT, 250059_AT	13	4.4	6.07E-08	7.83E-05
GOTERM_BP_FAT	GO0042743~hydrogen peroxide metabolic process	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	9.10E-08	1.27E-04
GOTERM_MF_FAT	GO0005506~iron ion binding	260298_AT, 260941_AT, 260035_AT, 254912_AT, 250576_AT, 266941_AT, 253998_AT, 264567_S_AT, 264404_AT, 250651_AT, 254092_AT, 267626_AT, 254550_AT, 260148_AT, 265102_AT, 256099_AT, 250059_AT, 249934_AT, 247091_AT, 252238_AT, 260950_S_AT, 258920_AT, 266191_AT, 254836_AT, 260957_AT, 257143_AT, 253502_AT	27	9.2	1.43E-07	1.84E-04
GOTERM_BP_FAT	GO0034614~cellular response to reactive oxygen species	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	1.90E-07	2.65E-04
GOTERM_BP_FAT	GO0034599~cellular response to oxidATive stress	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	2.19E-07	3.05E-04
GOTERM_MF_FAT	GO0005509~calcium ion binding	254338_S_AT, 249934_AT, 247091_AT, 257405_AT, 252238_AT, 260941_AT, 260035_AT, 254912_AT, 266941_AT, 253998_AT, 253968_AT, 264567_S_AT, 264391_AT, 266191_AT, 254092_AT, 246302_AT, 248898_AT, 265102_AT, 259064_AT, 250059_AT,	21	7.1	3.43E-07	4.42E-04

		254774_AT				
GOTERM_MF_FAT	GO0016209~antioxidant activity	249934_AT, 247091_AT, 252238_AT, 260941_AT, 260035_AT, 266941_AT, 253998_AT, 254912_AT, 264567_S_AT, 266191_AT, 254092_AT, 265102_AT, 250059_AT	13	4.4	4.22E-07	5.45E-04
GOTERM_MF_FAT	GO0009055~electron carrier activity	251196_AT, 249934_AT, 247091_AT, 252238_AT, 258359_S_AT, 260941_AT, 260035_AT, 254912_AT, 266941_AT, 253998_AT, 250576_AT, 264567_S_AT, 264404_AT, 266191_AT, 250651_AT, 266356_AT, 254092_AT, 267626_AT, 254836_AT, 257143_AT, 253502_AT, 265102_AT, 256099_AT, 250059_AT	24	8.1	4.52E-07	5.83E-04
GOTERM_BP_FAT	GO0006800~oxygen and reactive oxygen species metabolic process	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	1.11E-06	1.55E-03
GOTERM_BP_FAT	GO0042542~response to hydrogen peroxide	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	2.62E-06	3.66E-03
GOTERM_BP_FAT	GO0000302~response to reactive oxygen species	249934_AT, 266191_AT, 247091_AT, 252238_AT, 260941_AT, 265102_AT, 260035_AT, 250059_AT, 266941_AT, 253998_AT, 264567_S_AT	11	3.7	1.58E-05	2.20E-02
GOTERM_BP_FAT	GO0055114~oxidATion reduction	251196_AT, 260298_AT, 260941_AT, 260035_AT, 254912_AT, 253998_AT, 266941_AT, 250576_AT, 264567_S_AT, 264404_AT, 250651_AT, 254092_AT, 267626_AT, 260148_AT, 265102_AT, 256099_AT, 250059_AT, 249934_AT, 248519_AT, 247091_AT, 252238_AT, 260950_S_AT, 258359_S_AT, 253696_AT, 266191_AT, 254836_AT, 260957_AT, 257143_AT, 253309_AT, 253502_AT, 250958_AT	31	10.5	1.76E-05	2.46E-02
Cluster 2	**Enrichment Score 4.8**	**Affymetrix ID**	**Count**	**%**	**P-Value**	**FDR**
GOTERM_MF_FAT	GO0005199~structural constituent of cell wall	256352_AT, 255140_X_AT, 245875_AT, 257679_AT, 258008_AT, 257041_AT, 246652_AT, 250683_X_AT, 250682_X_AT	9	3.1	5.01E-08	6.47E-05
GOTERM_BP_FAT	GO0009664~plant-type cell wall organizATion	259525_AT, 255140_X_AT, 245875_AT, 265443_AT, 261099_AT, 257041_AT, 246652_AT, 247991_AT, 250683_X_AT, 250682_X_AT	10	3.4	3.20E-07	4.46E-04
Cluster 3	Enrichment Score 4.1	**Affymetrix ID**	Count	%	P-Value	FDR
GOTERM_BP_FAT	GO0007047~cell wall organizATion	259525_AT, 260758_AT, 265443_AT, 263229_S_AT, 261099_AT, 253763_AT, 251434_AT, 263560_S_AT, 247871_AT, 262198_AT, 250802_AT, 245546_AT, 265246_AT, 264682_AT, 248406_AT, 247991_AT	16	5.4	5.43E-08	7.57E-05
GOTERM_BP_FAT	GO0045229~external encapsulATing structure organizATion	259525_AT, 260758_AT, 265443_AT, 263229_S_AT, 261099_AT, 253763_AT, 251434_AT, 263560_S_AT, 247871_AT, 262198_AT, 250802_AT, 245546_AT, 265246_AT, 264682_AT, 248406_AT, 247991_AT	16	5.4	7.59E-08	1.06E-04
Cluster 4	**Enrichment Score 3.6**	**Affymetrix ID**	**Count**	**%**	**P-Value**	**FDR**
GOTERM_MF_FAT	GO0030599~pectinesterase activity	250802_AT, 265246_AT, 250801_AT, 267287_AT, 248408_AT, 254056_AT, 248406_AT, 246229_AT, 262198_AT	9	3.1	4.66E-06	6.01E-03
Cluster 5	**Enrichment Score 1.8**	**Genes**	**Count**	**%**	**P-Value**	**FDR**
GOTERM_BP_FAT	GO0009664~plant-type cell wall organizATion	259525_AT, 255140_X_AT, 245875_AT, 265443_AT, 261099_AT, 257041_AT, 246652_AT, 247991_AT, 250683_X_AT, 250682_X_AT	10	3.4	3.20E-07	4.46E-04

