Gene Expression Analysis in Response to Salt Stress in Wheat (*Triticum aestivum*) and Cytogenetic Derivatives of Wheat and the salt tolerant wheat grass, *Lophopyrum elongatum*

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Abstract

Gene Expression Analysis in Response to Salt Stress in Wheat (*Triticum aestivum*) and Cytogenetic Derivatives of Wheat and the salt tolerant wheat grass, *Lophopyrum elongatum*

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Comparative gene expression analysis was carried out to identify salt-stress responsive genes in *T. aestivum* and cytogentic stocks derived from *T. aestivum* x *Lophopyrum elongatum* crosses. A microarray consisting of 5728 cDNA amplicon probes was used in two gene expression profiling experiments. The first experiment examined the transcriptional profile of roots of the *T. aestivum* cultivar Norstar treated with 150 mM NaCl + 15mM $CaCl_2$ over a time course of 72 hours. The microarray analysis with Norstar revealed that there are 229 genes with significantly altered expression in salt treated plants. The second experiment compared gene expression profiles in wheat and wheat derivatives with different degrees of salt tolerance. Comparisons were made of roots of 150 mM NaCl + 150 mM $CaCl_2$ treated Chinese spring wheat, the amphiploid derived from a Chinese Spring Wheat x *L elongatum* cross and the disomic substitution line 3E(3A) of Chinese Spring in which chromosome 3A of wheat was replaced with chromosome 3E of *L. elongatum*. The analysis revealed that there are 212 genes that are significantly regulated in at least one genotype under salt stress and 42 genes have differences in regulation under salt stress between genotypes indicated by significant genotype by treatment interaction. Microarray analysis provided a practical tool for monitoring salt responsive genes in both experiments.

Dedications and Acknowledgements

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1.0 Introduction

Soil salinity is a major contributor to worldwide losses in agricultural productivity. Bread wheat (*Triticum aestivum*) is an important agronomical crop that is moderately salt tolerant and under conditions of salt stress has decreased biomass and yield. There is a range of salt tolerance among related grass species, with *Lophopyrum elongatum* being one of the most highly salt tolerant. Differences in salt tolerance among plant species is correlated with the ability of a plant to extrude Na^+ from tissues, maintain a favorable K^+/Na^+ ratio, and maintain water uptake and growth with high external salt concentrations. Increased salt tolerance among some species is thought to be mediated through pre-activation or enhanced activation of signaling pathways that lead to faster or/and enhanced response under salt stress (Hasegawa et al., 2000). Salt tolerance is a multigenic trait that has been partially characterized by identifying genes that change expression in response to salt stress. Due to the large number of genes involved it has been a challenge to understand the processes that mediate the salt stress response and to identify the genes that contribute to improved tolerance. Due to the complexity of the salt tolerance trait, progress in producing new cultivars with increased salt tolerance among agronomically important crops has been slow. Genome wide analysis can make an important contribution towards the identification of genes involved in stress response and the discovery of the signaling pathways that regulate their expression.

1.1 Salt Tolerance and Agriculture

Salinity stress causes large yearly losses in the world's agricultural productivity (Tester and Davenport, 2003; Schoups et al., 2005). There are many mechanisms by which salt can accumulate in soils, the major contributors are from rain water where there are low levels of dissolved salt which can accumulate over time and rock weathering (Rengasamy 2006). Salts can also be elevated in soils when there wind blown from marine environments, through evapo-transpiration of ground water with dissolved salts, where rainfall is too low to leach salt from the soil, in areas where there is poor quality irrigation water and also areas of agriculture where perennial vegetation has been cleared and annual crops and pastures have been planted leading to altered water tables and evapo-transpiration rates (Tester and Davenport, 2003; Rengasamy, 2006). Salinity affects over 800 million hectares, which accounts for 6% of land in the world (Munns, 2005). Irrigated agricultural land accounts for only 15% of farmed lands but is estimated to produce one third of world's food and 20% of it is affected by salinity (Munns, 2005). Salinization is becoming an increasing problem through current irrigation and land clearing practices (Tester and Davenport, 2003; Munns, 2005). With a predicted 50% increase in the world's population from 2001 to 2050, improved productivity of crops is needed to ensure food security, which will require increasing yields in normal and salinized lands (Blumwald et al., 2000; Flowers, 2004; Yamaguchi and Blumwald, 2005). The development of increased salt tolerance in crop species would aide in providing sustainability on increasingly salinized soils and may allow additional areas with marginal soils to be cultivated. Progress in producing salt-tolerant crop species has been

slow to date due to the complexity of this multigenic trait (Bohnert et al., 2001; Flowers, 2004).

Among plants there is a strikingly broad spectrum of salt tolerance from salt-sensitive glycophytes to salt-tolerant halophytes that are able to grow at concentrations exceeding 200 mM NaCl; some cultivated species such as sugar beet have salt tolerance that overlaps with that of halophytes (Greenway and Munns, 1980; Blumwald et al., 2000). Differences in salt tolerance also occur within species (Flowers, 2004). The negative effect of salinity is a combination of osmotic and ionic stress factors which can lead to reduced water uptake, reduced nutrient availability of essential ions such a K^+ and injury to cells by salt entering the transpiration stream, all of which are detrimental to plant growth (Grattan and Grieve, 1999; Blumwald et al., 2000; Tester and Davenport, 2003; Schoups et al., 2005). The main mechanisms of salt tolerance are the minimization of salt uptake by roots and sequestration of toxic ions in vacuoles of older tissues and the establishment of osmotic and ionic homeostasis (Hasegawa et al., 2000; Munns, 2005). Halophytic species do not have enzymes that are less sensitive to Na^+ and Cl^- ions (Hasegawa et al., 2000; Yokoi et al., 2002). It appears the increased salt-tolerance is attributed to improved osmotic adjustment and sequestration of toxic ions into vacuoles by enhanced activation and/or persistence of biochemical mechanisms through enhanced perception of stress and its integration into signaling–response pathways (Hasegawa et al., 2000; Bohnert et al., 2001).

1.2 Sodium and Chloride Uptake

Root membranes provide a selective barrier that controls what enters and exits cells. Under normal conditions plants maintain a high K^+/Na^+ ratio in tissues, alterations to this ratio under salt stress results in ion-specific stresses. Unlike K^+, Na^+ is not essential for cellular processes and under elevated concentrations it is toxic to cytosolic enzymes as it competes with K^+ for binding sites (Blumwald et al., 2000; Tester and Davenport, 2003). Thus, maintenance of cytosolic K^+ ions above a critical threshold under salt stress is critical in preventing toxic side affects associated with NaCl stress. As there is a negative electrical potential across the plasma membrane (~ -180 mV), the entrance of Na^+ will mainly be passive whereas it is less likely for Cl^- to gain entrance under these conditions unless there are low Cl^- concentrations in the cytosol (Blumwald et al., 2000; Munns, 2005). During the initial phase of salt shock the influx of Na^+ will reduce the membrane potential providing a means for Cl^- to enter down the chemical gradient, once steady state conditions and the membrane potential have been reestablished Cl^- influx will require coupling for transport, probably via unidentified Cl^-/H^+ symporters (Hasegawa et al., 2000).

Major influx of Na^+ in roots has been associated with non-selective cation channels that are weakly voltage-dependent (Davenport and Tester, 2000) or voltage independent (Amtmann and Sanders, 1999). Evidence also suggests influx of Na^+ can occur through both low and high-affinity transporters under high levels of NaCl. Under high external concentration of Na^+, Na^+ ions compete with K^+ for uptake and are able to gain entrance into the cytosol through common transport systems facilitated by the

difficulty in discriminating between Na^+ and K^+ as they have similar radii and charge (Grattan and Grieve, 1999; Blumwald et al., 2000; Yokoi et al., 2002). When the wheat low affinity cation transporter LCT1 is expressed in salt-sensitive yeast cells it results in the accumulation of high levels of Na^+ ions and decreased levels of K^+ ions under salt stress (Amtmann et al., 2001). This demonstrates that under saline conditions the transporter can mediate Na^+ influx and possibly K^+ efflux. Increased external K^+ and Ca^{2+} concentrations inhibited Na^+ uptake and rescued cell growth (Amtmann et al., 2001). The down regulation of the high affinity K^+-transporter *HKT1* in wheat transgenic lines leads to a subsequent decrease in Na^+ accumulation under high salt as compared to control plants suggesting a role for *HKT1* in Na^+ uptake (Laurie et al., 2002). In barley, NaCl treatment not only leads to an influx of Na^+ it also results in an efflux of H^+, K^+ and NH^{4+} for which the mechanism is unknown (Shabala et al., 2003). This result indicates that Na^+ ions can deplete K^+ pools by not only competing for uptake but also by stimulating efflux.

1.3 Minimizing Na^+ and Cl^- Toxicity

Na^+ and Cl^- ions that enter the cytosol are effluxed through the plasma membrane and compartmentalized into the vacuole reducing cytosolic toxicity. Sequestering Na^+ and Cl^- into vacuoles relieves cytosolic toxicity and results in an osmotic pressure that is counter balanced with the cytosolic accumulation of K^+ and organic solutes enabling the maintenance of water uptake from saline solutions (Hasegawa et al., 2000; Munns, 2005). Salt tolerance is improved by the accumulation of salt in the vacuoles of older leaves and away from young tissues that are at their photosynthesizing peak (Munns, 2005).

Unlike animals, plants do not have Na^+-ATPase or Na^+/K^+-ATPase and rely on the proton motive force produced by H^+-ATPase and H^+-pyrophosphatases to drive transport of other metabolites and ions (Blumwald et al., 2000; Hasegawa et al., 2000). In roots, the export of the majority of Na^+ and Cl^- ions that enter cells back into the medium is mediated by plasma membrane Na^+/H^+ transporters that couple downhill H^+ transport generated by H^+-ATPases, with uphill Na^+ extrusion (Blumwald et al., 2000; Hasegawa et al., 2000). In *Arabidopsis thaliana* the activity and transcription of SOS1 a plasma membrane Na^+/H^+ exchanger is regulated by SOS2 a serine/threonine protein kinase and SOS3 a calcineurin B-like calcium-binding protein. In the presence of salt-induced elevated cytosolic Ca^{2+} levels, SOS3 activates the protein kinase activity of SOS2, which in turn phosphorylates and activates SOS1 increasing Na^+/H^+ exchange activity (Halfter et al., 2000; Shi et al., 2000; Qiu et al., 2002; Shi et al., 2002). In addition to activating SOS1, mutant analysis of *sos2* and *sos*3 implicates SOS2-SOS3 in transcriptional regulation of SOS1 , additionally only in the shoots and not in the roots of sos2 mutants is the transcriptional up-regulation of SOS1 abolished under NaCl stress, implying that there are additional root-specific kinases that can increase transcription of SOS1 in sos2 mutants (Shi et al., 2000) .

In Arabidopsis Na^+/H^+ antiporters are coded for by a six member gene family, *AtNHX1-6* (Yokoi et al., 2002). AtNHX1 and AtNHX2 are localized to the tonoplasts of plant cells; their transcripts have been found in both shoot and roots (Yokoi et al., 2002). *AtNHX1, AtNHX2* and *AtNHX5* were shown to suppress Na^+ sensitivity of a yeast strain

that is deficient in *ScNHX1* which encodes a yeast vacuolar Na^+/H^+ exchanger indicating they are orthologs of yeast *ScNHX1* and compartmentalize Na^+ ions (Rus et al., 2001; Yokoi et al., 2002). Transcription of *AtNHX1* and *AtNHX2* is increased under osmotic stress and abscisic acis (ABA) treatment whereas *AtNHX5* induction is NaCl specific (Yokoi et al., 2002). Transcription of the *T. aestivum TNXH1* gene, is induced by salt stress in wheat and encodes a cation/proton antiporter that is able to suppress *nhx1* yeast sensitivity to hygromycin which is toxic in the cytosol but not if sequestered into vacuoles; hygromycin sequestration is NXH1-mediated (Brini et al., 2005).

1.4 Alleviation of Na^+ Toxicity by Ca^{2+}

High levels of Na^+ leads to a Ca^{2+} deficiency by reducing Ca^{2+} uptake, replacing cell wall and membrane-bound Ca^{2+} which reduces membrane selectivity of other cations and by reducing Ca^{2+} translocation to shoots (Maathuis et al., 2003; Munns, 2005). The addition of Ca^{2+} to the media is known to ameliorate plant Na^+ toxicity under salt stress. When Ca^{2+} was added to NaCl at 1/10 the molar concentration of NaCl , barley plants recovered maximal losses in root growth and almost eliminated efflux of H^+, K^+ and NH_4^+ from the roots (Shabala et al., 2003). Elevated Ca^{2+} concentrations have a blocking effect on Na^+ uptake through non-selective and low-affinity cation channels (Davenport and Tester, 2000; Amtmann et al., 2001; White and Davenport, 2002; Tester and Davenport, 2003), in addition, increasing Ca^{2+} concentrations shifts the equilibrium under high salinity to favor the uptake of K^+ over Na^+, increases membrane integrity and reduces K^+ leakage from roots (Grattan and Grieve, 1999) all of which result in a more favorable K^+/Na^+ ratio in plants. Ca^{2+} is also an important secondary messenger

molecule in salt stress plants and the molecular basis for calcium-mediated extrusion of sodium from cells has been identified in the SOS pathway. SOS mutants exhibit a K^+ deficiency and Na^+ sensitivity that can be overcome by the addition of external Ca^{2+} (Zhu et al., 1998; Halfter et al., 2000). Analysis of the SOS pathway indicate that Ca^{2+} has a role in Na^+ and K^+ homeostasis mediated by SOS3. The role of calcium is important in salt tolerance not only in inhibiting the uptake of Na^+ but also in signaling under salt stress.

Supplementing NaCl treatments with Ca^{2+} in salt stress experiments is a more realistic stress. In salt stress conditions under actual agricultural conditions, most crops are subjected to a $Na^+/(Na^+ + Ca^{2+})$ ratio range of 0.1- 0.7 in irrigation water or soil solutions, with the ratio increasing through evaporation with selective precipitation of Ca^{2+} over Na^+ (Grattan and Grieve, 1999). Moreover, the addition of salt to growth mediums reduces Ca^{2+} activity and without adequate supplementation of Ca^{2+} the effects seen by salt treatments may be due to impaired root membrane function (Munns, 2005) .

1.5 Salt Tolerance in Wheat, *L. elongatum* and their Derivatives

Within members of the tribe Triticeae that encompasses the major grain crops, barley, rye and wheat there is a wide spectrum of salt tolerance (Gorham et al., 1985). The wild wheat grass, *L. elongatum* is a highly salt-tolerant close relative of bread wheat, *T. aestivum* and occurs naturally in the saline environment of the littoral zones of the Mediterranean region (McGuire and Dvorak, 1981; Dvořák and Ross, 1986). Several lines have been established from crosses of the diploid *L. elongatum* (2n=2x=14, genome

EE) and hexaploid wheat, *T. aestivum* cv. Chinese Spring (2n= 6x= 42, genomes AA BB DD) in an endeavor to identify physiological and genetic mechanisms conferring salt-tolerance. This has been facilitated by the genomic polyploidy of wheat, as the addition and substitution of alien chromosomes from related species into its genome is tolerated (Dvořák et al., 1988; Schachtman et al., 1989; Omeilian et al., 1991). The cross of *L. elongatum* with *T. aestivum* cv. Chinese Spring was used to produce an octaploid amphiploid that contains the genetic material of both species (2n= 8x= 56, AA BB DD EE). The amphiploid has been found to have significantly increased salt tolerance compared to its parent Chinese Spring, (Dvořák and Ross, 1986; Dvořák et al., 1988; Schachtman et al., 1989; Omeilian et al., 1991) although it did not have the full tolerance of *L. elongatum* (Dvořák and Ross, 1986).

For the salt-tolerance of the amphiploid to become apparent, application of sufficient salt-stress is required (Omeilian et al., 1991). Under conditions of no stress the performance of both Chinese Spring and its amphiploid are alike, both having similar plant dry weight and seed yield (Dvořák and Ross, 1986). Under saline conditions the increased salt-tolerance of the amphiploid is reflected in the significantly greater plant biomass and seed production than its wheat parent (Dvořák et al., 1988; Schachtman et al., 1989; Omeilian et al., 1991). Field studies conducted with varying salinity levels over three years demonstrated that the amphiploid was far superior to Chinese Spring in plant height, biomass, grain yield and grain weight (Omeilian et al., 1991). When grown in solution cultures of 100 mM NaCl the amphiploid plants are able to produce twice the amount of plant dry matter and seeds as compared to Chinese Spring and 30 times as

much in 250 mM NaCl solution (Dvořák et al., 1988). The superiority of the amphiploid to Chinese Spring under imposed salinity indicates that adaptations to salt of *L. elongatum* are expressed in the genetic background of wheat (Dvořák et al., 1988; Schachtman et al., 1989).

The enhanced tolerance of the amphiploid under salt stress is correlated with a higher inclusion of K^+ and exclusion of Na^+ and Cl^- ions (Schachtman et al., 1989; Omeilian et al., 1991). When grown in salt solutions the amphiploid transports less Na^+ and Cl^- to the shoots, accumulates significantly less of these ions in its leaves and is able to maintain higher water content resulting in lower concentrations of Na^+ and Cl^- relative to Chinese Spring. The shoots of the amphiploid also have higher K^+ levels indicating a greater capacity to discriminate between Na^+ and K^+ (Schachtman et al., 1989). These characteristics result in a higher Na^+/K^+ ratio in the leaves. In the roots, there is little difference in the concentrations of Na^+, Cl^- and K^+ between the amphiploid and Chinese Spring (Schachtman et al., 1989).

Other derivatives of the cross between *L. elongatum* and Chinese Spring are also available providing a valuable resource for examining the effects of single chromosome pairs or arms in conferring salt-tolerance. Each of *L. elongatum's* seven chromosomes have been introduced into Chinese Spring via disomic substitution and addition lines, in which a chromosome pair of *L. elongatum* is substituted for one of Chinese Spring's or are added to the genome of Chinese Spring, respectively. A disomic addition line has one extra pair of *L. elongatum* chromosomes added to the wheat genetic background.