Appendix V**: Enriched GO categories for genes showing lower response to salt in primed plants compared to control plants in the selected background (FDR≤0.05).**
The top 300 genes from the list Control>Primed are compared against A) genes salt specific background and B) roots specific background.

A) Control>Primed on salt specific background

Cluster1	Enrichment Score 5.2	Count	%	P-Value	Affymetrix ID	FDR
GOTERM_BP_FAT	GO0009723~response to ethylene stimulus	29	9.7	1.69E-11	257918_AT, 250287_AT, 257919_AT, 260237_AT, 249333_AT, 264415_AT, 254926_AT, 266821_AT, 248448_AT, 257927_AT, 247492_AT, 252193_AT, 255250_AT, 247213_AT, 252408_AT, 261470_AT, 258516_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 260856_AT, 250622_AT, 261763_AT, 260581_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	2.34E-08
GOTERM_BP_FAT	GO0010033~response to organic substance	57	19.1	3.13E-08	259561_AT, 249333_AT, 261648_AT, 266821_AT, 250207_AT, 248448_AT, 252193_AT, 255250_AT, 253666_AT, 261033_AT, 248618_AT, 261470_AT, 250435_AT, 258516_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 261763_AT, 247655_AT, 260581_AT, 260429_AT, 247543_AT, 261037_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 264415_AT, 258277_AT, 250455_AT, 267607_S_AT, 261892_AT, 262092_AT, 254926_AT, 247052_AT, 260205_AT, 257927_AT, 254014_AT, 247492_AT, 257206_AT, 249890_AT, 251705_AT, 256093_AT, 263539_AT, 247213_AT, 263783_AT, 252408_AT, 247925_AT, 264202_AT, 261443_AT, 250622_AT, 250292_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT	4.33E-05
GOTERM_BP_FAT	GO0009873~ethylene mediATed signaling pAThway	19	6.4	8.92E-08	257918_AT, 250287_AT, 261470_AT, 249333_AT, 264415_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 266821_AT, 260856_AT, 248448_AT, 257927_AT, 250622_AT, 247492_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	1.23E-04
GOTERM_MF_FAT	GO0003700~transcription factor activity	52	17.4	2.52E-07	253070_AT, 248160_AT, 261648_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 248618_AT, 261470_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 247655_AT, 267140_AT, 260581_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT, 266455_AT	3.23E-04
GOTERM_BP_FAT	GO0009719~response to endogenous stimulus	47	15.8	2.95E-07	257918_AT, 250287_AT, 257919_AT, 260237_AT, 249333_AT, 264415_AT, 261648_AT, 258277_AT, 250455_AT, 267607_S_AT, 262092_AT, 254926_AT, 247052_AT, 266821_AT, 260205_AT, 257927_AT, 250207_AT, 248448_AT, 247492_AT, 252193_AT, 251705_AT, 263539_AT, 255250_AT, 247213_AT, 253666_AT, 261033_AT, 248618_AT, 252408_AT, 261470_AT, 258516_AT, 247925_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 261443_AT, 250622_AT, 261763_AT, 250292_AT, 260581_AT, 260429_AT, 248794_AT, 262211_AT, 247543_AT, 261037_AT, 253259_AT	4.09E-04
GOTERM_BP_FAT	GO0006350~transcription	45	15.1	1.26E-06	257918_AT, 250287_AT, 253070_AT, 255937_AT, 264415_AT, 261564_AT, 261892_AT, 259293_AT, 266821_AT, 247052_AT, 260205_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 249890_AT, 251705_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 261033_AT, 263783_AT, 261470_AT, 256159_AT, 260203_AT, 249940_AT, 260278_AT, 266052_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 250292_AT, 252679_AT, 260429_AT, 248794_AT, 262211_AT, 247543_AT, 266455_AT, 253259_AT	1.75E-03
GOTERM_BP_FAT	GO0000160~two-component signal transduction system (phosphorelay)	19	6.4	1.97E-06	257918_AT, 250287_AT, 261470_AT, 249333_AT, 264415_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 266821_AT, 260856_AT, 248448_AT, 257927_AT, 250622_AT, 247492_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	2.73E-03