Disomic substitution lines have a *L. elongatum* chromosome pair substituted into the genetic background of wheat. (Disomic substitution lines are designated as DS3E(3A) in which a 3E pair of chromosomes has substituted a 3A pair from wheat. There are 21 possible disomic substitution combinations and 7 possible disomic addition lines). All of disomic addition lines and disomic substitution lines (except DS4E(4A)) were tested under salt-stress conditions and were found to have reduced dry weight and seed yield as compared to the amphiploid (Dvořák et al., 1988; Omeilian et al., 1991). This implied that more than one chromosome would need to be transferred to obtain the same degree of salt-tolerance as the amphiploid. While there is reduced salt-tolerance as compared to the amphiploid, disomic substitution lines 2E(2D), 3E(3A), 7E(7A), 7E(7B) and 7E(7D) have increased tolerance relative to Chinese Spring when grown in solution culture of 250 mM NaCl (Dvořák et al., 1988). Through diallel crosses between disomic addition lines the authors established that chromosome 3E, 4E and 7E appear to have an additive effect on both plant dry weight and on seed yield (Dvořák et al., 1988). Field studies demonstrated that all disomic substitution lines except 6E increased salt tolerance, with chromosome 3E having the greatest effect in all treatments and accounting for half of the difference observed between the amphiploid and Chinese Spring. Individual contributions of disomic substitution lines 1E, 2E, 5E and 7E accounted for the other half of the variance in salt tolerance (Omeilian et al., 1991).

1.6 Microarray in Stress Response Pathways

Different methodologies can be applied in molecular biology to study transcriptional levels of genes. Northern blot analysis utilizes synthesized nucleic-acid probes which hybridize to membrane-bound target mRNA to study gene transcriptional

levels. RT-PCR uses reverse transcriptase to produce cDNA either using target specific primers or by a Poly-A primer followed by subsequent rounds of PCR amplification with gene specific primers. The yield of the resulting target DNA is proportional to the original mRNA transcript level. Northern blots and RT-PCR are effective methods to study transcriptional levels of few genes as probes or primers must be synthesized for each target and both methodologies are relatively inexpensive.

Microarray technology enables the global expression patterns of numerous genes to be examined simultaneously. Microarray probes are either oligonucleotides or PCR products derived from cDNA clones that are fixed to a glass slide, cDNA from the mRNA pools of two given samples of interest are labeled with different fluorophores and hybridized to the probes. The two fluorophores such as Cy3 and Cy5 have differing excitation and emission wavelength profiles enabling the two hybridized samples to be distinguished and compared relative to each other. This provides information about the relative abundance of a given transcript in a sample for each probe on the microarray. To study the effect of an experimental treatment on transcriptional levels the microarray design usually involves the comparison of a cDNA population derived from control tissue and a population derived from a treated sample. Increases or decreases in mRNA levels are measured as the ratio of florescent signals from the two samples. That is, higher florescence emissions for a treated sample emissions than that of the control sample indicate gene induction under treatment conditions.

Microarray analysis is being used in plant molecular biology to study the responses under differing treatments and is providing useful insight into regulation of

genes that would otherwise not be obtained by the use of small scale transcript profiling. Several experiments have used microarray technology to look at the transcriptional profiles under salt stress. In Maize, a 7943 cDNA microarray experiment was conducted by Wang et al (2003) to examine responses in root tissues to salt stress over time-course of 1 hour to 72 hours. They found that 11% of genes responded to salt stress in at least one time point and there was transient expression with a burst at 3hr followed by a decline. Clustering analysis revealed that there is succession of regulation of salt responsive genes in multiple signaling and response cascades. Kawasaki et al (2001) used a 1728 cDNA microarray to analyze the transcriptional profiles of two rice lines with differing salt tolerance levels over 15min to 1-week time course. Pokkali, a moderately salt-tolerant line, was found to alter transcript levels within 15 min after the beginning of salt stress and 10% of the genes were found to have altered expression at 1 hr. Transcriptional regulation persisted but became less pronounced over time. In contrast, the salt-sensitive line IR29 died within 24 of salt stress, transcription comparison showed that response to salt stress was delayed and a recovery period found in Pokkali after 3hr salt stress was not observed in IR29. Microarray experiments have also been used to compare responses to multiple abiotic stresses. Microarray analysis conducted by Seki et al (2002) on the expression profile of 7000 full-length cDNA probes from *Arabidopsis thaliana* under drought, cold and high salinity stresses found a total of 524 genes had that highly altered expression. Twenty-two genes were altered under all three stresses. Cold stress had the fewest number of up or down regulated genes in common with the other stresses. The greatest overlap was between high-salinity and drought, with 141 genes having similar altered expression, which indicates a significant

overlap in signaling pathways for these stresses. Using an 8,100 oligonucleotides probe set, the effects of cold, salt and osmotic stress (mannitol) were monitored in *Arabidopsis thaliana* by Kreps et al (2002). The study found that the expressions of 30% of genes were altered by the stresses with the majority being stimulus specific. However, among genes that were affected by more than one stress, the greatest overlap in response was again found to be between osmotic stress and salt. There were also tissue specific differences in expression profile comparisons between roots and leaves (Kreps et al., 2002).

Although much information has been gathered on the mechanisms of salt-tolerance and the transcription of specific genes, until recently knowledge about the coordination of genes elucidating a salt response was lacking due to the unavailability of a technology to study global expressions patterns. With the availability of microarray technology comparisons of global expression patterns under limitless experimental conditions and the use of different tissues provides the means for novel gene discovery and increased understanding of the regulation of gene expression.

The physiological increased salt tolerance of an amphiploid produced from a cross of *L. elongatum* and Chinese spring is associated with addition of *L. elongatum* chromosomes into wheat thus providing a genetic system to understand the multigenic trait and identification of genes that are involved in increased tolerance. By the use of microarray technology multiple genes involved in coordinating a response can be examined simultaneously

1.7 Objectives

Due the genetic complexity of salt tolerance, microarray technology has the advantage of characterizing changes in mRNA levels for thousands of genes simultaneously. Two microarray experiments were conducted using microarray chips consisting of 5728 cDNA amplicons from wheat to examine the effects of salt treatment on gene expression. The first experiment used RNA samples from wheat cultivar Norstar collected at different time points throughout the application of salt stress to look at the alterations of mRNA levels and to identify which genes are altered and at what stage in response to salt stress in wheat. The second microarray experiment compared the expression profiles of the wheat cultivar Chinese Spring and two cryogenic derivatives that have varying degrees of salt tolerance; one an octaploid amphiploid from the cross of *L. elongatum* with *T. aestivum* cv. Chinese Spring containing the genetic material of both species (2n= 8x= 56, AA BB DD EE) and a disomic substitution line with chromosome pair 3A of Chinese Spring being replaced by the chromosome pair 3E from *L. elongatum.* The amphiploid was chosen to characterize salt tolerance as it has been found to have significantly increased salt tolerance compared to its parent Chinese Spring, (Dvořák and Ross, 1986; Dvořák et al., 1988; Schachtman et al., 1989; Omeilian et al., 1991) and disomic substitution lines of chromosome 3E in field studies were found to account for half of the difference in salt tolerance between the amphiploid and Chinese Spring (Omeilian et al., 1991) . Comparison of the cryogenic lines and Chinese Spring provides insight into the genes involved in conferring a greater salt tolerance to the amphiploid as compared to Chinese Spring. By also using DS3E(3A) genes controlled by genes or

located on chromosome 3E that provide increased salt tolerance can be identifed by comparing to both the amphiploid and Chinese spring while differences in gene expression between DS3E(3A) and the amphiploid may indicate that these genes found elsewhere in the *L. elongatum* genome

2.0 Material and Methods

2.1 Root Growth Inhibition by NaCl

Triticum aestivum cultivar Norstar seeds were surface sterilized, stratified for three days at 4°C in the dark and then germinated at 22°C on sterile wet filter paper supported by near vertical slant boards moistened with sterile tap water in a growth chamber. Seedling were grown for ten days and then transferred into 10 L hydroponic tanks with modified Hoagland's solution (Table 1). After 36 days of growth in hydroponic solution the tanks were subjected to NaCl + $CaCl_2$ treatments using a 7:1 molar ratio of Na^+ to Ca^{2+}. The control tank contained only modified Hoagland's solution. Treatments tanks had 50 mM NaCl + 7.14 mM $CaCl_2$; 100 mM NaCl + 14.29 mM $CaCl_2$ and 150 mM NaCl + 21.43 $CaCl_2$ mM added to modified Hoagland's solution (pH 6.02-6.10). The growth chamber was maintained at 22°C/15°C day/night temperatures with a cycle of 11 hours light and of 13 hours dark. There were eight plants per tank. The length of the longest root was measure at 6 hours, 24 hours, 48 hours and 144 hours after the commencement of salt treatment for each plant. The average growth for the longest root was used to determine the salt concentration at which there is 50% inhibition of the root growth rate.

Table 1. Modified Hoagland's Solution

	Nutrient	Solution Concentration
Macronutrients	$NH_4H_2PO_4$	1.00 mM
	KNO_3	3.00 mM
	$Ca(NO_3)_2$	4.50 mM
	$MgSO_4$	0.75 mM
	KH_2PO_4	0.50 mM
	Fe-EDTA	50.00 mg/L
Micronutrients	KCL	50.00 µM
	H_3BO_3	50.00 µM
	$MnSO_4$	10.00 µM
	$ZnSO_4$	2.00 µM
	$CuSO_4$	2.00 µM
	H_2MoO_4	1.50 µM

2.2 Plant Material and Growth Conditions

A time course experiment for salt stress response was done with the hexaploid wheat (*T. aestivum)* cultivar Norstar. A second time course study for the comparison of three genotypes was done with *T. aestivum* cv Chinese Spring, an octaploid amphiploid developed from a cross between *Lophopyrum elongatum* (Host) A. Love (syn Elytrigia elongata, Thinopyrum elongatum, Agropyron elongatum) and *T. aestivum* and thirdly, the disomic substitution line 3E(3A) in which chromosome 3A of wheat has been replaced with chromosome 3E from *L. elongatum*. Chinese Spring wheat will be referred to as CS, the amphiploid as AgCS, and the disomic substitution line as DS(3E)3A. The seeds for both experiments were surface sterilized and stratified at 4°C for three days. Seeds were then placed on moistened filter paper in petri dishes, and germinated in a growth chamber. Light was provided with fluorescent and incandescent bulbs with a long day

light cycle; 16 hours lights: 8 hours dark. The light was provided by gradient with the first seven hours having 42 μmoles/m^2/s and the following nine hours being 93.8 μmoles/m^2/s. The growth chamber was maintained at 22 ± 1°C / 20± 1°C: day/night temperatures.

After three to five days of growth on Petri dishes, when roots were sufficiently long, the seedlings were transferred to six hydroponic tanks containing modified Hoagland solution, pH 5.8-6.0 (Table 1). The pH of the solution was adjusted to 5.8- 6.0 by the addition of KOH or HCL. Solution levels were replenished daily with deionized water if needed and changed every seven days with fresh modified Hoagland's solution. For the genotype-comparison array, each tank contained seedlings from each genotype. The plants were grown under the same light and temperature conditions as in the germination period.

2.3 Norstar Time-Course Array Plant Treatment

When plants reached day 18 after germination the growth solution was changed. Hoagland's solution was used for three control tanks, in the three treatment tanks the growth solution was replaced with Hoagland's solution supplemented with 150mM of NaCl and 15mM of $CaCl_2$. Three biological replicates of ten plants each were harvested at 6 hrs, 24 hrs and 72 hrs for salt-treated plants and at 24 hrs for control plants. The plants were removed from each tank by cutting the roots from shoots and immediately freezing each sample in liquid nitrogen. Samples were stored at –80°C.

2.4 Genotype-Comparison Array Plant Treatment

Nine days after transfer to the hydroponic tanks, aluminum mesh dividers were placed between the roots of different genotypes to prevent entanglement. At twenty-six days from the beginning of germination, the salt- treatment was applied. The hydroponic tanks were rinsed and fresh modified Hoagland solution was used in three of the tanks to be used to grow control plants in three biological replicates. The other three tanks were subjected to a salt treatment by replacing the solution with modified Hoagland plus 150mM NaCl and 15mM $CaCl_2$, producing three "salt treated" biological replicates. The solution levels for both the control and treatment tanks were replenished daily with deionized water. For the Microarray samples, plants were harvested after 3 days of treatment and the roots and the shoots separated and frozen in liquid nitrogen and stored at –80°C.

2.5 RNA Extraction and Purification

For the Norstar time-course microarray, RNA from roots of three biological replicates each comprising ten salt treated plants or ten control plants were used. Samples were taken at 6 hrs, 24 hrs and 72 hrs of "salt treated" plants and one-day control plants. RNA in the genotype-comparison microarray analysis was extracted from the root tissue of each genotype CS, DS(3E)3A and AgCS from each of the three biological replicates of control plants and plants that were "salt treated" for 72 hrs. Each biological replicate sample included four to eleven plants.

Two to four grams of root material was homogenized in liquid nitrogen, the RNA was extracted using TRIZOL reagent according to the manufacturer's protocol (Invitrogen Life Technologies) with the inclusion of the optional "proteoglycan and polysaccharide purification" step. The resulting RNA pellets were dissolved in DEPC treated water. The RNA quality was assessed by agarose gel electrophoresis and from the A260/A280 ratio of the spectrophotometer readings. The RNA samples were purified with RNeasy columns (Qiagen) according to the manufacturer's protocol and re-quantified using A260 spectrophotometer readings.

2.6 *Lophopyrum elongatum* EST Sequence Comparison to *Triticum aestivum*

To determine if there was adequate sequence similarity between *L. elongatum* and *T. aestivum* to conduct microarray experiments of a *T. aestivum* cDNA amplicon microarray, the nucleotide sequences of 89 *Lophopyrum elongatum* EST sequences were compared by Blastx (Autchul el al 1997) to the wheat EST database from the Genome Canadian supported program in Functional Genomics of Abiotic Stress (FGAS).

2.7 Microarray Construction

The microarray chips were constructed in the laboratory of Dr. P. Gulick (Concordia University, Montreal, Canada) by Dr. A. Dryanova and Dr. A. Monroy. The array consisted of 5728 cDNAs, 26% of the clones are from the Genome Canada program Functional Genomics of Abiotic Stress (FGAS) and 74% from the from the NSF-USDA (USA) wheat EST collection. The array included 1630 features that were selected by data mining of FGAS and the NSF wheat EST databases with predicted regulatory gene sequences from *Arabidopsis thaliana* and rice. The potential regulatory sequences

include RNA-binding proteins, protein kinases, protein phosphatases, E3 ubquitin ligases, glycosylphosphatidylinositol anchored proteins, GTP binding proteins and 11 families of transcription factors. All candidate genes were resequenced. The remaining cDNA amplicons in the microarray were random clones from FGAS and from a unigene set of NSF wheat EST clone collections.

The cDNA inserts for each EST were amplified from the plasmids with universal M13 forward and M13 reverse oligonucleotide primers by PCR. PCR products were subjected to quality assessment by gel electrophoresis, and then purified by Montage PCR 96 plates (Millipore Corp., USA). Purified products were completely dried under vacuum and dissolved in Pronto Universal Spotting solution (Corning Life Sciences). The resulting cDNA solutions were spotted on Corning UltraGAPS slides (Corning Life Sciences) using a SDDC-2 Virtek arrayer (Engineering Services Inc., Toronto) equipped with Stealth SMP3 pins (Telechem International) in a 48 pin configuration. Each clone product was spotted once per microarray slide.

2.8 Target Preparation Norstar Time-Course Array

For reverse transcription, 1.5 µl of 50 µM oligo dT primer (24 mer) was added to 30 µg of total RNA for a final volume of 19 µL. The mixture was heated at 70°C for 10 minutes, cooled on ice for 2 minutes and centrifuged briefly. A 9.1µl aliquot of reaction master mix was added for a final concentration of 1 X first-strand buffer (Superscript III, Invitrogen), 5 mM DTT, 1 X aa-dNTP and 1.3 U of RNaseOUT (Invitrogen). To each sample mixture 200 U of superscript III RT was then added, mixed and then incubated at

46°C for 3 hours. RNA was removed from the resulting cDNA by hydrolysis by incubating at 70°C for 10 minutes with 3.0 µl of 2.5 M NaOH. The hydrolysis reaction was then neutralized with 15 µl of 2 M HEPES.

The cDNA was cleaned by mixing with 4µl of 3M sodium acetate (pH 5.2), followed by 105µl of 99% ethanol and incubated at -70°C for a minimum of 30 minutes and then centrifuged at room temperature for 20 minutes. The supernatant was removed and the pellet was gently washed using 350 µl of 70% ethanol. The ethanol was removed and the pellet was dried at room temperature.

Target labeling Cy3 and Cy5 dyes were re-suspended in 73µl of DMSO and stored at -20°C in the dark. The cDNA pellet was dissolved in 4.5 µl of 0.1M $Na(CO_3)_2$, 4.5µl of the appropriate dye was added and incubated in the dark at room temperature for one hour. In the Norstar time course array, Cy5 was used for common reference labeling and Cy3 for experimental sample (salt-treated and non treated control samples). Samples are incubated in the dark for 1 hour at room temperature.

Each Cy3 and Cy5 labeled sample was purified by adding 35µl of 0.1M sodium acetate (pH 5.2) and 250 µL of PB buffer and applying the sample to QIAquick PCR purification column (Qiagen) and following the manufacturer's protocol. Two modifications were applied to the protocol: instead of one wash with 750µl of PE buffer two washes with 400µl of PE buffer are used and samples were eluted using 40µl of EB buffer that was added to the column and left for 3 minutes at 50 °C before centrifugation

for one minute, the eluate was then applied to the column again and incubated for 3 minutes at 50 °C before the final centrifugation of the cy-labeled cDNA.

2.9 Pre-hybridization Norstar Time-Course Array

An aliquot of 100 µl of pre-warmed DIG Easy Hybridization buffer (Roche) was applied onto a chip and covered with a Hybri-Slip. The slide was placed in a hybridization box and incubated at 42°C 30-60 minutes. The slide was then removed from the box and placed into a 50 ml Falcon tube containing 0.1X SSC. The cover slip was removed inverted a couple of times and the slide transferred for rinsing into a 50 ml Falcon tube containing MilliQ water and inverted. To dry the slide, it was transferred to a 50 ml Falcon tube with the bottom stuffed with a piece of Kimwipes and then centrifuged dry at 1000 rpm for 2 minutes.

2.10 Hybridization Norstar Time-Course Array

The experimental design for the hybridizations for the Norstar time-course used a common reference design in which each experimental sample was compared to a common reference sample that consisted of pooled RNA from the three replicates of control 24 hrs non treated plants. Hybridizations were carried out by Dr. M. Monroy. Three hybridizations corresponding to each of three biological replicates were done for control plants and salt treated plants for 6 hr, 24 hrs and 72 hrs.

The Cy3 and Cy5 labeling reactions were combined together in one tube for each sample and then concentrated using a speedvac without heat, 100 µl of DIG Easy Hybridization buffer (Roche) was added, heated to 95°C for 2 minutes and cooled at RT for 5 minutes. The hybridization solution was applied to the microarray slide which was then covered with a hybrid-slip and incubated overnight at 37°C. The slides were washed in 50 ml falcon tubes containing 1X SSC and 0.1 %SDS until cover slips could be removed easily. The slides were washed three times for 10 minutes at 50 °C with 1X SSC and 0.1% SDS and rinsed two times for 5 minutes with 0.1 X SSC at RT. Slides were dried by placing them in a 50 ml falcon tube with kimwipes at the bottom and centrifuging at 1000 rpm for 2 minutes.

2.11 Microarray Hybridizations and Scanning for the Genotype-Comparison Array

All microarray hybridizations for the genotype-comparison microarray were conducted at Queens University (http://www.queensu.ca/microarray/). The microarray experiment used a common reference design in which all the samples from each genotype were compared to a common reference sample consisting of a pool of RNA from the control CS replicates. This design facilitates multiple comparisons among genotypes.

The target cDNA was generated from 20 µg total RNA by the 3DNA Array 900 Detection kit, and hybridized according to the manufacturer's protocol (Genisphere Inc., Hatfield, PA). Microarray slides were imaged at a resolution of 10 µm using a ScanArray4000 scanner interfaced with QuantArray software (Version 3.0, GSI

Lumonics, Wilmington, MA). The laser power range was 72 to 90% at a PMT voltage of 70%.

2.12 Data Analysis

Microarray imaging data for the genotype-comparison array and the Norstar time-course array was submitted to the website expressyourself (Luscombe et al., 2003) which performs background corrections and LOWESS and LOESS normalizations (Cleveland and Grosse, 1991). A "relative level" of fluorescent signal for each feature was calculated as the ratio of the fluorescence signal value of the control or treatment samples divided by the common reference sample fluorescence values. Relative levels of fluorescent signal for control and treated samples from the genotype-comparison array were analyzed by Student's *t* test in Excel and by two-way analysis of variance (ANOVA) using SPSS Statistics Software. In ANOVA, treatment (control or 150 mM NaCl + 15 mM $CaCl_2$ treatment) and genotype were independent variables for the genotype-comparison array. In the Norstar-time course array, one-way ANOVA was used for control and salt-treated time-points to identify genes with significant changes in expression. Features with significant ANOVA P values ($P<.05$) were analyzed by Student's *t*-test in pair-wise between treatment and control samples at each time-point. In both the Norstar time-course and genotype-comparison arrays, features with low signals were removed from the analysis, this was evaluated by calculating the sum of the Cy3 (control or treated samples) and Cy5 (common control reference sample) fluorescence emissions if the value was less than double the mean value plus one standard deviation of the background emission it was flagged. The mean ratios of the Cy3 florescence (from either the control or 150 mM NaCl + 15 mM $CaCl_2$ treated plants treatment samples) and

Cy5 florescence (common reference samples) emissions for each feature was used as a measure of the "relative level" of gene expression in each genotype under salt-treated or non-treated conditions. In both array experiments the "change in gene expression" under "salt treated" (150mM NaCl + 15mM $CaCl_2$) conditions was calculated as the ratio of the "relative level" of the treated sample over the "relative level" of the control sample for the given genotype.

Hierarchical Clustering (HCL) and *k*-mean clustering (KMC) analysis were performed for the genotype-comparison array using TM4 Tigr MeV suite (Saeed et al, 2003). For HCL and KMC analysis, log2 values for the change in gene of expression levels were used for genes that were found to have ANOVA treatment or treatment by genotype interaction significance and a 1.5 fold of greater change in gene expression in at least one genotype. KMC clustering analysis was also performed for the relative levels of control and 150 mM NaCl samples for genes that were found to have ANOVA treatment by genotype interaction significance.

3.0 Results and Discussion

3.1 Root Growth Inhibition by NaCl Treatment

Norstar plants were grown hydroponically and subjected to different concentrations of NaCl supplemented with calcium to assess growth inhibition. The length of the longest root was measured at 24 hrs, 48 hrs and 6 days to study the effect of root growth with different levels of NaCl. As expected control samples had the greatest growth rates, plants treated at 50 mM NaCl had slightly reduced growth and 100 mM and 150 mM NaCl treated samples had over 50% reduction in growth at each time-point (Fig 1). Growth inhibition did not have a linear relationship to NaCl concentration. Over the time course of the treatment the differences in growth between control plants and treated plants increased, especially between the control and the 100 mM and 150 mM NaCl treated plants. Plants treated with 150 mM NaCl and 21.43 mM $CaCl_2$ had approximately 50% root growth reduction and this concentration of salt was used for subsequent treatment comparisons of Chinese Spring with the wheat- *Lophopyrum* amphiploid, and the disomic substitution line. The 150 mM level of NaCl treatment is high enough to sufficiently challenge the amphiploid as it has been previously shown to have physiological superiority for plant weight and seed yield compared to wheat when grown at 100 mM (Dovrak et al, 1988; Omeilian et al., 1991) and does cause full cession of growth in wheat.

Figure 1.Root Growth in Norstar Under Salt Stress

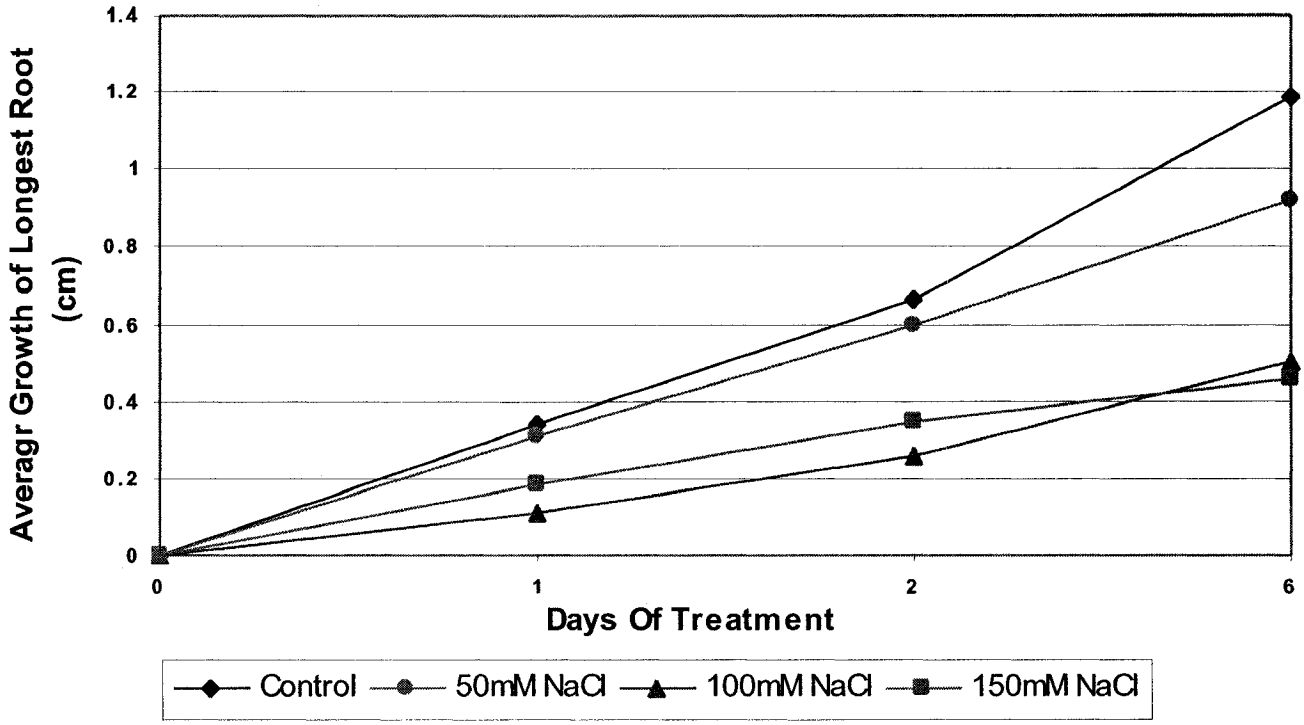

Figure 1. The effect of different concentrations of NaCl on root growth of the *T. aestivum* cultivar Norstar.

3.2 Norstar Time-Course Salt Treatment Microarray

The transcript levels in roots of the wheat cultivar Norstar (Nst) were characterized over a 72 hour time-course of a 150 mM NaCl + 15 mM $CaCl_2$ salt-stress. The array consisted of 5728 cDNA amplicons including 5170 unique genes. These include 1631 ESTs with high sequence similarity to several classes of signaling and regulatory associated genes. The cDNA used in the hybridization were from the roots of wheat cultivar Norstar (Nst) plants that were subjected to 150 mM NaCl + 15 mM $CaCl_2$ treatment for 6 hrs, 24 hrs and 72 hrs. The control sample for this array was plants harvested 24 hr after transfer to modified Hoagland's solution without salt.

A common reference experimental design was used. The common reference was a pool of the mRNA from three biological replicates of control plants (Fig 2). One-way ANOVA was performed on the expression data from all the arrays to identify genes that were significant ($P \leq 0.05$) for treatment effects. Of the 5360 genes analyzed in the Norstar time course array, 1238 genes had significant P values ($P \leq 0.05$). Genes with significant P values in ANOVA were further classified as 1) genes that also had at least a 2 fold or 1.5 fold changes in expression relative to the control and 2) genes that had a significant P value in pair wise comparison of individual time points in between treated samples and control samples and a ≥ 1.5 fold change between respective relative expression values.

Figure 2. Experimental Design of Norstar Time-Course Array

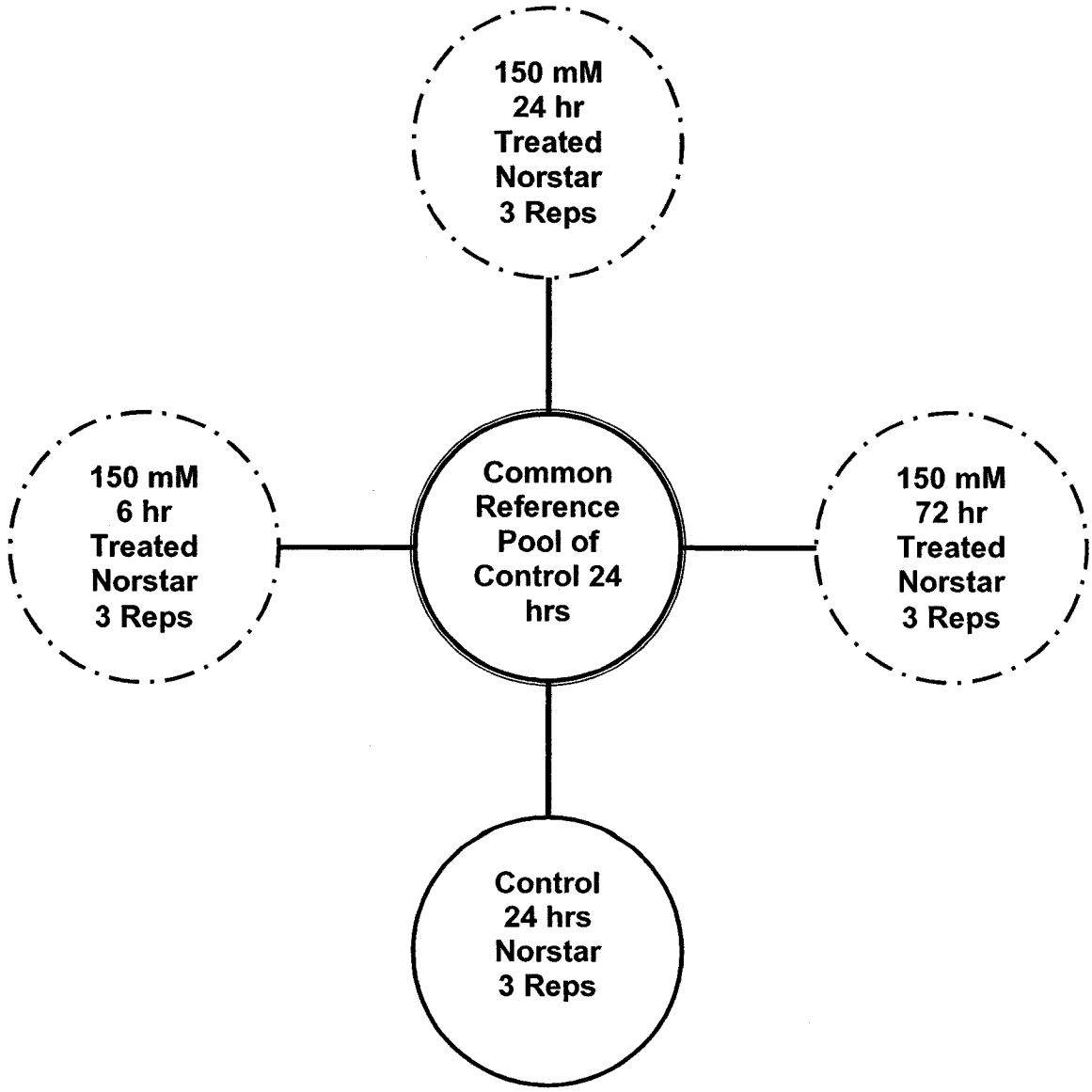

Figure 2. The experimental design is based on a series of pairwise comparisons between biological triplicates of 150 mM NaCl 6 hrs, 24 hrs, 72 hrs treated plants and 24 hrs control plants to a common reference. The common reference consists of pooled 24 hrs control Norstar RNA samples.

Among the genes that had significant P values in ANOVA, there were 59 genes found to have ≥ 2 fold changes in mRNA levels under salt stress and 229 genes that had ≥ 1.5 fold changes (Table 2). Relatively modest changes in expression that were sustained over several time points may be classified as significantly changed by ANOVA. To study a larger spectrum of regulated genes during the time-course of the experiment, genes with a significant P- value and a threshold value of at least a 1.5 fold change in expression level in salt treated plants compared to control treated plants for at least one time-point were included in the discussion. Fifty-one of the significant genes did not have control sample relative levels above the background cutoff and changes in gene expression ratio (treatment sample relative level/control relative level) under salt-stress at the different time-points could not be determined. However, as the relative levels for salt-stress treatment (treatment sample/common reference) at different time-points have ANOVA significance that is confirmed by *t*-test significance they are included in Supplemental Table 1 with all genes determined by ANOVA to be significant. Moreover, 18 of these genes did have ≥ 1.5 –fold differential expression of relative mRNA levels between time-points and are detailed below.

Norstar genes transcripts levels were found to have a biphasic pattern of gene expression changes, under NaCl stress. At six hrs of salt stress, the expression of 118 genes was significantly up or down regulated by at least 1.5 fold. At 24 hrs the expression of only 58 genes was significantly changed whereas at 72 hrs 133 genes were significantly changed in mRNA levels (Figure 3a). A biphasic mRNA response under salt stress has been previously been reported for early salt induced (ESI) genes in wheat

(Galvez et al., 1993). In addition, a similar pattern of expression under salt stress was also reported in Arabidopsis where a 4-fold reduction in the number of genes with significant changes in expression was noted between 3 hrs and 27 hrs (Kreps et al., 2002). However as no further time-points were studied it was not established whether there is a biphasic response to salt in Arabidopsis.

Among these genes there were 159 genes that were significantly regulated at only one time-point, 59 genes were exclusively up or down-regulated at 6 hrs, 20 genes at the 24 hrs time point and 80 genes at the 72 hrs (Figure 3b). There were 60 genes with changes at two time-points and 10 genes were significantly up or down-regulated at all three time-points. The number of induced genes is greater than the number of repressed at all the time points. A summary of all genes with a significant P value from ANOVA and a change $\geq$ 1.5 fold is provided in Table 2. Differences in the regulation of genes between time-points demonstrate that the identification of a gene as salt-stress regulated is dependent on the sampling time. Moreover, the low proportion of stress regulated genes that were detected at more than one time-point indicates that changes in gene expression in response to salt-stress are transient and dynamic.

In addition to the detection of changes of expression level at each time-point under salt-stress relative to untreated controls, a comparison of steady state mRNA levels between each time-point was done. Differentially regulated genes can be detected which have a $\geq$ 1.5-fold difference between the salt -stressed time-points and may not necessarily be $\geq$ 1.5 fold regulated between salt-treated and control samples, for example genes that are

moderately repressed (≤ 1.5 fold) at early time points and moderately induced (≤ 1.5 fold) at later time points relative to the controls, will show greater changes (≥ 1.5 fold) when comparison between treatment time points are made. Comparison of salt-treated samples between time-points identified 220 genes in total with a ≥ 1.5-fold difference in transcript levels between two of the salt treatment time-points and with significant P values in ANOVA and Student's *t*-test. There are 106 genes that have a ≥ 1.5 fold differences in RNA levels at 6 hrs of treatment compared to 2 4hrs of treatment. Between 6 hrs and 72 hrs there are 129 genes and between 24 hrs and 72 hrs there are 108 genes with a ≥1.5-fold difference in RNA levels (Table 2 and Fig 4.). Among the 220 genes that are regulated between time points, 139 genes also have ≥1.5-fold differences in gene expression levels in a least one salt treated time point compared to the control sample. This indicates significant differential regulation of RNA levels for those genes at different time-points. The remaining 91 genes only gave significant differences between time-points and not between treated and control plants.