GOTERM_MF_FAT	GO0030528~transcription regulATor activity	52	17.4	4.67E-06	253070_AT, 248160_AT, 261648_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 248618_AT, 261470_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 247655_AT, 267140_AT, 260581_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 265983_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT, 266455_AT	5.99E-03
GOTERM_BP_FAT	GO0006355~regulATion of transcription, DNA-dependent	40	13.4	5.72E-06	257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 266821_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 252193_AT, 249890_AT, 251705_AT, 255250_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 263783_AT, 248618_AT, 261470_AT, 258516_AT, 260278_AT, 266052_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 248794_AT, 262211_AT, 247543_AT, 253259_AT	7.91E-03
GOTERM_BP_FAT	GO0051252~regulATion of RNA metabolic process	40	13.4	6.53E-06	257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 266821_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 252193_AT, 249890_AT, 251705_AT, 255250_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 263783_AT, 248618_AT, 261470_AT, 258516_AT, 260278_AT, 266052_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 248794_AT, 262211_AT, 247543_AT, 253259_AT	9.03E-03
GOTERM_BP_FAT	GO0045449~regulATion of transcription	55	18.5	6.83E-05	253070_AT, 248160_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 261033_AT, 248618_AT, 261470_AT, 256159_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 267140_AT, 260581_AT, 260429_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 260205_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 250292_AT, 265983_AT, 252679_AT, 248794_AT, 262211_AT, 266455_AT, 253259_AT	9.45E-02
Cluster2	**Enrichment Score 3.6**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_BP_FAT	GO0009642~response to light intensity	11	3.7	1.18E-05	262148_AT, 250351_AT, 263150_AT, 246099_AT, 251166_AT, 247655_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	1.64E-02
GOTERM_BP_FAT	GO0009644~response to high light intensity	9	3.0	1.90E-05	262148_AT, 250351_AT, 263150_AT, 251166_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	2.62E-02
Cluster3	**Enrichment Score 3.0**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_BP_FAT	GO0009408~response to heAT	14	4.7	8.81E-06	262148_AT, 263150_AT, 260978_AT, 250296_AT, 262911_S_AT, 247925_AT, 250351_AT, 252515_AT, 251166_AT, 247655_AT, 247691_AT, 266294_AT, 250781_AT, 256430_AT	1.22E-02
GOTERM_BP_FAT	GO0009642~response to light intensity	11	3.7	1.18E-05	262148_AT, 250351_AT, 263150_AT, 246099_AT, 251166_AT, 247655_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	1.64E-02
GOTERM_BP_FAT	GO0009644~response to high light intensity	9	3.0	1.90E-05	262148_AT, 250351_AT, 263150_AT, 251166_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	2.62E-02