Figure 3. Distribution of Regulated Genes in the Norstar Time-Course Array

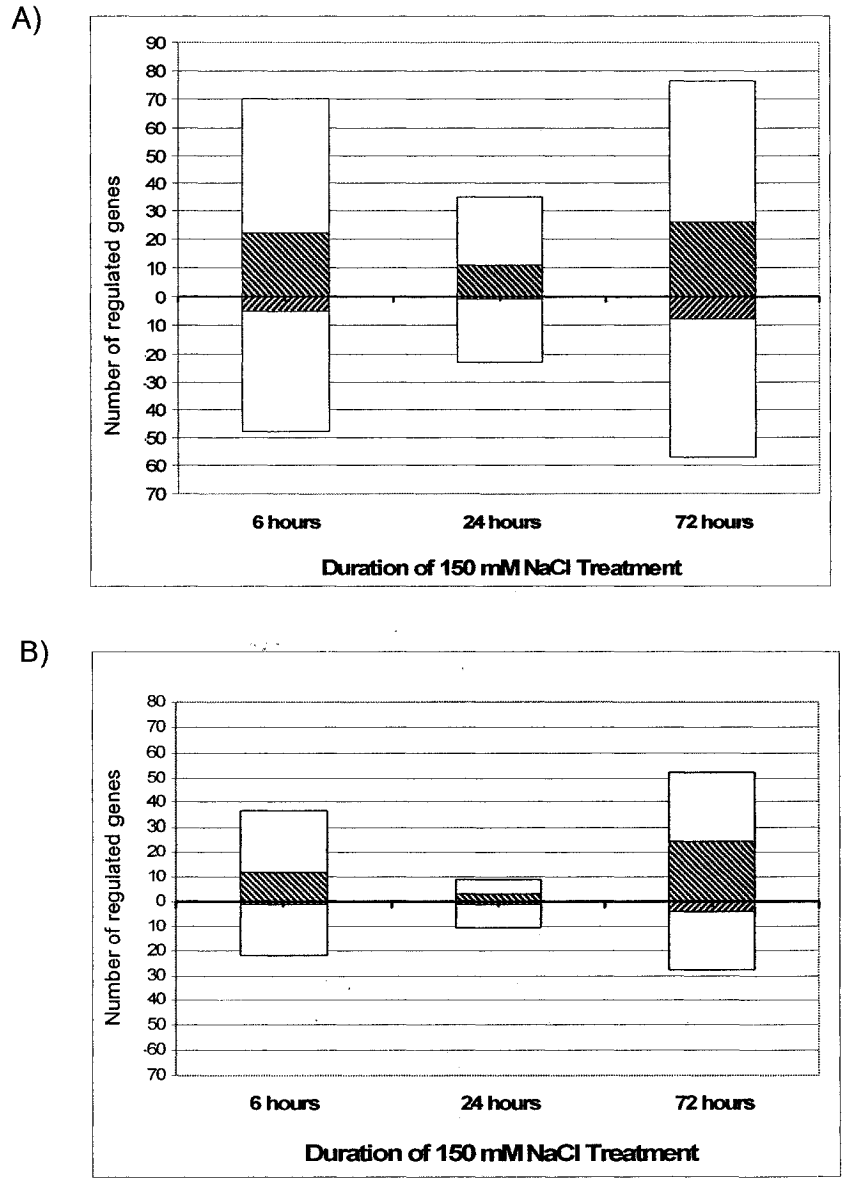

Figure 3. The distribution of genes with significant changes (ANOVA, $P \leq 0.05$; and $\geq$1.5-fold change in expression level at least one time-point) during a150mM NaCl treatment in Norstar wheat. Bars above the x-axis represent the number of up-regulated genes, bars below down-regulated. Shaded bars represent genes that have equal or greater than a 2-fold change A) The number of genes that are $\geq$ 1.5 fold regulated at 6 hours, 24 hours and 72 hours. B) The number of genes that are $\geq$ 1.5 fold regulated at only one time point (either at 6 hours, 24 hours or 72 hours) after salt stress has been applied.

Table 2. Norstar Time-Course Array of Significantly Regulated Genes

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr017_L19	14-3-3 protein homologue	1.38	0.89	**1.86**	**1.54**	0.74	**0.48**
Tr015_F07	22 kDa drought-inducible protein	0.81	1.35	1.02	**0.60**	0.80	1.32
Tr005_C05	2-oxoglutarate/malate translocator	**1.56**	1.47	1.46	1.07	1.07	1.01
Tr004_G16	33 kDa polypeptide of water-oxidizing complex of photosystem II	0.90	1.08	1.47	0.83	**0.61**	0.74
Tr017_H12	60S ribosomal protein L44	**0.65**	**0.63**	1.01	1.03	**0.64**	**0.62**
Tr016_D08	60S ribosomal protein, L10'	**1.50**	0.99	1.12	**1.52**	1.34	0.88
Tr007_L10	ABC transporter	ND	ND	ND	**0.66**	1.04	**1.56**
Tr002_B21	actin depolymerization factor-like protein	ND	ND	ND	0.87	**0.65**	0.74
Tr002_F05	actin depolymerization factor-like protein	**1.84**	1.08	1.32	**1.70**	1.39	0.82
Tr015_B14	actin depolymerization factor-like protein	0.92	1.14	0.75	0.81	1.23	**1.51**
Tr005_M06	ADH glutamate dehydrogenase	**2.34**	1.20	1.29	**1.95**	**1.82**	0.93
Tr009_H21	alanine aminotransferase	**0.63**	1.06	0.78	**0.59**	0.80	1.35
Tr005_A21	aldehyde dehydrogenase	**1.69**	1.36	1.08	1.25	**1.57**	1.26
Tr009_I03	alpha purothionin	0.85	1.00	**1.76**	0.85	**0.48**	**0.57**
Tr002_D21	alpha-amylase	1.15	0.87	1.35	1.32	0.86	0.65
Tr016_F23	alternative oxidase	0.88	**0.60**	**0.64**	1.46	1.37	0.93
Tr002_O19	AP2	0.81	1.15	1.25	0.71	**0.65**	0.92
Tr002_E01	AP2 domain transcription factor family	0.69	0.88	**0.64**	0.79	1.08	1.38
Tr011_D14	AP2 transcription factor	1.25	**1.54**	1.18	0.82	1.06	1.30
Tr014_J13	AP2 transcription factor	0.98	1.36	0.87	0.72	1.13	**1.58**
Tr014_L21	AP2 transcription factor	0.89	0.91	**0.63**	0.97	1.40	1.44
Tr014_L23	AP2 transcription factor	0.84	0.79	**0.59**	1.07	1.42	1.33
Tr014_P11	AP2 transcription factor (EBP)	0.86	0.77	**0.60**	1.11	1.44	1.29
Tr014_J20	AP2 transcription factor (CBF-like)	0.76	1.06	0.69	0.72	1.10	**1.53**
Tr014_J14	AP2 transcription factor (CBF-like)	1.20	0.88	0.80	1.36	**1.50**	1.11
Tr011_H04	AP2 transcription factor (DREB2)	**0.51**	0.90	**0.56**	**0.57**	0.91	**1.61**
Tr011_H06	AP2 transcription factor (DREB2)	**0.52**	0.91	**0.54**	**0.57**	0.95	**1.68**
Tr014_F03	AP2 transcription factor (DREB2)	**0.49**	1.02	**0.60**	**0.48**	0.82	**1.71**
Tr014_F05	AP2 transcription factor (DREB2)	**0.56**	0.96	**0.61**	**0.58**	0.91	**1.58**
Tr014_D21	AP2 transcription factor (DREBF2)	**0.66**	1.04	0.67	**0.63**	0.98	**1.55**
Tr014_J02	AP2 transcription factor (EREBP)	1.13	1.40	0.88	0.81	1.29	**1.59**
Tr014_J19	AP2 transcription factor (EREBP)	0.80	1.17	0.76	0.68	1.05	**1.53**
Tr014_J21	AP2 transcription factor (EREBP)	0.77	1.11	0.71	0.69	1.08	**1.56**
Tr013_K24	Ap2 transcription factor (EREBP)	ND	ND	ND	**0.33**	**0.50**	**1.51**
Tr011_F12	AP2 transcription factor (ERF1)	0.71	0.91	**0.58**	0.78	1.22	**1.56**
Tr014_N15	AP2 transcription factor (ERF3)	0.90	0.95	**0.63**	0.95	1.44	**1.51**
Tr011_B24	AP2 transcription factor (ERF4)	0.75	**0.56**	0.67	1.33	1.12	0.84
Tr014_J11	AP2 transcription factor (ERF4)	0.67	0.95	**0.64**	0.71	1.05	1.48
Tr014_J12	AP2 transcription factor (ERF4)	0.92	0.89	**0.59**	1.03	**1.54**	**1.50**
Tr014_J17	AP2 transcription factor (ERF4)	0.84	1.15	0.74	0.73	1.13	**1.55**
Tr001_H15	ARF-Aux/IAA transcription factor	0.78	0.85	**0.52**	0.91	**1.51**	**1.66**
Tr001_H21	ARF-Aux/IAA transcription factor	0.85	0.80	**0.62**	1.07	1.38	1.30
Tr003_F23	ARF-Aux/IAA transcription factor	**0.65**	0.73	**0.51**	0.88	1.26	1.43
Tr012_P09	ARF-Aux/IAA transcription factor	0.68	0.79	**0.48**	0.85	1.41	**1.65**

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr013_O23	ARF-Aux/IAA transcription factor	0.86	0.86	**0.58**	1.01	1.49	1.48
Tr002_E15	ARF-Aux/IAAtranscription factor	**0.63**	0.85	0.80	0.74	0.79	1.07
Tr002_G09	ARF-Aux/IAAtranscription factor	**0.64**	0.82	**0.61**	0.78	1.05	1.35
Tr016_D09	beta-glucosidase (with alternative splicing)	1.03	1.08	0.72	0.95	1.43	**1.51**
Tr016_N05	beta-tubulin	**0.60**	0.77	0.78	0.78	0.78	1.00
Tr001_P13	bHLH transcription factor	**1.99**	1.10	**1.66**	**1.81**	1.20	0.67
Tr003_C16	bHLH transcription factor	1.21	0.97	**1.51**	1.25	0.80	**0.64**
Tr013_E19	bHLH transcription factor	**0.59**	0.80	**0.59**	0.73	1.00	1.37
Tr013_O04	bHLH transcritpion factor	0.77	0.85	**0.62**	0.91	1.23	1.36
Tr008_I02	BRI1-KD interacting protein 109	0.84	1.18	0.67	0.71	1.26	**1.77**
Tr012_D01	bZIP transcription factor	**0.49**	ND	**0.49**	ND	1.00	ND
Tr011_H15	bZIP transcription factor	**0.52**	0.96	**0.50**	**0.54**	1.03	**1.91**
Tr017_P02	caffeic acid O-methyltransferase	**1.69**	1.15	**3.01**	1.46	**0.56**	**0.38**
Tr017_L04	CAHC_HORVU Carbonic anhydrase, chloroplast precursor (Carbonate dehydratase)	**1.53**	1.11	**1.61**	1.37	0.95	0.69
Tr013_F13	Calcium-dependent protein kinase	1.22	1.19	**2.00**	1.03	**0.61**	**0.59**
Tr012_J20	Calmodulin	1.30	**1.92**	**1.86**	0.68	0.70	1.03
Tr013_M22	Calmodulin	ND	ND	ND	ND	**1.57**	ND
Tr017_E22	CB2G_LYCES Chlorophyll a-b binding protein 3C, chloroplast precursor (LHCII type I CAB	0.89	0.94	**1.51**	0.95	**0.59**	0.62
Tr013_L09	CBL-interacting protein kinase	**2.12**	1.21	0.91	**1.75**	**2.32**	1.32
Tr002_N15	cellulose synthase	**0.62**	**0.65**	**0.63**	0.95	0.99	1.04
Tr002_D17	cellulose synthase-like protein OsCslE1	**1.74**	1.09	**1.82**	**1.60**	0.96	**0.60**
Tr015_D16	chitinase 2	1.00	**1.65**	1.39	**0.61**	0.72	1.19
Tr008_O18	chitinase II precursor	1.30	**1.96**	**2.96**	0.67	**0.44**	**0.66**
Tr017_C17	chitinase II precursor	1.41	**1.72**	**3.09**	0.82	**0.46**	**0.56**
Tr011_P18	chlorophyll a/b binding protein CP29	0.74	1.16	0.78	**0.64**	0.95	1.49
Tr017_P21	chlorophyll a/b-binding protein	**0.65**	**0.49**	1.13	1.33	**0.58**	**0.44**
Tr008_B03	cleavage and polyadenylation specificity factor	1.22	1.22	**1.53**	1.00	0.80	0.80
Tr016_D03	cold acclimation protein	**2.19**	1.20	1.17	**1.82**	**1.86**	1.02
Tr017_A14	cold acclimation protein	1.37	1.03	0.71	1.34	**1.92**	1.44
Tr016_E07	cold acclimation protein WCOR413 - wheat	**1.51**	1.45	0.87	1.04	**1.74**	**1.67**
Tr016_B09	cold acclimation protein WCOR80	**2.34**	1.31	0.97	**1.78**	**2.42**	1.36
Tr012_F16	cold regulated protein	**2.64**	1.38	1.11	**1.91**	**2.38**	1.24
Tr002_M20	cold-regulated	**1.70**	**1.69**	1.03	1.01	**1.66**	**1.65**
Tr002_N07	cold-responsive LEA/RAB-related COR protein	1.35	1.14	**1.59**	1.19	0.85	0.72
Tr012_F11	CONSTANS zinc finger transcription factor	ND	ND	ND	0.87	1.33	**1.53**
Tr013_C11	COR39 protein	**3.21**	**2.06**	0.88	**1.56**	**3.66**	**2.35**
Tr017_L22	cp31AHv protein	**0.66**	**0.55**	1.04	1.21	**0.64**	**0.53**
Tr001_L05	Cyclin dependent kinase C	ND	ND	ND	1.22	0.70	**0.58**

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr009_H09	cystatin 1	**1.59**	1.11	1.13	1.42	1.40	0.98
Tr005_L20	cysteine proteinase inhibitor	**2.09**	1.13	1.08	**1.85**	**1.93**	1.05
Tr016_F11	cytochrome	0.99	1.19	**2.41**	0.83	**0.41**	**0.50**
Tr004_F21	cytosolic 6-phosphogluconate dehydrogenase	**0.65**	0.72	0.69	0.90	0.95	1.06
Tr017_L14	cytosolic tRNA-Ala synthetase-like protein	**1.66**	1.05	**1.63**	**1.59**	1.02	**0.64**
Tr017_N15	dehydrin (having alternative splicing products)	1.47	0.99	0.73	1.48	**2.01**	1.36
Tr003_A11	dehydrin 3	**2.38**	**2.25**	1.03	1.06	**2.32**	**2.19**
Tr002_F09	dehydrin 5	**3.75**	**1.81**	1.48	**2.07**	**2.53**	1.22
Tr009_L16	dehydrin 5	**4.28**	**2.70**	1.29	**1.59**	**3.31**	**2.09**
Tr010_E22	dehydrin 5	**4.85**	**2.72**	1.21	**1.79**	**4.00**	**2.24**
Tr016_F17	dehydrin 5	**1.95**	**1.61**	0.88	1.21	**2.22**	**1.84**
Tr015_B22	dehydrin 8	1.46	1.02	0.75	1.43	**1.94**	1.35
Tr016_L07	dehydrin WZY1-1	**2.61**	**1.74**	1.08	**1.5**	**2.40**	**1.61**
Tr016_P20	dehydrin-/LEA group 2-like protein	**1.92**	1.17	1.17	**1.64**	**1.63**	1.00
Tr017_L02	delta-COP	**2.01**	**1.51**	**2.21**	1.34	0.91	0.68
Tr006_I09	dihydroflavonol 4-reductase	**0.65**	0.72	**0.60**	0.90	1.08	1.20
Tr004_O20	DNAJ heat shock N-terminal domain-containing protein	0.94	0.74	**0.65**	1.26	1.44	1.14
Tr009_P08	DnaJ like protein	0.68	0.96	**0.65**	0.71	1.04	1.47
Tr004_C02	early nodulin 75 precursor-like protein	1.10	**1.73**	1.29	**0.63**	0.85	1.34
Tr016_M08	elongation factor	1.49	0.96	1.05	**1.55**	1.43	0.92
Tr001_L18	elongation factor 1B gamma	1.45	0.90	1.13	**1.62**	1.29	0.79
Tr016_B07	emb|CAB85507.1~gene_id:MED24.17~strong similarity to unknown protein [Arabidopsis thaliana	**1.67**	1.14	1.29	1.47	1.30	0.88
Tr002_L13	endo ,4-beta-glucanase	1.08	0.95	**1.54**	1.14	0.70	**0.62**
Tr002_G23	EREBP transcription factor	**0.59**	1.11	**0.56**	**0.53**	1.07	**2.00**
Tr011_D12	ethylene responsive element binding factor3	1.20	**0.61**	0.84	**1.96**	1.43	0.73
Tr004_I03	expressed protein	1.41	1.32	**1.90**	1.07	0.74	0.70
Tr008_K03	expressed protein	0.80	1.32	1.30	**0.61**	**0.62**	1.02
Tr016_A04	expressed protein	0.77	1.11	**1.87**	0.70	**0.41**	**0.59**
Tr011_L21	far-red impaired response protein	**0.63**	0.69	**0.60**	0.90	1.04	1.16
Tr012_D19	F-box ubiquitin ligase	**1.58**	1.21	1.13	1.30	1.40	1.08
Tr005_N17	ferredoxin-NADP(H) oxidoreductase	1.02	1.16	**1.71**	0.88	**0.60**	0.68
Tr001_J20	glutamate-1-semialdehyde 2,1-aminomutase	1.23	**1.53**	1.12	0.81	1.11	1.37
Tr016_G11	glutathione transferase F4	**1.79**	1.35	1.36	1.32	1.32	0.99
Tr014_P20	glycine-rich RNA-binding protein	**1.61**	0.91	1.03	**1.78**	**1.56**	0.88
Tr002_J13	glycosyl hydrolase family 1/beta-glucosidase	**1.69**	1.14	**1.55**	1.48	1.09	0.74
Tr004_L15	GPI-anchored protein	1.39	1.09	0.81	1.28	**1.72**	1.34
Tr013_I20	GSK-like kinase	**1.57**	0.96	1.12	**1.64**	1.41	0.86
Tr016_D15	H2B2_WHEAT Histone H2B.2	0.77	**0.56**	**0.61**	1.37	1.25	0.91
Tr016_J04	H2B2_WHEAT Histone H2B.2	0.88	**0.66**	0.67	1.34	1.31	0.98

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr001_H20	haloacid dehalogenase hydrolase	**0.53**	0.83	0.71	**0.64**	0.75	1.17
Tr016_M23	heat shock protein 70	**0.58**	0.81	0.71	0.71	0.81	1.14
Tr016_G21	heat shock-related protein	**0.59**	**0.63**	**0.60**	0.93	0.98	1.06
Tr004_N21	histone H1flk	0.83	**0.63**	**0.58**	1.32	1.43	1.08
Tr015_L17	histone H2B	0.92	**0.60**	0.69	**1.53**	1.34	0.87
Tr015_M01	histone H4	1.22	0.75	1.07	**1.63**	1.14	0.70
Tr016_H09	histone H4	0.95	**0.55**	**0.56**	**1.73**	**1.71**	0.99
Tr016_I23	histone H4	0.67	**0.59**	0.77	1.13	0.86	0.76
Tr017_G05	histone H4	ND	ND	ND	ND	**1.92**	ND
Tr012_F14	hydroxycinnamoyl benzoyltransferase	0.92	**1.52**	1.32	**0.61**	0.70	1.15
Tr008_B01	hypothetical Arabidopsis thaliana chromosome 3, T18N14.110	**1.54**	1.28	1.21	1.20	1.27	1.06
Tr005_A10	hypothetical protein	0.70	**0.56**	0.73	1.25	0.95	0.76
Tr010_G05	hypothetical protein	1.30	**1.67**	1.10	0.78	1.18	**1.52**
Tr010_O12	hypothetical protein	1.20	0.76	1.09	**1.58**	1.11	0.70
Tr011_G07	hypothetical protein	0.90	1.34	0.83	0.67	1.09	**1.63**
Tr016_F09	Hypothetical protein	**0.48**	0.77	**0.43**	**0.63**	1.13	**1.79**
Tr016_K17	hypothetical protein	**1.61**	1.04	1.19	**1.54**	1.36	0.88
Tr016_L08	hypothetical protein	1.41	0.95	0.90	1.49	**1.57**	1.06
Tr016_O23	hypothetical protein	1.42	0.72	0.94	**1.98**	**1.51**	0.76
Tr017_L23	Hypothetical protein	0.97	0.68	1.23	1.42	0.78	**0.55**
Tr007_E19	hypothetical protein F18F4.150	0.77	1.18	0.74	**0.65**	1.05	**1.60**
Tr003_L21	IAA1 protein	0.75	0.80	**0.62**	0.94	1.19	1.28
Tr016_B15	IAA1 protein	0.76	0.74	**0.55**	1.04	1.39	1.33
Tr017_G12	inorganic pyrophosphatase	1.26	0.73	0.78	**1.73**	**1.61**	0.93
Tr016_J14	late embryogenesis abundant protein LEA14-A	**2.15**	1.46	**1.62**	1.47	1.33	0.90
Tr002_A03	leucine-rich repeat transmembrane protein kinase	**4.43**	**3.88**	1.15	1.14	**3.85**	**3.37**
Tr014_D17	light-harvesting complex IIa protein	0.74	1.19	0.80	**0.62**	0.92	1.48
Tr015_K06	lipase	1.10	1.04	**1.55**	1.05	0.71	0.67
Tr001_N14	lipid transfer protein	0.75	0.78	**0.46**	0.96	**1.62**	**1.68**
Tr006_C21	long cell-linked locus protein	**0.57**	0.86	**0.65**	0.67	0.88	1.32
Tr001_H09	LRR transmembrane protein kinase	ND	ND	ND	0.81	**0.41**	**0.50**
Tr012_L13	LRR transmembrane protein kinase	0.88	1.15	1.47	0.76	**0.60**	0.79
Tr012_P05	LRR transmembrane protein kinase	0.79	1.12	1.27	0.71	**0.63**	0.88
Tr013_J14	LRR transmembrane protein kinase	**2.03**	**2.53**	**1.79**	0.80	1.14	1.42
Tr017_L10	lysyl-tRNA synthetase	**0.64**	ND	1.06	ND	**0.61**	ND
Tr014_B10	MADS Box transcription factor	**0.60**	0.77	0.70	0.77	0.85	1.10
Tr014_F12	MADS Box transcription factor	ND	ND	ND	1.24	0.69	**0.55**
Tr001_N15	MAP kinase	**1.52**	1.13	1.45	1.34	1.05	0.78
Tr016_M04	maturase K	1.40	0.82	0.77	**1.71**	**1.81**	1.06
Tr015_K22	membrane protein	**2.01**	1.39	1.14	1.44	**1.77**	1.23
Tr016_D07	membrane protein	**4.23**	**2.02**	1.48	**2.09**	**2.86**	1.37
Tr017_A19	membrane protein	**3.60**	**1.96**	**1.50**	**1.84**	**2.40**	1.30
Tr005_E07	metallothionein	**1.56**	**1.67**	**1.80**	0.93	0.87	0.93

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr005_L18	metallothionein-like protein type 4	**1.69**	1.26	1.48	1.34	1.15	0.85
Tr005_E24	MtN19	**1.66**	1.07	1.30	**1.56**	1.28	0.82
Tr014_F24	multiple stress-associated zinc-finger protein	0.74	**0.64**	**0.63**	1.15	1.18	1.02
Tr001_D09	MYB transcription factor	**0.63**	0.94	0.82	**0.66**	0.76	1.14
Tr001_I02	MYB transcription factor	**0.22**	**0.60**	**0.13**	**0.37**	**1.70**	**4.53**
Tr001_J09	MYB transcription factor	1.20	1.28	**1.57**	0.94	0.77	0.82
Tr001_N23	MYB transcription factor	**0.61**	0.85	1.17	0.72	**0.52**	0.72
Tr012_G20	MYB transcription factor	**0.58**	0.98	**0.52**	**0.60**	1.11	**1.87**
Tr012_P19	MYB transcription factor	0.96	1.06	**1.61**	0.90	**0.60**	**0.66**
Tr013_A17	MYB transcription factor	1.44	1.01	0.88	1.42	**1.64**	1.15
Tr013_E20	MYB transcription factor	1.27	0.96	0.74	1.32	**1.71**	1.29
Tr016_P03	myosin heavy chain-like protein	1.22	1.09	**1.52**	1.11	0.80	0.72
Tr003_J21	NAC transcription factor	0.74	0.82	**0.59**	0.90	1.25	1.39
Tr013_N14	NAC transcription factor	0.72	0.77	**0.46**	0.94	**1.56**	**1.66**
Tr013_D21	NAM transcription factor	**1.53**	0.88	0.98	**1.75**	**1.56**	0.89
Tr013_G12	NAM transcription factor	1.36	0.89	1.04	**1.52**	1.30	0.85
Tr011_O06	NBS-LRR resistance gene-like protein ARGH06	0.71	1.16	0.78	**0.61**	0.92	1.49
Tr004_F23	No Blast Hit	**0.60**	1.04	0.76	**0.58**	0.79	1.37
Tr008_J04	No Blast Hit	0.74	1.27	0.85	**0.58**	0.86	1.49
Tr011_G24	No Blast Hit	**0.45**	1.08	**0.45**	**0.42**	1.01	**2.39**
Tr011_O05	No Blast Hit	0.67	0.94	**0.65**	0.71	1.02	1.44
Tr013_I24	No Blast Hit	ND	ND	ND	**0.58**	1.17	**2.04**
Tr015_M05	No Blast Hit	0.89	1.11	**1.58**	0.81	**0.57**	0.70
Tr016_H06	No Blast Hit	0.72	1.07	0.69	0.67	1.04	**1.55**
Tr016_H08	No Blast Hit	0.81	1.39	**3.80**	**0.59**	**0.21**	**0.37**
Tr017_I05	No Blast Hit	0.71	0.90	1.22	0.80	**0.58**	0.73
Tr017_L03	No Blast Hit	1.42	1.42	**6.84**	1.00	**0.21**	**0.21**
Tr017_L11	No Blast Hit	0.81	**0.62**	1.47	1.31	**0.55**	**0.42**
Tr017_L21	No Blast Hit	0.77	1.13	**5.55**	0.68	**0.14**	**0.20**
Tr011_P10	not received	ND	ND	ND	0.82	**0.51**	**0.63**
Tr014_N22	O-methyltransferase	**0.59**	0.93	0.99	**0.64**	**0.60**	0.94
Tr005_O14	O-methyltransferase	**0.59**	0.93	1.07	**0.64**	**0.55**	0.86
Tr017_P10	O-methyltransferase	0.88	**0.56**	1.07	**1.58**	0.82	**0.52**
Tr017_P06	o-methyltransferase ZRP4	1.01	1.20	**6.49**	0.84	**0.16**	**0.19**
Tr017_P08	o-methyltransferase ZRP4	1.43	0.74	1.11	**1.94**	1.29	**0.66**
Tr008_A22	OTU-like cysteine protease family protein	1.07	0.83	**0.64**	1.29	**1.66**	1.29
Tr005_H08	p68 RNA helicase	**1.92**	**2.42**	**1.88**	0.79	1.02	1.29
Tr015_O24	phosphatidylinositol 4-kinase	1.49	1.05	0.98	1.42	**1.52**	1.07
Tr016_P09	phosphatidylinositol 4-kinase	1.28	1.01	**1.60**	1.26	0.80	**0.64**
Tr013_L08	Phospholipase C	0.70	1.14	**0.54**	**0.62**	1.31	**2.12**
Tr017_D13	photosystem I subunit N	1.10	1.19	**1.61**	0.92	0.68	0.74
Tr015_O03	photosystem-1 F subunit precursor	**0.65**	1.17	0.69	**0.55**	0.94	**1.70**
Tr005_G20	plant metallothionein-like protein	**0.54**	0.88	0.90	**0.62**	**0.60**	0.97
Tr017_L18	plastid ribosomal protein L35	**1.88**	1.09	**1.54**	**1.74**	1.22	0.71

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr017_C12	polyprotein	1.17	0.98	0.69	1.19	**1.69**	1.41
Tr001_D04	Polyubiquitin	0.83	1.14	**1.56**	0.73	**0.53**	0.73
Tr014_A23	Polyubiquitin	0.69	1.14	0.98	**0.60**	0.70	1.17
Tr017_N03	potassium transporter	0.85	1.39	**4.83**	**0.61**	**0.18**	**0.29**
Tr003_H18	potassium transporter, (HAK5/POT5)	1.47	1.37	0.93	1.07	**1.58**	1.47
Tr017_I07	promoter-binding factor-like protein	0.88	1.27	**3.46**	0.69	**0.25**	**0.37**
Tr006_P12	protein	**0.64**	0.77	0.99	0.84	**0.65**	0.77
Tr001_L01	Protein kinase	**2.18**	1.27	**2.43**	**1.71**	0.90	**0.53**
Tr001_L11	Protein kinase	1.45	0.81	1.06	**1.78**	1.37	0.77
Tr003_E22	Protein kinase	0.70	0.81	**0.66**	0.86	1.07	1.24
Tr003_G21	Protein kinase	ND	ND	ND	1.13	0.71	**0.63**
Tr012_I10	Protein kinase	0.76	0.97	**0.66**	0.78	1.15	1.48
Tr013_D13	Protein kinase	0.78	1.17	0.75	**0.66**	1.04	**1.56**
Tr013_I17	Protein kinase	0.85	0.92	**0.62**	0.93	1.37	1.47
Tr006_E19	protein kinase	**0.61**	0.70	**0.65**	0.87	0.94	1.09
Tr016_F24	protein kinase	0.95	1.00	**3.21**	0.94	**0.29**	**0.31**
Tr014_N18	Protein Kinase (CBL-interacting)	**1.61**	1.14	1.15	1.41	1.40	0.99
Tr014_N16	Protein Kinase (Serine/threonine)	**1.66**	1.11	1.27	1.49	1.31	0.87
Tr009_J12	protein T19E23.7 [imported]	**0.51**	0.80	**0.62**	**0.65**	0.84	1.30
Tr005_N13	proteinase inhibitor II	1.31	1.27	**1.60**	1.03	0.82	0.79
Tr012_F04	proteinase inhibitor-protein bsi1	**1.57**	**1.99**	**2.22**	0.79	0.71	0.89
Tr013_O02	receptor kinase	1.07	ND	**0.40**	ND	**2.68**	ND
Tr001_D12	receptor-like protein kinase	0.75	0.94	1.17	0.79	**0.64**	0.80
Tr013_C20	receptor-like protein kinase	**1.55**	**1.52**	1.01	1.02	**1.53**	**1.51**
Tr017_G21	receptor-like protein kinase	1.18	0.71	1.06	**1.65**	1.11	0.67
Tr016_C06	ribosomal protein	1.47	0.92	1.09	**1.60**	1.35	0.84
Tr017_P11	ribosomal protein L17-like protein	0.92	0.69	1.45	1.34	**0.64**	**0.48**
Tr016_F05	ribulose-1,5-bisphosphate carboxylase activase	**0.63**	0.83	0.92	0.76	0.69	0.91
Tr017_N21	ribulose-1,5-bisphosphate carboxylase small subunit	0.72	1.06	**1.52**	0.68	**0.47**	0.70
Tr015_A14	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.81	1.06	**1.57**	0.76	**0.51**	0.68
Tr015_C20	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.96	1.13	**2.67**	0.85	**0.36**	**0.43**
Tr015_G03	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.74	1.38	**5.19**	**0.54**	**0.14**	**0.27**
Tr015_O08	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.82	1.01	1.31	0.81	**0.62**	0.77
Tr016_D01	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	1.01	1.25	**2.00**	0.81	**0.51**	**0.62**
Tr016_D05	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	1.02	1.15	**2.52**	0.89	**0.41**	**0.46**
Tr016_J01	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	1.01	1.27	**3.15**	0.79	**0.32**	**0.40**
Tr016_J23	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.94	1.18	**3.22**	0.79	**0.29**	**0.37**

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr017_A05	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.93	1.00	**2.27**	0.93	**0.41**	**0.44**
Tr017_B02	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	0.84	0.97	**1.58**	0.87	**0.53**	**0.61**
Tr015_C18	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	0.76	1.06	**1.72**	0.72	**0.44**	**0.61**
Tr015_C24	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	0.91	0.86	1.44	1.05	**0.63**	**0.60**
Tr015_E06	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	0.85	1.07	1.32	0.80	**0.65**	0.81
Tr016_D13	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	0.86	1.23	**2.05**	0.69	**0.42**	**0.60**
Tr016_N10	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	0.68	0.88	**1.59**	0.78	**0.43**	**0.55**
Tr016_N19	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	ND	ND	ND	ND	**0.48**	ND
Tr017_G24	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	1.28	0.98	**1.77**	1.30	0.72	**0.55**
Tr017_P17	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	1.46	1.22	**2.75**	1.20	**0.53**	**0.44**
Tr016_B18	ribulosebiphosphate carboxylase	1.14	1.01	**2.25**	1.13	**0.51**	**0.45**
Tr017_H16	ribulosebiphosphate carboxylase	0.88	1.02	1.47	0.86	**0.60**	0.69
Tr015_A05	ribulosebisphosphate carboxylase	0.75	1.06	1.29	0.71	**0.58**	0.83
Tr003_D07	RING zinc finger protein	ND	ND	ND	ND	**0.65**	ND
Tr010_D21	RING-H2 zinc finger protein	1.38	0.93	**1.50**	1.48	0.92	**0.62**
Tr013_J13	RING-H2 zinc finger protein	0.90	0.92	**1.67**	0.99	**0.54**	**0.55**
Tr016_P05	RNA polymerase II, 28841-29486	1.17	1.37	**2.31**	0.85	**0.51**	**0.60**
Tr001_H02	RNA recognition motif- protein	1.46	1.19	**1.58**	1.22	0.92	0.76
Tr012_D05	RNA-binding glycine-rich protein	**1.75**	1.04	1.13	**1.68**	**1.55**	0.92
Tr012_H21	RNA-binding glycine-rich protein	**1.57**	0.96	1.02	**1.63**	**1.54**	0.94
Tr012_N09	RNA-binding glycine-rich protein	1.43	0.91	1.08	**1.57**	1.32	0.84
Tr012_N13	RNA-binding glycine-rich protein	1.17	**1.56**	1.25	0.75	0.94	1.25
Tr012_O08	RNA-binding glycine-rich protein	**1.52**	0.99	1.08	**1.53**	1.41	0.92
Tr013_A11	RNA-binding glycine-rich protein	**1.98**	1.03	1.19	**1.92**	**1.67**	0.87
Tr013_J24	RNA-binding glycine-rich protein	**1.53**	**1.99**	**2.23**	0.77	0.68	0.89
Tr013_K17	RNA-binding glycine-rich protein	**1.62**	1.20	1.20	1.36	1.35	1.00
Tr001_D02	RNA-binding protein	**1.52**	1.01	1.33	**1.50**	1.14	0.76
Tr013_N19	RNA-binding protein	**1.50**	0.97	1.07	**1.55**	1.40	0.91
Tr017_M20	RNA-binding protein	1.35	0.75	0.81	**1.80**	**1.68**	0.93
Tr008_D11	RRM-containing protein SEB-4	**0.57**	0.71	0.80	0.79	0.71	0.89
Tr008_G05	seed maturation protein	**0.50**	ND	**0.52**	ND	0.97	ND
Tr007_D22	selenium binding protein	1.49	1.12	0.97	1.33	**1.52**	1.15
Tr017_C10	senescence-associated protein-like	**2.29**	**1.59**	1.11	1.44	**2.07**	1.43
Tr016_K23	signal peptidase protein-like protein	1.09	0.69	0.87	**1.58**	1.25	0.79
Tr013_F02	S-locus protein kinase	0.79	**0.62**	**0.58**	1.29	1.37	1.07
Tr001_J23	S-receptor kinase	**0.60**	0.82	1.20	0.72	**0.50**	0.69
Tr005_G07	S-receptor kinase	1.34	1.48	**1.56**	0.91	0.86	0.95
Tr002_I18	steroid sulfotransferase	**3.70**	**2.04**	1.42	**1.82**	**2.60**	1.43