B) Control> Primed on roots background

Cluster1	Enrichment Score 7.3	Count	%	P-Value	Affymetrix ID	FDR
GOTERM_BP_FAT	GO0009723~response to ethylene stimulus	29	9.7	1.18E-14	257918_AT, 250287_AT, 257919_AT, 260237_AT, 249333_AT, 264415_AT, 254926_AT, 266821_AT, 248448_AT, 257927_AT, 247492_AT, 252193_AT, 255250_AT, 247213_AT, 252408_AT, 261470_AT, 258516_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 260856_AT, 250622_AT, 261763_AT, 260581_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	1.64E-11
GOTERM_BP_FAT	GO0010033~response to organic substance	57	19.1	1.18E-12	259561_AT, 249333_AT, 261648_AT, 266821_AT, 250207_AT, 248448_AT, 252193_AT, 255250_AT, 253666_AT, 261033_AT, 248618_AT, 261470_AT, 250435_AT, 258516_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 261763_AT, 247655_AT, 260581_AT, 260429_AT, 247543_AT, 261037_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 264415_AT, 258277_AT, 250455_AT, 267607_S_AT, 261892_AT, 262092_AT, 254926_AT, 247052_AT, 260205_AT, 257927_AT, 254014_AT, 247492_AT, 257206_AT, 249890_AT, 251705_AT, 256093_AT, 263539_AT, 247213_AT, 263783_AT, 252408_AT, 247925_AT, 264202_AT, 261443_AT, 250622_AT, 250292_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT	1.63E-09
GOTERM_BP_FAT	GO0009719~response to endogenous stimulus	47	15.8	1.59E-10	257918_AT, 250287_AT, 257919_AT, 260237_AT, 249333_AT, 264415_AT, 261648_AT, 258277_AT, 250455_AT, 267607_S_AT, 262092_AT, 254926_AT, 247052_AT, 266821_AT, 260205_AT, 257927_AT, 250207_AT, 248448_AT, 247492_AT, 252193_AT, 251705_AT, 263539_AT, 255250_AT, 247213_AT, 253666_AT, 261033_AT, 248618_AT, 252408_AT, 261470_AT, 258516_AT, 247925_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 261443_AT, 250622_AT, 261763_AT, 250292_AT, 260581_AT, 260429_AT, 248794_AT, 262211_AT, 247543_AT, 261037_AT, 253259_AT	2.20E-07
GOTERM_MF_FAT	GO0003700~transcription factor activity	52	17.4	1.80E-10	253070_AT, 248160_AT, 261648_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 248618_AT, 261470_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 247655_AT, 267140_AT, 260581_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 265983_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT, 266455_AT	2.31E-07
GOTERM_BP_FAT	GO0009873~ethylene mediATed signaling pAThway	19	6.4	3.78E-10	257918_AT, 250287_AT, 261470_AT, 249333_AT, 264415_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 266821_AT, 260856_AT, 248448_AT, 257927_AT, 250622_AT, 247492_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	5.23E-07
GOTERM_BP_FAT	GO0000160~two-component signal transduction system (phosphorelay)	19	6.4	1.88E-08	257918_AT, 250287_AT, 261470_AT, 249333_AT, 264415_AT, 245173_AT, 256009_AT, 264202_AT, 257917_AT, 266821_AT, 260856_AT, 248448_AT, 257927_AT, 250622_AT, 247492_AT, 248794_AT, 247543_AT, 262211_AT, 253259_AT	2.60E-05
GOTERM_MF_FAT	GO0030528~transcription regulATor activity	52	17.4	3.32E-08	253070_AT, 248160_AT, 261648_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 248618_AT, 261470_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 247655_AT, 267140_AT, 260581_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 265983_AT, 263584_AT, 248794_AT, 262211_AT, 253259_AT, 266455_AT	4.26E-05
GOTERM_BP_FAT	GO0006350~transcription	45	15.1	4.34E-08	257918_AT, 250287_AT, 253070_AT, 255937_AT, 264415_AT, 261564_AT, 261892_AT, 259293_AT, 266821_AT, 247052_AT, 260205_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 249890_AT, 251705_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 261033_AT, 263783_AT, 261470_AT, 256159_AT, 260203_AT, 249940_AT, 260278_AT, 266052_AT, 264202_AT,	6.00E-05