Table 2. Continued

Microarray ID	Annotation	Change in Expression level			Difference in Expression between time-points		
		Tr.Nst.6/ Ct.Nst	Tr.Nst.24/ Ct.Nst	Tr.Nst.72/ Ct.Nst	Tr.Nst.6/ Tr.Nst.24	Tr.Nst.6/ Tr.Nst.72	Tr.Nst.24 / Tr.Nst.72
Tr005_M24	subtilisin-like serine proteinase	ND	ND	ND	**1.60**	1.41	0.88
Tr007_O12	sucrose synthase 2	0.70	1.15	0.98	**0.61**	0.71	1.17
Tr002_D05	sucrose:sucrose 1-fructosytransferase	1.35	0.87	1.11	**1.56**	1.22	0.78
Tr014_K01	tetratricopeptide repeat protein	1.12	**1.58**	1.21	0.71	0.93	1.31
Tr004_I01	thaumatin-like protein	1.06	**2.12**	**1.95**	**0.50**	**0.54**	1.09
Tr010_P13	timing of CAB expression 1	1.33	1.07	0.86	1.24	**1.55**	1.24
Tr016_P24	translation initiation factor 5A	1.39	0.90	1.36	**1.54**	1.02	**0.66**
Tr007_B20	triose phosphate translocator	0.78	1.19	1.26	**0.66**	**0.62**	0.94
Tr007_J22	triosephosphate isomerase 1	**0.61**	**0.59**	0.69	1.03	0.89	0.86
Tr004_F16	type 1 membrane protein -like	0.91	1.42	1.07	**0.64**	0.85	1.33
Tr017_P15	type 1 non-specific lipid transfer protein precursor	1.41	1.11	**1.63**	1.27	0.87	0.68
Tr015_J06	ubiquinol-cytochrome C reductase complex ubiquinone-binding protein (QP-C)	**0.61**	0.95	**0.64**	**0.64**	0.95	1.48
Tr001_J04	ubiquitin/ribosomal protein CEP52	0.69	0.85	**0.65**	0.81	1.06	1.31
Tr014_C15	ubiquitin-conjugating enzyme	1.15	**0.65**	0.81	**1.78**	1.43	0.80
Tr014_M08	ubiquitin-conjugating enzyme	**1.50**	0.94	1.12	**1.59**	1.34	0.84
Tr017_B11	ubiquitin-conjugating enzyme	**1.59**	0.86	0.85	**1.84**	**1.88**	1.02
Tr017_F17	ultraviolet-B-repressible protein	**1.51**	1.06	1.23	1.42	1.23	0.86
Tr004_F03	unknown protein	0.81	1.46	0.85	**0.55**	0.95	**1.72**
Tr004_L12	unknown protein	ND	ND	ND	1.02	**0.60**	**0.59**
Tr005_D09	unknown protein	0.84	0.73	1.11	1.16	0.76	**0.66**
Tr005_E15	unknown protein	**0.58**	0.98	0.72	**0.59**	0.80	1.36
Tr006_J15	unknown protein	**0.62**	0.95	0.71	**0.65**	0.87	1.33
Tr009_B01	unknown protein	0.71	1.13	0.77	**0.62**	0.92	1.47
Tr015_A04	unknown protein	0.76	0.99	**1.60**	0.76	**0.47**	**0.62**
Tr015_I08	unknown protein	ND	ND	ND	0.76	**0.37**	**0.49**
Tr016_A14	unknown protein	**1.72**	**1.56**	1.05	1.10	**1.64**	1.48
Tr016_M01	unknown protein	1.48	1.28	0.88	1.15	**1.67**	1.45
Tr017_P19	unknown protein	1.05	0.99	**1.77**	1.06	**0.59**	**0.56**
Tr008_N04	wheat aluminum induced protein wali 3	1.38	**2.25**	**1.81**	**0.61**	0.76	1.24
Tr010_J20	wheat aluminum induced protein wali 3	1.19	**1.60**	1.48	0.75	0.81	1.08
Tr003_O12	WRKY transcription factor	0.81	**0.65**	0.70	1.26	1.16	0.92
Tr002_D01	xyloglucan endotransglycosylase	**1.63**	1.09	**1.51**	1.49	1.08	0.72
Tr017_P14	Zhi1_GP4_P32-35	**1.73**	1.07	**1.88**	**1.63**	0.92	**0.57**
Tr017_P16	Zhi2_GP13_P32-35	**1.66**	0.95	**1.97**	**1.75**	0.84	**0.48**
Tr017_P18	Zhi3_65_P32-35	**1.78**	1.05	1.16	**1.70**	**1.54**	0.90
Tr017_P20	**Zhi4_GP4_P32-34**	**1.40**	**0.89**	**1.16**	**1.56**	**1.20**	**0.77**

Table 2. Genes that had significant changes in expression determined by ANOVA ($P \leq 0.05$) with a $\geq$ 1.5-fold change in expression with 150 mM NaCl stress in at least one treated time point compared to control plants or that have a $\geq$ 1.5-fold change between time points. Data is alphabetically ordered by the gene annotation. Boldface indicates values that are significant as determined by ANOVA and have $\geq$ 1.5-fold changes in expressed transcript levels of treatment/control at least one time point or between relative levels for treated time points. Abbreviations are as follows: Tr= 150 mM NaCl treated, Ct= control treatment, Nst=Norstar, 6= 6 hours, 24=24 hours and 72 =72 hours, ND=no data.

Figure 4. Differentially Regulated Genes in Norstar in Comparisons Between Time-Points

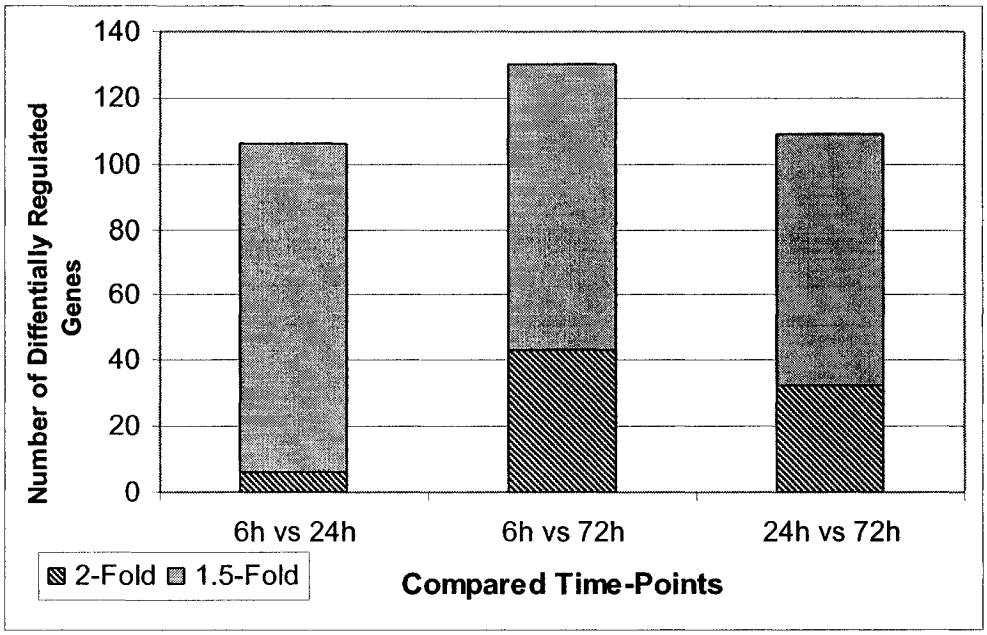

Figure 4. The Number of genes between 150 mM salt-stressed time points that were significant for ANOVA and *t*-test and that had a ≥1.5-fold difference in transcript levels.

3.3 Sequence Comparison between *L. elongatum* and Wheat.

Eighty-nine *L. elongatum* EST nucleotide sequences were compared by blastn to the FGAS database of 80,000 wheat EST sequences. Twenty-eight *L. elongatum* sequences did not have a blast hit in wheat. Sixty-one sequences were found to have nucleotide sequence similarity ranging from 84% to 100%, with 97% sequence similarity between *L. elongatum* and *T. aestvium* having the largest number of hits with 12 sequences. The average sequence identity between *L. elongatum* and wheat was 94.16%. The high sequence conservation between *L. elongatum* and wheat that indicates that mRNA derived from *L. elongatum* would likely hybridize with *T. aestivum* cDNA amplicons on a microarray. Previous work has shown that probes from *L. elongatum* hybridize to northern blots of *T. aestivum* RNA (Galvez et al., 1993) . While there were 28 *L.elongatum* ESTs that BLAST failed to identify a homolog for this may be due to limited size of the FGAS EST database. The pairs of sequences with over 95% sequence identity, indicates the high sequence similarity between the two species, that is the degree of similarity between homeologs in the A, B and D genomes of *T. aestivum* and barley (*Hordeum vulgaris*) (Ridha Farajalla and Gulick, 2007).

3.4 Gene Expression Profiles of CS, DS3E(3A) and AgCS

The wild species, *L. elongatum* is more salt-tolerant than its relative *T. aestivum.* The amphiploid produced from a cross of the *T. aestivum* and *L. elongatum* is highly salt tolerant, but less tolerant than *L. elongatum* (Dvořák et al., 1988). Several *T. aestivum* disomic substitution lines in which a chromosome pair in *T. aestivum* has been

exchanged for a pair from *L. elongatum* have also shown increased salt tolerance relative to wheat, with chromosome pair 3E having the most significant effect (Omeilian et al., 1991). To examine the gene expression profiles during salt stress and to identify genes that could account for the differences in salt-tolerance between Chinese Spring, the amphiploid and disomic substitution line DS3E(3A), each genotype was treated with saline and non-saline (control) hydroponic solutions and the mRNA profiles from roots after three days of treatment were examined by microarray. The microarray slides used were the same configuration as those used in the Norstar time-course array experiment described above.

Chinese Spring (CS), its amphiploid (AgCS) and disomic substitution 3E (3A) line (DS3E(3A)) plants were grown hydroponically and subjected to a control treatment or 150 mM NaCl + 15 mM $CaCl_2$ treatment for three days. To allow comparisons between all the genotypes a common reference consisting of a pool from the control Chinese Spring replicates was used for all of the hybridizations (Fig.5). Of the 5728 features, 5230 had expression levels in at least one genotype above background values. Two-way ANOVA was used to identify genes with significant differences ($P \leq 0.05$) in expression due to treatment effect, genotype effect and genotype by treatment effect. Change in gene expression was calculated as the ratio of the relative levels of the fluorescence signal of the "salt-treated" samples relative to the control for the same genotypes' control samples relative levels ((salt treated sample treated fluorescence/ common reference fluorescence)/ (Control fluorescence/ common reference fluorescence)).

From two- way ANOVA 2057 genes had significant ($P \leq 0.05$) differences in expression for at least one factor. Of these, 868 were significant for genotype effects, 1442 had significant treatment effects and 305 genes had genotype by treatment interaction effects. A number of genes had significant effects for more than one factor, the list of genes with significant effects is provided in Supplemental Table 2. There were 212 genes with a significant P value ($P \leq 0.05$) and a 2 fold or greater change in gene expression under salt treatment in at least one genotype. In Table 3 and Figure 6 the details of 212 genes with a significant P value and at least a two-fold change in gene expression under salt stress is provided. Two hundred and seven genes were significant for treatment effects which included 59 of the 60 genes that had significant genotype effects and 37 of the 42 genes that had genotype-by-treatment interaction effects.

Among the 212 genes with $\geq$ 2-fold changes in expression, 128 genes had increased expression and 85 genes had decreased expression. Twenty six percent of the genes with significant changes in expression are annotated as regulatory or components of signal transduction pathways. The Venn diagrams in Figure 6 represent the distribution genes within each cultivar with significant ANOVA P values ($P \leq 0.05$) and $\geq$ 2 -fold salt-stress induction and/or repression. Few genes that are $\geq$ 2 fold up or down regulated have significantly changed expression in all three genotypes demonstrating that there are significant differences in regulation at the transcriptional level during salt stress in CS, DS3E(3A) and AgCS. In each genotype, the number of induced genes is greater than the number which were repressed. CS had the largest number of $\geq$ 2 -fold up and

down regulated genes among the three genotypes. CS not only has the highest total number of highly regulated genes but also the greatest number of salt stress regulated genes that are unique to one genotype, some of these genes have altered expression levels in the other genotypes but the changes do not pass the 2 fold cut-off threshold. There are 34 genes that are induced or repressed in all three genotypes under salt stress and the change in gene expression is generally much larger than two-fold especially for induced genes in all three genotypes (up to 8.15 fold induction). Among this group there are genes that encode proteins that have been previously identified as being induced by osmotic stress, some of which have been shown to have protective functions under stress such as dehydrins, catalases and disease resistance genes (Munns, 2005). There are also genes involved with regulatory functions and cell signaling pathways.

3.5 Genotype-Comparison Array; Cluster Analysis of the Change in Gene Expression Levels

To further analyze the change in gene expression levels of the CS, AgCS and DS 3A (3E) genotypes Hierarchal clustering (HCL) and *k*-means clustering (KMC) with Euclidian distances was performed on the change of gene expression levels for 201 of the 212 regulated genes using the program Tigr MeV (Saeed et al., 2003). HMC clusters are built iteratively by nearest neighbor joining, in which genes with the most similar patterns of expression are grouped and averaged to build a series of branches (dendogram) of genes that are grouped based on similarity expression. For KMC clusters the mean expression of each cluster is calculated and genes are moved within the number of clusters iterated until the mean of a gene's expression profile is closest to mean of the

cluster. Results are provided in figure 7 and Table 3. Only genes with detectable expression in all three genotypes could be included in the analysis, thus 11 that were not detected in all genotypes at all time points were removed from the data set. Most genes were repressed or induced in all three genotypes; the majority of the genes with differences in expression between CS, DS3E(3A) and AgCS had differences in the magnitude of change in the expression. The KMC analysis was set to cluster genes into 12 clusters, cluster 1 contained 24 genes that had similar repression levels in all three genotypes and cluster 12 contained 20 genes that had similar induction levels in all three genotypes. Genes found in these two clusters respond the same in all three genotypes; therefore the alteration of mRNA levels of these genes in response to salt stress likely does not contribute to the specific differences in salt tolerance found in the genotypes but rather represent a common response to salt stress in all of the genotypes.

Other clusters represent genes with greater differences in expression levels between genotypes which may lead to the differences in the degrees of salt tolerance between the genotypes (Figure 7 and Table 3). Clusters 4, 6, 7 and 9 represent genes that are highly up regulated in one or more genotypes and only have small differences in the other genotype(s), 40% of the 201 genes fall into these clusters. Genes in cluster 4 (12 genes) had higher induction in DS3E(3A) than CS and AgCS. In clusters 6 (33 genes) and 7 (16 genes) gene induction is higher in CS than the other two genotypes, these genes may account for increased salt tolerance of DS3E(3A) and AgCS. DS3E(3A) also has greater similarity in gene expression change to AgCS suggesting that these genes may lie on or be regulated by chromosome 3E. In cluster 7 the change in gene expression is

negatively correlated with salt tolerance levels, indicating that these genes deserve further characterization into the roles they may play among the degree of salt tolerance between the three genotypes. In cluster 9 (27 genes) AgCS has higher induction than CS and DS3E(3A), the latter two had similar expression levels. Clusters 5, 8 and 11 have the fewest number of genes but have largest difference in gene expression levels between genotypes. Cluster 5 (Tr015_K18, Tr001_G24, Tr013_K19, Tr003_G20 and Tr003_K07) represents genes that have the largest difference between AgCS and the other two genotypes, there are only small changes of gene expression in CS and DS3E(3A) while AgCS has strong induction (2.02-5.85 fold) (table 3). Of these, one is annotated as a hypothetical protein, 2 genes are annotated as transcription factors, one as a protein kinase and the other as a receptor protein kinase indicating that 4 of the genes are involved in gene regulation. They are strong candidates for further study as they may account for the higher salt tolerance found in AgCS compared to CS. As DS3E(3A) has a similar pattern of expression to CS for these genes and not AgCS it suggests that these genes are not regulated by 3E but elsewhere in the E genome of *L. elogantum* .

In cluster 8 (3 genes) there is induction in CS while in the other genotypes there is slight repression creating a greater difference in gene expression levels. The genes in this cluster are good candidates for further investigation as both DS3E(3A) and AgCS behave in a similar manner and suggest that these genes may account for increased salt tolerance of both genotypes as compared to CS and that the three genes may be under the regulation or found on chromosome 3E. In cluster 11 (6 genes) all genes are induced in CS and DS3E(3A) but genes in AgCS have little change or are slightly repressed (0.9-1.5 fold) once again leading to a greater difference of gene expression between CS,

DS3E(3A) and AgCS and indicating these genes are not regulated by or found on chromosome 3E.

Down-regulated genes with differences between genotypes fall into clusters 2, 3 and 10. Genes in clusters 2 (2 genes) have a much greater magnitude of repression in all three genotypes as compared to cluster 3 (27 genes) but both have similar repression patterns; CS and AgCS repression levels are similar, while the same genes in DS3E(3A) are more strongly repressed. In Cluster 10, there is greater repression in CS while repression in DS3E (3A) and AgCS is similar and smaller. In CS, DS3E(3A) and AgCS several of the significantly regulated genes respond in the same fashion to salt stress as can be seen from the clustering of changes in gene expression levels but a greater number of genes have altered levels of expression between the genotypes. Genes that have differences in regulation between AgCS and CS are strong candidates to examine the resulting difference in salt tolerance between AgCS and CS. In addition, the clustering results indicates the diversity of gene regulation within gene families as the majority of gene families are found in more than one cluster that includes genes with identical annotations indicating that there is also differential regulation of gene isoforms within the genotypes. The differences seen in regulation of genotypes indicate that is an orchestration of regulation of genes that may be the basis of differences in salt tolerance found between the different genotypes and not necessarily the action of a few genes.

Figure 5. Experimental Design of Genotype-Comparison Array

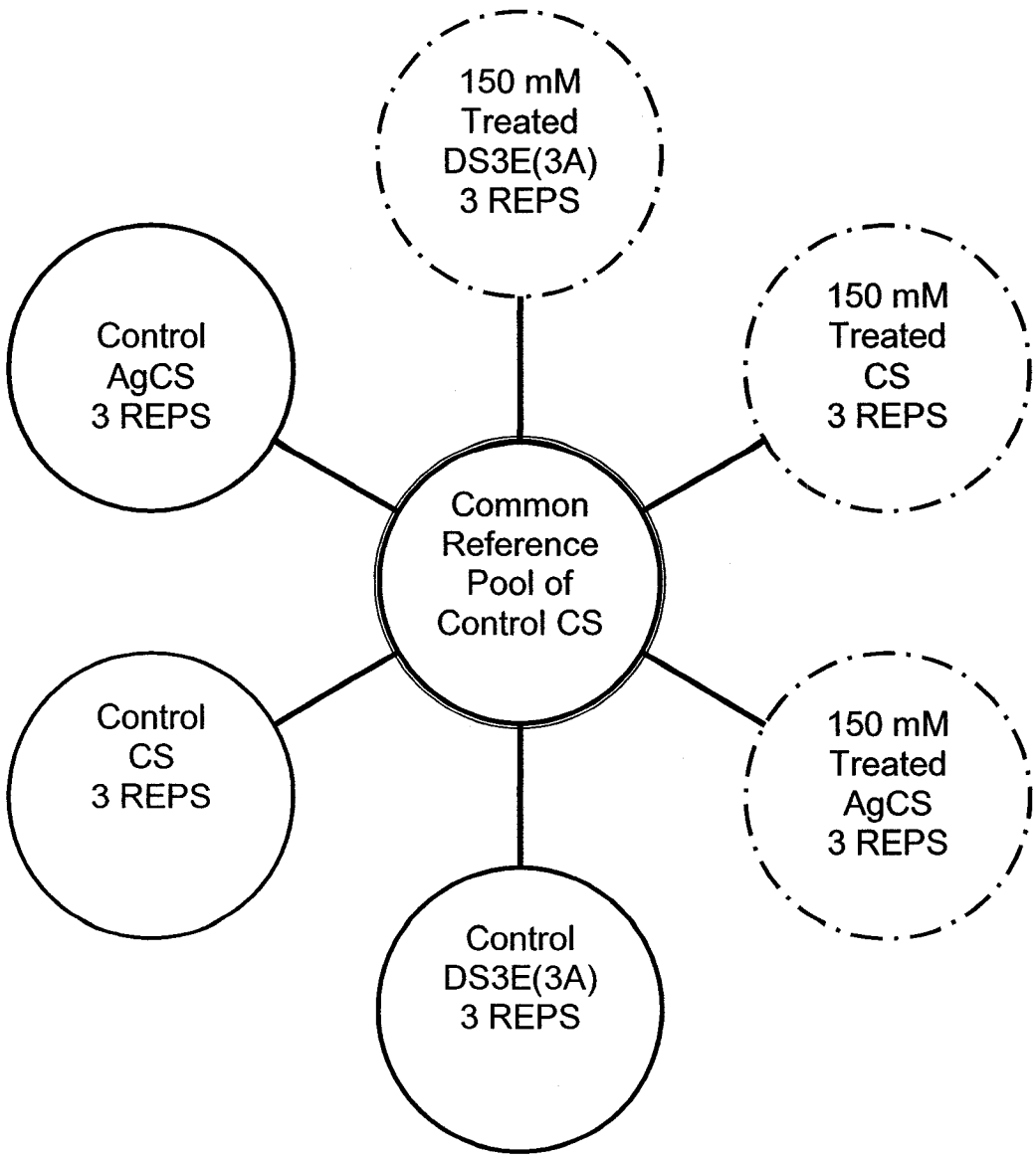

Figure 5. The experimental design is based on of series of pair wise comparisons between biological triplicates of 150mM NaCl treated and control treated plants of Chinese Spring, *T. aestivum x L. elongatum* amphiploid and DS3E(3A) to a common reference. The common reference consists of pooled control Chinese Spring RNA samples.

Table 3. Genotype-Comparison Microarray ≥ 2-fold ANOVA significant genes

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr005_B14	2115353B lipid transfer protein	**2.26**	**2.44**	1.14	**0.005**	**0.001**	0.089	11
Tr005_C05	2-oxoglutarate/malate translocator	**2.75**	**2.37**	1.72	0.997	**0.015**	0.791	7
Tr003_P10	abscisic acid- and stress-induced protein	**2.69**	1.52	1.79	**0.029**	**0**	0.109	6
Tr011_E03	actin	0.53	0.62	**0.50**	0.709	**0.002**	0.874	10
Tr016_J06	actin	0.61	0.62	**0.46**	0.721	**0**	0.458	1
Tr007_G20	actin 1	**0.47**	0.71	0.53	0.08	**0**	0.149	10
Tr005_A03	adenosine 5'-phosphosulfate reductase	**0.49**	0.58	0.63	0.379	**0.001**	0.627	10
Tr004_D18	alcohol dehydrogenase	**0.33**	1.29	0.51	0.296	**0.023**	**0.048**	10
Tr005_A15	aldehyde dehydrogenase	**2.52**	1.79	1.55	0.137	**0**	0.112	6
Tr002_L23	aldose 1-epimerase family	**2.04**	1.34	1.14	0.003	**0**	**0.018**	6
Tr004_N20	amylogenin	**2.45**	1.54	1.23	0.039	**0**	**0.015**	6
Tr011_D14	AP2 transcription factor	1.19	**2.55**	0.96	0.117	**0.01**	**0.042**	11
Tr014_J02	AP2 transcription factor (EREBP)	**2.12**	1.18	1.68	0.951	**0.001**	0.097	6
Tr001_H15	ARF-Aux/IAA transcription factor	**0.49**	**0.45**	0.70	0.068	**0.001**	0.449	3
Tr002_M15	ARF-Aux/IAAtranscription factor	**0.36**	0.65	**0.43**	0.127	**0**	0.118	1
Tr015_E11	arginase	1.74	1.29	**2.60**	0.247	**0.01**	0.372	9
Tr015_C11	asparaginase	**2.07**	1.59	1.39	0.103	**0.002**	0.424	6
Tr004_O12	beta-glucosidase	**0.33**	**0.42**	**0.41**	**0.023**	**0**	0.374	1
Tr002_A05	bHLH protein family	0.58	**0.36**	0.50	0.38	**0**	0.317	1
Tr001_A13	bHLH transcription factor	0.51	**0.49**	0.81	0.062	**0**	**0.05**	3
Tr003_N19	bHLH transcription factor	0.71	**0.45**	0.95	0.85	**0.01**	0.134	3
Tr005_G23	branched-chain alpha keto-acid dehydrogenase E1 alpha subunit	1.72	1.27	**2.41**	0.116	**0.01**	0.465	9
Tr017_L04	CAHC_HORVU Carbonic anhydrase, chloroplast precursor (Carbonate dehydratase)	0.83	**0.49**	0.58	0.691	**0.001**	0.178	3
Tr002_C09	calcium-dependent protein kinase	0.59	**0.46**	**0.50**	0.064	**0**	0.552	1
Tr013_G11	Calmodulin	**0.30**	ND	ND	**0.016**	**0**	ND	ND
Tr008_O13	calreticulin precursor	**0.47**	0.79	0.58	0.06	**0.002**	0.304	10
Tr007_E17	catalase	**4.94**	**5.32**	**3.52**	**0.041**	**0**	0.269	12
Tr015_E05	catalase	**4.27**	**6.32**	**4.12**	0.081	**0**	0.129	12
Tr002_N15	cellulose synthase	**0.49**	**0.46**	0.73	0.872	**0**	0.157	3
Tr015_D16	chitinase 2	**2.80**	1.82	**2.33**	0.615	**0**	0.269	7
Tr008_O18	chitinase II precursor	**2.94**	**3.24**	**2.63**	**0.016**	**0**	0.644	12
Tr017_C17	chitinase II precursor	**4.67**	**3.01**	**3.66**	**0.014**	**0**	0.43	12
Tr016_C22	cold acclimation protein	**0.46**	ND	ND	**0.025**	**0.014**	ND	ND
Tr016_D03	cold acclimation protein	**4.50**	**2.98**	**4.86**	0.336	**0**	0.351	12
Tr016_E07	cold acclimation protein WCOR413 - wheat	**2.85**	**2.01**	**2.03**	0.068	**0.001**	0.649	7
Tr017_C08	cold acclimation proteinprotein beta form	**2.14**	1.76	**2.29**	**0**	**0**	0.089	9
Tr017_N08	cold acclimation proteinprotein beta form	**2.66**	**2.16**	1.87	0.455	**0.001**	0.581	7

Table3. Continued

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr012_F16	cold regulated protein	**3.89**	1.64	**2.09**	0.065	**0**	0.128	7
Tr016_B08	cold regulated protein	**2.99**	1.50	1.67	0.269	**0.032**	0.6	6
Tr002_M20	cold-regulated	**4.79**	**4.56**	ND	**0.047**	**0.001**	0.985	12
Tr007_J24	copper chaperone	**2.06**	1.14	1.76	**0.01**	**0**	**0.007**	6
Tr013_C11	COR39 protein	**2.72**	**2.87**	**3.01**	0.559	**0**	0.934	12
Tr008_L13	CPRD2	1.87	1.02	**2.21**	0.467	**0.003**	0.09	9
Tr001_F12	cupin domain-containing protein	**0.37**	**0.42**	0.55	0.577	**0**	0.64	1
Tr004_F21	cytosolic 6-phosphogluconate dehydrogenase	**0.33**	**0.50**	0.50	0.266	**0**	0.184	1
Tr010_F13	defective chloroplasts and leaves protein-related / DCL protein-related	1.89	**2.30**	**2.10**	**0.021**	**0**	0.745	4
Tr007_G16	dehydrin	**3.49**	**5.27**	**3.99**	**0.002**	**0**	0.417	12
Tr003_A11	dehydrin 3	**5.16**	**6.42**	**3.66**	**0.028**	**0**	0.268	12
Tr002_F09	dehydrin 5	**2.35**	1.86	1.90	0.303	**0**	0.564	7
Tr009_L16	dehydrin 5	ND	**3.60**	**3.16**	**0.006**	**0**	0.78	12
Tr010_E22	dehydrin 5	ND	**4.10**	ND	0.129	**0.036**	ND	ND
Tr016_F17	dehydrin 5	**4.21**	**3.63**	**3.96**	**0.029**	**0**	0.95	12
Tr016_L07	dehydrin WZY1-1	**5.59**	**3.82**	**2.85**	0.085	**0**	0.35	12
Tr016_P20	dehydrin-/LEA group 2-like protein	1.32	**2.19**	1.70	0.111	**0.001**	0.468	4
Tr010_L05	diacylglycerol kinase	ND	ND	**0.43**	0.219	**0.042**	ND	ND
Tr013_J05	disease resistance protein	**0.48**	**0.30**	**0.39**	0.424	**0**	0.4	1
Tr011_O03	DNA excision repair protein ERCC3-like	ND	ND	**0.37**	0.016	**0.009**	ND	ND
Tr004_O20	DNAJ heat shock N-terminal domain-containing protein	0.74	**0.49**	0.77	0.39	**0.004**	0.26	3
Tr015_A01	DNAJ heat shock N-terminal domain-containing protein	**2.11**	1.41	1.40	0.197	**0.008**	0.29	6
Tr007_C17	early nodule-specific protein	**2.03**	ND	ND	**0.031**	**0.048**	ND	ND
Tr001_J08	elongation factor 1-alpha	**0.49**	**0.41**	**0.50**	0.769	**0**	0.746	1
Tr007_G11	endochitinase	**8.15**	**3.71**	**3.61**	**0.005**	**0**	**0.038**	12
Tr015_H23	exostosin family protein	**0.49**	0.92	1.11	0.884	0.122	**0.032**	10
Tr003_P12	fasciclin-like arabinogalactan-protein (FLA11)	**0.44**	1.10	ND	0.139	**0.048**	**0.028**	10
Tr008_I05	ferredoxin-NADP+ reductase	**0.41**	**0.45**	0.69	0.189	**0**	0.063	3
Tr004_O07	ferredoxin-related	**0.40**	0.58	0.55	0.052	**0**	0.377	10
Tr004_L02	formate dehydrogenase	**0.23**	**0.11**	**0.23**	0.841	**0**	0.219	2
Tr008_B02	fructose-bisphosphate aldolase	**0.46**	0.60	**0.44**	0.002	**0**	0.297	1
Tr010_F20	fructose-bisphosphate aldolase	**0.33**	0.58	**0.35**	0.032	**0**	0.103	1
Tr004_I23	fructosyltransferase	0.56	**0.49**	**0.43**	0.742	**0**	0.383	1
Tr007_F14	gamma-TIP-like protein	0.61	**0.45**	0.80	0.126	**0**	**0.022**	3
Tr005_C01	germin precursor	**2.65**	**2.05**	0.96	0.3	**0.045**	**0.367**	11
Tr016_G11	glutathione transferase F4	**3.34**	1.53	1.35	**0.032**	**0**	**0.011**	6
Tr002_B03	glycosyl hydrolase family 1	**0.39**	**0.27**	0.54	0.253	**0**	0.1	1
Tr001_H20	haloacid dehalogenase hydrolase	**0.41**	**0.43**	0.76	0.303	**0**	0.143	3

Table3. Continued

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr007_M14	haloacid dehalogenase-like hydrolase family protein	0.57	**0.33**	0.52	0.665	**0**	0.203	1
Tr016_G21	heat shock-related protein	**0.34**	**0.47**	**0.44**	0.147	**0**	0.476	1
Tr002_H03	hexose transporter	**2.99**	1.25	**2.07**	0.284	**0.001**	0.103	6
Tr004_H18	HGWP repeat containing protein-like	1.60	**2.12**	1.76	0.149	**0.001**	0.597	4
Tr004_N21	histone H1flk	0.59	**0.45**	**0.48**	0.061	**0**	0.538	1
Tr008_O20	histone-lysine N-methyltransferase	1.94	1.50	**2.13**	0.609	**0**	0.56	9
Tr002_G13	homeobox protein HAT22	1.43	1.61	**2.10**	0.714	**0.001**	0.511	9
Tr002_O11	homeodomain leucine zipper protein	**0.41**	0.78	0.80	0.115	**0.031**	0.325	10
Tr008_L05	homeodomain leucine zipper protein	**2.11**	1.14	**0.47**	0.159	0.914	**0.043**	8
Tr007_I13	hypersensitive-induced reaction protein 3	**2.09**	1.22	1.24	0.174	**0.003**	0.154	6
Tr004_H08	hypothetical protein	0.94	0.90	**0.48**	0.099	**0.013**	**0.043**	10
Tr008_J07	hypothetical protein	**2.70**	**2.61**	**2.33**	0.958	**0**	0.823	7
Tr010_D08	hypothetical protein	**5.70**	**4.05**	**3.04**	**0.033**	**0**	0.132	12
Tr010_K07	hypothetical protein	ND	ND	**2.45**	0.505	**0.038**	ND	ND
Tr011_O10	hypothetical protein	**2.01**	0.95	1.94	0.15	**0.003**	**0.024**	6
Tr015_J04	hypothetical protein	1.35	0.89	**2.32**	**0.019**	**0.001**	**0.004**	5
Tr015_K18	hypothetical protein	**2.02**	1.41	1.88	**0.038**	**0**	0.289	9
Tr017_K21	hypothetical protein	1.67	1.76	**2.23**	0.467	**0.011**	0.93	9
Tr001_D21	IAA1 protein	**0.42**	0.70	ND	**0.012**	**0**	**0.036**	10
Tr003_L21	IAA1 protein	**0.45**	0.66	0.76	0.132	**0**	**0.039**	10
Tr001_P24	inorganic pyrophosphatase	ND	ND	**0.50**	0.326	**0.022**	ND	ND
Tr017_H22	isovaleryl-CoA dehydrogenase	**2.45**	1.50	1.60	0.109	**0.003**	0.504	6
Tr002_A20	Kelch repeats protein family	0.68	**0.38**	0.66	0.568	**0.001**	0.128	3
Tr002_K13	Kelch repeats protein family	0.73	**0.42**	0.67	0.453	**0.005**	0.197	3
Tr001_E23	Kelch repeats-actin binding protein	0.65	**0.42**	1.27	0.19	**0.018**	**0.019**	3
Tr003_E08	Kelch Ubiquitin ligase	0.69	**0.34**	0.76	0.172	**0**	**0.023**	3
Tr002_A03	leucine-rich repeat transmembrane protein kinase	**2.86**	**4.60**	**5.60**	**0.008**	**0**	0.274	12
Tr001_N14	lipid transfer protein	0.62	**0.49**	0.63	**0.049**	**0**	0.445	3
Tr014_D09	lipoxygenase	**0.45**	0.71	0.64	0.761	**0.005**	0.371	10
Tr005_J03	LRR receptor-like protein kinase	**0.43**	0.64	0.67	0.136	**0.004**	0.373	10
Tr001_B19	LRR transmembrane protein kinase	**0.48**	0.59	0.65	0.334	**0**	0.548	10
Tr014_F12	MADS Box transcription factor	**0.33**	ND	0.75	0.692	**0.007**	**0.044**	10
Tr014_H04	MADS-box protein TaVRT-1	1.62	**2.02**	1.79	0.059	**0**	0.746	4
Tr006_B15	MATE efflux family protein-like	**0.49**	**0.41**	0.74	0.685	**0.001**	0.175	3
Tr015_K22	membrane protein	**2.08**	1.90	**2.29**	0.14	**0**	0.676	9
Tr016_D07	membrane protein	1.82	**2.03**	**2.87**	0.914	**0**	0.092	9
Tr017_A19	membrane protein	1.95	1.76	**2.49**	0.653	**0**	0.499	9
Tr005_E07	metallothionein	**2.90**	**2.55**	1.57	0.528	**0.008**	0.686	7
Tr006_L15	metallothionein	**2.22**	1.60	**2.23**	0.149	**0**	0.207	9