					256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 250292_AT, 252679_AT, 260429_AT, 248794_AT, 262211_AT, 247543_AT, 266455_AT, 253259_AT	
GOTERM_BP_FAT	GO0006355~regulATion of transcription, DNA-dependent	40	13.4	4.52E-08	257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 266821_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 252193_AT, 249890_AT, 251705_AT, 255250_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 263783_AT, 248618_AT, 261470_AT, 258516_AT, 260278_AT, 266052_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 248794_AT, 262211_AT, 247543_AT, 253259_AT	6.26E-05
GOTERM_BP_FAT	GO0051252~regulATion of RNA metabolic process	40	13.4	5.82E-08	257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 266821_AT, 257927_AT, 248448_AT, 251166_AT, 247492_AT, 252193_AT, 249890_AT, 251705_AT, 255250_AT, 258975_AT, 256430_AT, 250781_AT, 254074_AT, 263783_AT, 248618_AT, 261470_AT, 258516_AT, 260278_AT, 266052_AT, 264202_AT, 256009_AT, 245173_AT, 257917_AT, 260856_AT, 250622_AT, 248896_AT, 265983_AT, 248794_AT, 262211_AT, 247543_AT, 253259_AT	8.05E-05
GOTERM_BP_FAT	GO0009725~response to hormone stimulus	37	12.4	1.78E-06	257918_AT, 250287_AT, 257919_AT, 260237_AT, 249333_AT, 264415_AT, 261648_AT, 258277_AT, 267607_S_AT, 262092_AT, 254926_AT, 266821_AT, 247052_AT, 257927_AT, 248448_AT, 247492_AT, 252193_AT, 255250_AT, 247213_AT, 253666_AT, 252408_AT, 248618_AT, 261470_AT, 258516_AT, 247925_AT, 264202_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 250622_AT, 261763_AT, 260581_AT, 248794_AT, 262211_AT, 247543_AT, 253259_AT	2.46E-03
GOTERM_BP_FAT	GO0045449~regulATion of transcription	55	18.5	2.17E-06	253070_AT, 248160_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 250781_AT, 258975_AT, 254074_AT, 261033_AT, 248618_AT, 261470_AT, 256159_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 267140_AT, 260581_AT, 260429_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 261892_AT, 259293_AT, 247052_AT, 260205_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 250292_AT, 265983_AT, 252679_AT, 248794_AT, 262211_AT, 266455_AT, 253259_AT	3.00E-03
GOTERM_MF_FAT	GO0003677~DNA binding	54	18.1	3.50E-05	253070_AT, 248160_AT, 261648_AT, 261564_AT, 266821_AT, 248448_AT, 251166_AT, 252193_AT, 255250_AT, 258975_AT, 250781_AT, 254074_AT, 248618_AT, 261470_AT, 260203_AT, 249940_AT, 258516_AT, 260278_AT, 245173_AT, 256009_AT, 257917_AT, 260856_AT, 247655_AT, 267140_AT, 260581_AT, 247543_AT, 257918_AT, 250287_AT, 257919_AT, 260237_AT, 255937_AT, 264415_AT, 250445_AT, 261892_AT, 259293_AT, 247052_AT, 257927_AT, 247492_AT, 249890_AT, 251705_AT, 256430_AT, 263783_AT, 252408_AT, 266052_AT, 264202_AT, 250622_AT, 248896_AT, 265983_AT, 254062_AT, 263584_AT, 248794_AT, 262211_AT, 266455_AT, 253259_AT	4.50E-02
Cluster2	**Enrichment Score 4.1**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_BP_FAT	GO0009644~response to high light intensity	9	3.0	2.07E-06	262148_AT, 250351_AT, 263150_AT, 251166_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	2.86E-03
GOTERM_BP_FAT	GO0009642~response to light intensity	11	3.7	2.19E-06	262148_AT, 250351_AT, 263150_AT, 246099_AT, 251166_AT, 247655_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	3.02E-03
GOTERM_BP_FAT	GO0009628~response to abiotic stimulus	42	14.1	1.59E-04	257641_S_AT, 257919_AT, 252123_AT, 259161_AT, 255937_AT, 260978_AT, 261648_AT, 250296_AT, 251827_AT, 267607_S_AT, 262911_S_AT, 254926_AT, 252515_AT, 250207_AT, 246099_AT, 251166_AT, 252193_AT, 247691_AT, 255250_AT, 250781_AT, 256430_AT, 254074_AT, 262148_AT, 263150_AT, 248618_AT, 252408_AT, 260203_AT, 258516_AT, 245627_AT, 247925_AT, 250351_AT, 250558_AT, 251625_AT, 247655_AT, 247431_AT, 266097_AT, 260581_AT, 259925_AT, 250415_AT, 266294_AT, 261037_AT, 265569_AT	2.20E-01
Cluster3	**Enrichment Score 3.7**	**Count**	**%**	**P-Value**	**Affymetrix ID**	**FDR**
GOTERM_BP_FAT	GO0009644~response to high light intensity	9	3.0	2.07E-06	262148_AT, 250351_AT, 263150_AT, 251166_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	2.86E-03

GOTERM_BP_FAT	GO0009642~response to light intensity	11	3.7	2.19E-06	262148_AT, 250351_AT, 263150_AT, 246099_AT, 251166_AT, 247655_AT, 261648_AT, 247691_AT, 266294_AT, 261037_AT, 265569_AT	3.02E-03
GOTERM_BP_FAT	GO0009408~response to heAT	14	4.7	2.33E-06	262148_AT, 263150_AT, 260978_AT, 250296_AT, 262911_S_AT, 247925_AT, 250351_AT, 252515_AT, 251166_AT, 247655_AT, 247691_AT, 266294_AT, 250781_AT, 256430_AT	3.22E-03

Appendix VI complete list of genes with differences between control and priming detected by ChIP-Diff for H3K27me3overlapping between 24h and 10 days roots samples.

Gene ID	Description	Direction 24h	CoordinATes 24h	Length 24h	Direction 10d	CoordinATes 10d	Length 10d
AT1G05291	unknown protein	down	Chr11541600-1541800	200	down	Chr11541000-1541200	200
AT1G09380	integral membrane family protein / nodulin MtN21-relATed	down	Chr13028400-3028600	200	down	Chr13029400-3029600	200
AT1G12190	F-box family protein	down	Chr14134000-4134800	800	down	Chr14134200-4134600	400
AT1G12260	ANAC007 (*ARABIDOPSIS* NAC 007); transcription factor	down	Chr14162200-4162800	600	down	Chr14162600-4163000	400
AT1G18710	AtMYB47 (myb domain protein 47); DNA binding / transcription factor	down	Chr16449800-6450400	600	down	Chr16450200-6450400	200
AT1G19800	TGD1 (TRIGALACTOSYLDIACYLGLYCEROL 1); lipid transporter	down	Chr16845400-6845600	200	down	Chr16845400-6845600	200
AT1G30795	hydroxyproline-rich glycoprotein family protein	down	Chr110935400-10935600	200	down	Chr110935400-10935600	200
AT1G31875	unknown protein	down	Chr111442400-11443000	600	down	Chr111442600-11442800	200
AT1G47370	Toll-Interleukin-Resistance (TIR) domain-containing protein	down	Chr117367400-17368000	600	down	Chr117367800-17368000	200
AT1G47786	acyl-protein thioesterase-relATed	down	Chr117598000-17598400	400	down	Chr117598600-17598800	200
AT1G51460	ABC transporter family protein	down	Chr119080000-19080600	600	down	Chr119080000-19080200	200
AT1G51490	BGLU36 (BETA GLUCOSIDASE 36); cATalytic/ cATion binding / hydrolase, hydrolyzing O-glycosyl compounds	down	Chr119095200-19095400	200	down	Chr119095000-19095400	400
AT1G52070	jacalin lectin family protein	down	Chr119364800-19365000	200	down	Chr119364800-19365000	200
AT1G52140	unknown protein	down	Chr119405600-19407400	1800	down	Chr119406800-19407000	200
AT1G52410	TSA1 (TSK-ASSOCIATING PROTEIN 1); calcium ion binding / protein binding	down	Chr119519800-19520600	800	down	Chr119520000-19520200	200
AT1G56650	PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1); DNA binding / transcription factor	down	Chr121232400-21233000	600	down	Chr121232800-21233000	200
AT1G57830	Toll-Interleukin-Resistance (TIR) domain-containing protein	down	Chr121420800-21421200	400	down	Chr121420800-21421200	400
AT1G59722	unknown protein	down	Chr121945600-21946600	1000	down	Chr121944000-21944200	200
AT1G61630	equilibrATive nucleoside transporter, putATive (ENT7)	down	Chr122743400-22744200	800	down	Chr122743600-22744200	600
AT1G61750	unknown protein	down	Chr122805000-22805200	200	down	Chr122805000-22805200	200
AT1G62030	DC1 domain-containing protein	up	Chr122925000-22925200	200	up	Chr122924800-22925200	400
AT1G65342	unknown protein	down	Chr124270800-24271000	200	down	Chr124270600-24270800	200
AT1G67710	ARR11 (RESPONSE REGULATOR 11); transcription factor/ two-component response regulATor	down	Chr125379600-25380000	400	down	Chr125379600-25379800	200
AT1G69090	F-box family protein	down	Chr125976600-25977400	800	down	Chr125977000-25977200	200
AT1G69120	AP1 (APETALA1); DNA binding / protein binding / protein	down	Chr125984600-25985400	800	down	Chr125984600-25984800	200

	heterodimerizATion/ transcription activATor/ transcription factor						
AT1G75920	family II extracellular lipase 5 (EXL5)	down	Chr128505200-28505600	400	down	Chr128505200-28505400	200
AT2G14710	F-box family protein	down	Chr26298400-6298600	200	down	Chr26298400-6298600	200
AT2G14960	GH3.1	up	Chr26452000-6452400	400	up	Chr26451800-6452000	200
AT2G16220	F-box family protein	down	Chr27031600-7032200	600	down	Chr27031800-7032000	200
AT2G21890	CAD3 (CINNAMYL ALCOHOL DEHYDROGENASE HOMOLOG 3); binding / cATalytic/ oxidoreductase/ zinc ion binding	down	Chr29330400-9330800	400	down	Chr29330400-9330800	400
AT2G22180	hydroxyproline-rich glycoprotein family protein	down	Chr29430000-9430400	400	down	Chr29430200-9430400	200
AT2G23170	GH3.3; indole-3-acetic acid amido synthetase	up	Chr29864200-9864600	400	up	Chr29864400-9864600	200
AT2G23171	unknown protein	down	Chr29867000-9867200	200	down	Chr29867400-9867600	200
AT2G24205	Encodes a ECA1 gametogenesis relATed family protein	down	Chr210292000-10292200	200	down	Chr210291600-10291800	200
AT2G25697	unknown protein	down	Chr210946200-10946800	600	down	Chr210946600-10946800	200
AT2G25700	ASK3 (*ARABIDOPSIS* SKP1-LIKE 3); protein binding / ubiquitin-protein ligase	down	Chr210949600-10950200	600	down	Chr210950000-10950200	200
AT2G26580	YAB5 (YABBY5); transcription factor	down	Chr211305000-11305600	600	down	Chr211302800-11303800	1000
AT2G30300	nodulin-relATed	down	Chr212921400-12921600	200	down	Chr212921800-12922000	200
AT2G30760	unknown protein	down	Chr213107200-13107400	200	down	Chr213107400-13107600	200
AT2G31083	CLE5 (CLAVATA3/ESR-RELATED 5); protein binding / receptor binding	down	Chr213250400-13251600	1200	down	Chr213253000-13253200	200
AT2G32870	meprin and TRAF homology domain-containing protein / MATH domain-containing protein	down	Chr213944400-13944600	200	down	Chr213944400-13944600	200
AT2G34790	MEE23 (MATERNAL EFFECT EMBRYO ARREST 23); FAD binding / cATalytic/ electron carrier/ oxidoreductase	down	Chr214675400-14676200	800	down	Chr214676000-14676200	200
AT2G39010	PIP2E (PLASMA MEMBRANE INTRINSIC PROTEIN 2E); wATer channel	down	Chr216290600-16291200	600	down	Chr216290800-16291200	400
AT3G03200	anac045 (*Arabidopsis* NAC domain containing protein 45); transcription factor	down	Chr3737800-738200	400	down	Chr3738000-738200	200
AT3G09390	MT2A (METALLOTHIONEIN 2A); copper ion binding	down	Chr32888600-2889000	400	down	Chr32888400-2888800	400
AT3G16360	AHP4 (HPT PHOSPHOTRANSMITTER 4); histidine phosphotransfer kinase/ transferase, transferring phosphorus-containing groups	down	Chr35553400-5553600	200	down	Chr35553800-5554000	200
AT3G18010	WOX1 (WUSCHEL relATed homeobox 1); transcription factor	down	Chr36159800-6160400	600	down	Chr36160200-6160400	200
AT3G18550	BRC1 (BRANCHED 1); transcription factor	down	Chr36381400-6383200	1800	down	Chr36382600-6382800	200

AT3G20160	geranylgeranyl pyrophosphATe synthase, putATive / GGPP synthetase, putATive / farnesyltranstransferase, putATive	up	Chr37039200-7039800	600	up	Chr37039800-7040000	200
AT3G21840	ASK7 (*ARABIDOPSIS* SKP1-LIKE 7); protein binding / ubiquitin-protein ligase	down	Chr37694800-7695200	400	down	Chr37694800-7695200	400
AT3G21850	ASK9 (*ARABIDOPSIS* SKP1-LIKE 9); protein binding / ubiquitin-protein ligase	down	Chr37697800-7698200	400	down	Chr37696400-7696600	200
AT3G22080	meprin and TRAF homology domain-containing protein / MATH domain-containing protein	down	Chr37779800-7780000	200	down	Chr37779800-7780000	200
AT3G25710	BHLH32 (BASIC HELIX-LOOP-HELIX 32); DNA binding / transcription factor	down	Chr39368800-9369400	600	down	Chr39369200-9369400	200
AT3G29260	short-chain dehydrogenase/reductase (SDR) family protein	down	Chr311217200-11218000	800	down	Chr311217800-11218000	200
AT3G29970	germinATion protein-relATed	down	Chr311745200-11746000	800	down	Chr311745800-11746200	400
AT3G44780	unknown protein	down	Chr316320600-16321600	1000	down	Chr316321000-16321200	200
AT3G45560	zinc finger (C3HC4-type RING finger) family protein	down	Chr316722800-16723400	600	down	Chr316723200-16723400	200
AT3G50480	HR4 (HOMOLOG OF RPW8 4)	down	Chr318732200-18732800	600	down	Chr318732400-18732600	200
AT3G51200	auxin-responsive family protein	down	Chr319019400-19019800	400	down	Chr319019400-19019600	200
AT3G55700	UDP-glucoronosyl/UDP-glucosyl transferase family protein	down	Chr320672000-20672200	200	down	Chr320671800-20672200	400
AT4G00300	fringe-relATed protein	up	Chr4126800-127200	400	up	Chr4126400-126600	200
AT4G01420	CBL5 (CALCINEURIN B-LIKE PROTEIN 5); calcium ion binding	down	Chr4579800-580200	400	down	Chr4579800-580000	200
AT4G01520	anac067 (*Arabidopsis* NAC domain containing protein 67); transcription factor	down	Chr4657200-657400	200	down	Chr4657600-657800	200
AT4G04840	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	down	Chr42450000-2451000	1000	down	Chr42450200-2450400	200
AT4G04890	PDF2 (PROTODERMAL FACTOR 2); DNA binding / transcription factor	down	Chr42480200-2481000	800	down	Chr42480200-2480400	200
AT4G10220	unknown protein	down	Chr46362200-6363200	1000	down	Chr46363400-6363600	200
AT4G10350	ANAC070 (*Arabidopsis* NAC domain containing protein 70); transcription factor	down	Chr46414400-6414800	400	up	Chr46414200-6414400	200
AT4G11170	disease resistance protein (TIR-NBS-LRR class), putATive	down	Chr46811800-6813000	1200	down	Chr46812400-6812600	200
AT4G12510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	down	Chr47416600-7417200	600	down	Chr47416600-7417000	400
AT4G12520	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	down	Chr47420600-7421000	400	down	Chr47420800-7421000	200
AT4G13420	HAK5 (HIGH AFFINITY K+ TRANSPORTER 5); potassium ion transmembrane transporter/	up	Chr47797800-7798200	400	up	Chr47797600-7797800	200

	potassium sodium symporter						
AT4G19730	glycosyl hydrolase family 18 protein	down	Chr410732800-10733400	600	down	Chr410733000-10733400	400
AT4G19740	cATalytic/ cATion binding / chitinase/ hydrolase, hydrolyzing O-glycosyl compounds	down	Chr410737600-10739200	1600	down	Chr410738400-10738600	200
AT4G22030	F-box family protein	down	Chr411674000-11674200	200	down	Chr411674000-11674200	200
AT4G22070	WRKY31; transcription factor	down	Chr411691000-11691200	200	down	Chr411691000-11691200	200
AT4G28840	unknown protein	down	Chr414240000-14240800	800	down	Chr414240200-14240400	200
AT4G29033	Encodes a defensin-like (DEFL) family protein.	down	Chr414308600-14309400	800	down	Chr414308600-14308800	200
AT4G30590	plastocyanin-like domain-containing protein	down	Chr414935400-14935600	200	down	Chr414935400-14935600	200
AT5G01310	unknown protein	up	Chr5125400-125600	200	down	Chr5124200-124400	200
AT5G11520	ASP3 (ASPARTATE AMINOTRANSFERASE 3); L-aspartATe2-oxoglutarATe aminotransferase	down	Chr53688000-3688400	400	down	Chr53688000-3688400	400
AT5G15160	bHLH family protein	down	Chr54920800-4921400	600	down	Chr54921000-4921200	200
AT5G15210	ATHB30 (*ARABIDOPSIS* THALIANA HOMEOBOX PROTEIN 30); transcription factor	up	Chr54938000-4938200	200	up	Chr54937800-4938200	400
AT5G15800	SEP1 (SEPALLATA1); DNA binding / transcription factor	down	Chr55152800-5153400	600	down	Chr55153000-5153200	200
AT5G17100	unknown protein	down	Chr55625200-5625600	400	down	Chr55625200-5625600	400
AT5G17810	WOX12 (WUSCHEL relATed homeobox 12); DNA binding / transcription factor	down	Chr55880200-5881800	1600	down	Chr55881200-5881800	600
AT5G17960	DC1 domain-containing protein	up	Chr55946200-5947200	1000	down	Chr55945200-5945400	200
AT5G24070	peroxidase family protein	down	Chr58134800-8135000	200	down	Chr58134800-8135000	200
AT5G24820	aspartyl protease family protein	up	Chr58523800-8524000	200	up	Chr58523400-8523800	400
AT5G24910	CYP714A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	down	Chr58570800-8571000	200	down	Chr58570800-8571200	400
AT5G25390	SHN2 (shine2); DNA binding / transcription factor	down	Chr58819000-8820000	1000	down	Chr58819800-8820000	200
AT5G25620	YUC6 (YUCCA6); FAD binding / NADP or NADPH binding / flavin-containing monooxygenase/ monooxygenase/ oxidoreductase	down	Chr58937200-8937600	400	down	Chr58939000-8939400	400
AT5G25990	unknown protein	down	Chr59076400-9076600	200	down	Chr59075800-9076000	200
AT5G35770	SAP (STERILE APETALA); transcription factor/ transcription regulATor	down	Chr513938800-13939800	1000	down	Chr513939400-13939800	400
AT5G39560	unknown protein	up	Chr515842200-15842800	600	up	Chr515842400-15842600	200
AT5G40040	60S acidic ribosomal protein P2 (RPP2E)	up	Chr516029800-16030200	400	up	Chr516029600-16029800	200
AT5G40790	unknown protein	down	Chr516329200-16330000	800	down	Chr516329600-16329800	200
AT5G42590	CYP71A16; electron carrier/ heme binding / iron ion binding /	down	Chr517032600-17033200	600	down	Chr517032800-17033000	200

	monooxygenase/ oxygen binding						
AT5G43120	tetrATricopeptide repeAT (TPR)-containing protein	up	Chr517312800-17313200	400	up	Chr517313000-17313400	400
AT5G45200	disease resistance protein (TIR-NBS-LRR class), putATive	down	Chr518287800-18288800	1000	down	Chr518289000-18289200	200
AT5G46350	WRKY8; transcription factor	down	Chr518801000-18801400	400	down	Chr518800400-18800800	400
AT5G57785	unknown protein	down	Chr523407600-23408400	800	down	Chr523408200-23408600	400
AT5G60010	FAD binding / calcium ion binding / electron carrier/ iron ion binding / oxidoreductase/ oxidoreductase, acting on NADH or NADPH, with oxygen as acceptor / peroxidase/ superoxide-generATing NADPH oxidase	down	Chr524164600-24165400	800	down	Chr524164600-24164800	200
AT5G60970	TCP5 (TEOSINTE BRANCHED1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR 5); transcription factor	down	Chr524534400-24535000	600	down	Chr524534600-24534800	200