Table3. Continued

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr005_L18	metallothionein-like protein type 4	**6.25**	**3.92**	**4.14**	0.068	**0**	0.289	12
Tr006_N01	mitochondrial processing peptidase alpha-chain precursor	**0.48**	0.79	0.77	0.443	**0.003**	0.221	10
Tr002_J23	monosaccharide transporter 3	**2.19**	1.35	1.27	0.156	**0.001**	0.06	6
Tr005_E24	MtN19	**2.31**	1.66	1.61	0.798	**0**	0.304	6
Tr001_G24	MYB transcription factor	0.78	ND	**2.32**	**0.043**	**0.037**	**0.011**	5
Tr003_J21	NAC transcription factor	0.51	**0.49**	0.72	**0.001**	**0**	**0.008**	3
Tr012_K14	NAC transcription factor	1.58	**2.17**	**2.06**	0.072	**0**	0.23	4
Tr003_C04	NAM transcription factor	**2.07**	**2.25**	**2.18**	0.755	**0**	0.949	4
Tr003_C06	NAM transcription factor	1.85	1.68	**2.12**	**0.038**	**0.001**	0.889	9
Tr004_K16	NAS5_HORVU Nicotianamine synthase	**0.14**	**0.06**	**0.07**	0.414	**0**	0.239	2
Tr015_G15	NBS-LRR protein	1.93	1.46	**2.19**	0.436	**0**	0.228	9
Tr006_N08	No Blast Hit	ND	ND	**2.29**	0.427	**0.007**	ND	ND
Tr008_J04	No Blast Hit	**2.53**	**4.19**	1.45	0.797	**0**	**0.016**	11
Tr015_G21	No Blast Hit	1.84	1.70	**2.42**	0.468	**0**	0.288	9
Tr015_M06	No Blast Hit	ND	ND	**2.18**	0.188	**0.018**	ND	ND
Tr016_G09	No Blast Hit	**2.14**	1.74	1.69	0.06	**0**	0.357	6
Tr016_M12	No Blast Hit	0.67	**0.42**	0.54	0.796	**0.001**	0.371	3
Tr017_G22	No Blast Hit	0.68	**0.49**	0.53	0.222	**0**	0.48	3
Tr017_L11	No Blast Hit	**2.06**	0.72	1.00	0.387	0.834	**0.036**	8
Tr005_B23	nodulin 3	1.74	**2.49**	1.50	0.337	**0**	0.221	4
Tr010_A15	non-LTR retroelement reverse transcriptase	**0.46**	**0.27**	**0.36**	0.139	**0**	0.353	1
Tr017_P10	O-methyltransferase	**2.06**	0.68	0.91	0.08	0.854	**0.034**	8
Tr004_P11	oxalate oxidase	**3.60**	1.68	1.63	0.168	**0.01**	0.421	7
Tr005_I09	oxalate oxidase	**3.39**	**3.11**	**3.57**	**0**	**0**	0.774	12
Tr005_H08	p68 RNA helicase	**2.98**	**2.65**	**4.32**	**0.025**	**0**	0.234	12
Tr007_P22	pectin-glucuronyltransferase	**2.28**	1.44	1.14	0.166	**0.002**	0.077	6
Tr006_D12	pg1	0.97	**0.40**	0.79	0.114	**0.014**	0.061	3
Tr009_G23	phenylalanine ammonia-lyase	**2.32**	1.41	1.22	**0.012**	**0.001**	0.061	6
Tr016_M03	phosphatidylinositol 4-kinase	**0.40**	**0.44**	**0.47**	0.983	**0**	0.769	1
Tr017_K19	phosphatidylinositol 4-kinase	ND	1.09	**0.44**	**0.036**	0.102	**0.031**	10
Tr005_P11	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplast precursor	1.67	1.24	**2.26**	0.349	**0.01**	0.498	9
Tr002_C07	phosphoenolpyruvate kinase	**0.42**	0.63	0.58	0.887	**0**	0.389	10
Tr010_G22	phosphoethanolamine N-methyltransferase	**0.43**	0.75	0.62	0.404	**0**	0.211	10
Tr005_J16	phospholipase	0.55	**0.48**	0.58	0.422	**0**	0.776	3
Tr004_H22	phospoenolpyruvate carboxylase	**0.46**	**0.42**	0.78	0.121	**0**	0.241	3
Tr016_L10	photosystem II type I chlorophyll a/b binding protein	**2.33**	1.42	1.67	0.531	**0.003**	0.338	6
Tr004_N14	plasma membrane H+ ATPase	**0.49**	0.64	0.73	**0.027**	**0**	0.241	10
Tr002_M24	polygalacturonase-inhibiting protein	**0.45**	0.51	0.90	**0.002**	**0**	**0.017**	3
Tr001_D08	Polyubiquitin	**0.47**	**0.44**	0.59	**0.029**	**0**	0.362	1
Tr016_N17	proline-rich protein	1.35	**2.23**	1.75	**0.030**	**0**	0.112	4

Table3. Continued

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr003_A09	Protein kinase	**2.27**	1.57	1.97	**0.001**	**0**	0.299	9
Tr013_G04	Protein kinase	1.32	1.34	**2.09**	0.496	**0.006**	0.39	9
Tr013_K19	Protein kinase	0.89	0.70	**5.85**	**0.001**	**0.002**	**0**	5
Tr014_O11	Protein kinase	**2.36**	1.34	1.39	**0.011**	**0**	**0.021**	6
Tr014_N16	Protein Kinase (Serine/threonine)	**0.48**	0.90	0.65	0.369	**0.007**	0.21	10
Tr005_N13	proteinase inhibitor II	1.41	**2.00**	1.38	0.056	**0.002**	0.267	4
Tr011_G17	pyruvate kinase, cytosolic isozyme	**0.46**	0.58	0.57	0.154	**0**	0.61	10
Tr005_K05	Ran binding protein-1	**2.71**	**2.43**	**3.22**	**0**	**0**	0.493	7
Tr001_B17	Receptor protein kinase	0.53	1.02	**0.49**	**0.012**	**0.007**	**0.026**	10
Tr003_G20	Receptor protein kinase	1.46	1.04	**2.59**	0.059	**0.01**	**0.047**	5
Tr003_E06	receptor protein kinase ARK3	1.61	1.62	**2.00**	0.165	**0.004**	0.82	9
Tr002_N12	receptor protein kinase PERK1-like protein	1.88	**2.34**	1.76	**0.048**	**0**	0.219	4
Tr012_M02	receptor-like kinase	1.96	1.25	**2.22**	0.116	**0**	**0.032**	9
Tr001_J18	receptor-like protein kinase	**0.49**	**0.36**	0.68	0.314	**0**	0.104	3
Tr016_F02	Ribosome recycling factor, chloroplast precursor	**0.49**	0.66	0.99	0.926	**0.006**	0.066	10
Tr016_E21	ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit	**2.03**	1.45	1.50	0.136	**0.001**	0.4	6
Tr001_N17	RNA-binding glycine-rich protein	**0.42**	**0.47**	**0.46**	0.416	**0**	0.917	1
Tr012_N13	RNA-binding glycine-rich protein	**3.08**	1.34	1.64	0.446	**0**	**0.023**	6
Tr013_J24	RNA-binding glycine-rich protein	**2.84**	**2.29**	1.91	0.364	**0**	0.057	7
Tr001_B20	RNA-binding protein	**2.10**	**2.70**	1.57	**0.006**	**0**	0.162	4
Tr004_F20	saccharopin dehydrogenase-like protein	**2.30**	**2.02**	**2.88**	0.247	**0.004**	0.907	9
Tr007_D22	selenium binding protein	**2.48**	1.28	1.92	**0.016**	**0.002**	0.19	6
Tr017_C10	senescence-associated protein-like	**2.07**	1.23	1.35	**0.024**	**0**	0.058	6
Tr004_C05	Ser/Arg-related nuclear matrix protein	**2.59**	1.50	1.67	0.32	**0.003**	0.346	6
Tr016_K18	Serine/threonine Kinase	0.80	**0.49**	0.82	0.068	**0.006**	0.162	3
Tr002_I18	steroid sulfotransferase	1.77	1.89	**2.34**	0.619	**0**	0.543	9
Tr002_G02	sulfotransferase family	0.61	**0.48**	**0.47**	0.636	**0**	0.47	1
Tr002_G22	sulfotransferase family	0.57	0.59	**0.46**	0.762	**0**	0.772	1
Tr002_I08	sulfotransferase family	**0.41**	**0.45**	0.74	0.067	**0**	**0.006**	3
Tr004_I01	thaumatin-like protein	**5.50**	1.93	**2.83**	**0.002**	**0**	**0.004**	7
Tr001_D24	TIR1/COI1 E3 ubiquitn ligase	**2.45**	1.92	1.76	0.927	**0.001**	0.618	7
Tr005_M01	tonoplast intrinsic protein	**0.50**	**0.39**	0.58	0.37	**0**	0.369	1
Tr012_L14	transcription activator REB	**2.32**	1.19	1.71	**0.003**	**0.001**	0.139	6
Tr005_P20	transcription factor MYB24	**2.99**	1.39	1.88	0.153	**0.002**	0.203	6
Tr012_E14	transcriptional activator	**2.15**	1.03	1.24	**0**	**0.002**	**0.013**	6
Tr014_I16	transducin/WD-40 repeat protein	**2.36**	1.06	1.43	0.11	**0.002**	**0.018**	6
Tr004_O03	triosephosphat-isomerase	**0.47**	0.70	0.60	**0.017**	**0**	0.098	10
Tr004_K15	trypsin inhibitor (Bowman-Birk) - two	**3.26**	1.35	1.55	**0.007**	**0.001**	**0.048**	6

Table3. Continued

Microarray ID	Annotation	Change in Expression level			ANOVA P- Values			KMC Cluster #
		Tr.CS/ Ct.CS	Tr.DS3E(3A)/ Ct.DS3E(3A)	Tr.AgCS/ Ct.AgCS	Geno	Treat	GbyT	
Tr004_F16	type 1 membrane protein -like	1.60	**2.31**	1.60	0.181	**0**	0.229	4
Tr017_N23	type 1 non-specific lipid transfer protein precursor	1.47	**2.34**	1.01	**0.027**	**0.007**	0.052	11
Tr005_F01	tyrosine-specific protein phosphatase protein	**2.38**	1.77	1.78	0.077	**0.001**	0.494	6
Tr016_C21	unknown	0.64	**0.47**	0.74	0.595	**0.001**	0.315	3
Tr005_E15	unknown protein	**0.50**	0.52	0.70	0.483	**0.001**	0.757	3
Tr007_A16	unknown protein	**2.43**	**3.24**	**3.70**	0.209	**0**	0.174	12
Tr008_C17	unknown protein	ND	ND	**2.40**	**0.007**	**0.019**	ND	ND
Tr008_G03	unknown protein	**0.35**	**0.27**	**0.30**	0.118	**0**	0.537	1
Tr015_E03	unknown protein	**2.14**	1.98	**2.46**	0.587	**0**	0.848	9
Tr016_A14	unknown protein	**2.21**	1.79	**2.66**	0.58	**0**	0.463	9
Tr016_M01	unknown protein	1.73	1.50	**2.24**	**0.035**	**0**	0.292	9
Tr004_D10	vacuolar proton-ATPase	**3.17**	**2.18**	1.38	0.191	**0.012**	0.193	7
Tr008_N04	wheat aluminum induced protein wali 3	**4.24**	**2.11**	1.90	0.955	**0**	**0.045**	7
Tr010_J20	wheat aluminum induced protein wali 3	**3.85**	**2.45**	**2.05**	0.474	**0**	0.102	7
Tr003_F01	WRKY transcription factor	ND	**2.33**	0.89	0.075	**0.008**	**0.011**	11
Tr003_I13	WRKY transcription factor	**2.21**	1.17	1.30	**0.023**	**0**	**0.003**	6
Tr003_K07	WRKY transcription factor	**2.82**	**2.53**	**3.54**	0.405	**0**	0.704	12
Tr002_K15	WRKY transcription factor	1.26	1.00	**2.02**	0.078	**0.008**	**0.038**	5
Tr003_E07	zinc finger transcription factor ZF1	1.94	1.52	**2.26**	**0.047**	**0**	0.563	9
Tr001_N07	zinc-finger protein	1.87	1.37	**2.24**	0.643	**0.012**	0.771	9

Table 3. Genes that have a ≥ 2-fold change in expression with treatment of 150mM NaCl for three days relative to untreated controls in at least one genotype and with a significant P value (P≤ 0.05) in two way ANOVA analysis_ for treatment effects or treatment by genotype interaction. Genes are listed alphabetically by annotation. **Boldface** indicates values that have a ≥ 2-fold for change in expression for relative transcript levels of treatment/control in each genotype and significant ANOVA P-values (P≤ 0.05), ND=no data. Abbreviations are as follows: Ct=Control, Tr=Treatment, CS=Chinese spring, DS3E(3A) = Disomic substitution 3E(3A), AgCS= Amphiploid, Geno =genotype significance, Treat= Treatment significance, GbyT= genotype by treatment interaction significance.

Figure 6. Distribution of Genes with ≥ 2 -fold Changes among Three Genotypes

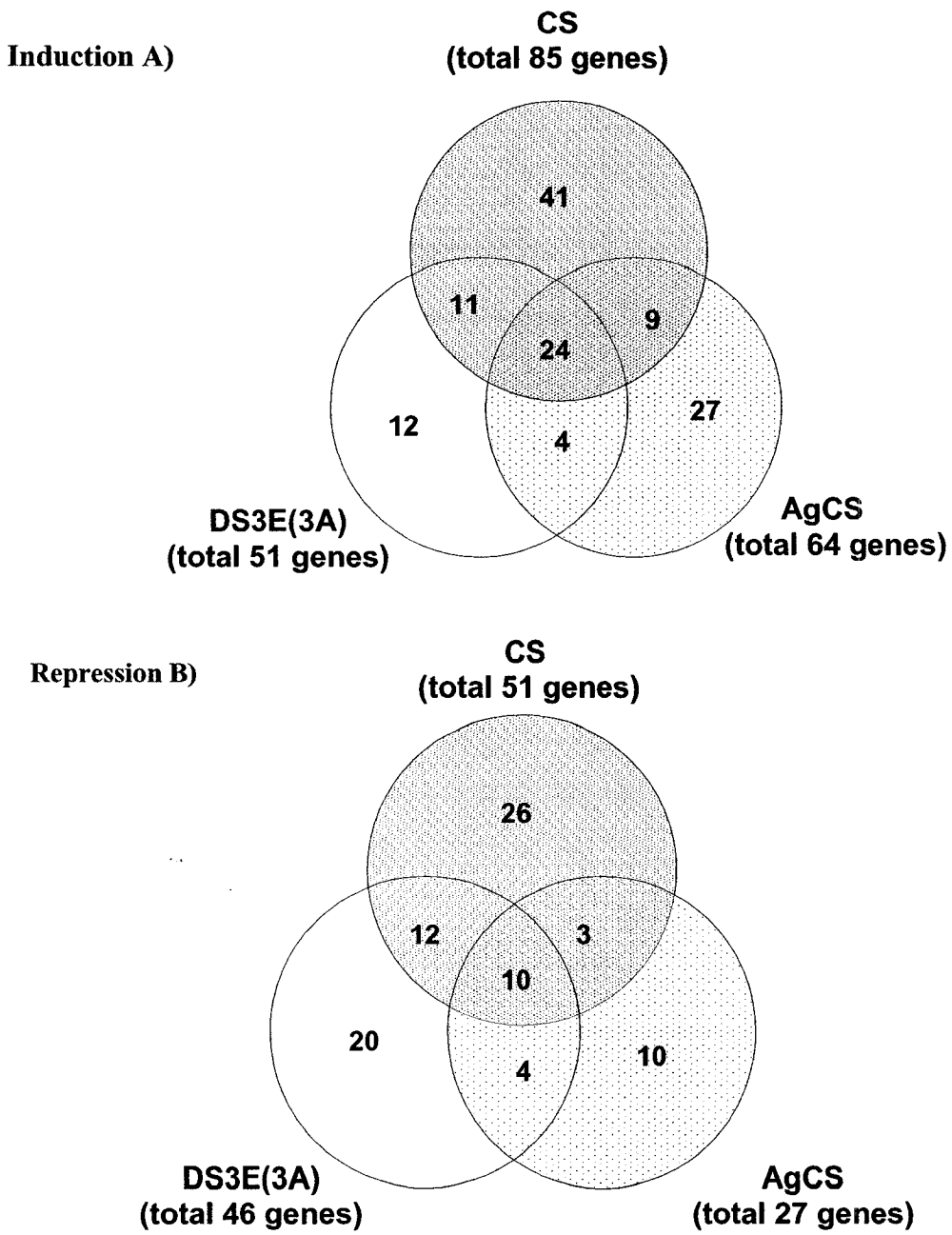

Figure 6. Distribution of 212 genes with at least a two-fold change in expression in at least one genotype. Areas of overlap represent genes that have a two-fold change in more than one genotype. A) Represents gene that have a 2-fold or greater induction under salt stress conditions. B) Represents genes that have a 2-fold or greater repression under salt stress conditions.

Figure 1. HMC and KMC Analysis of ≥ 2-Fold Genotype-Comparisons Array Genes

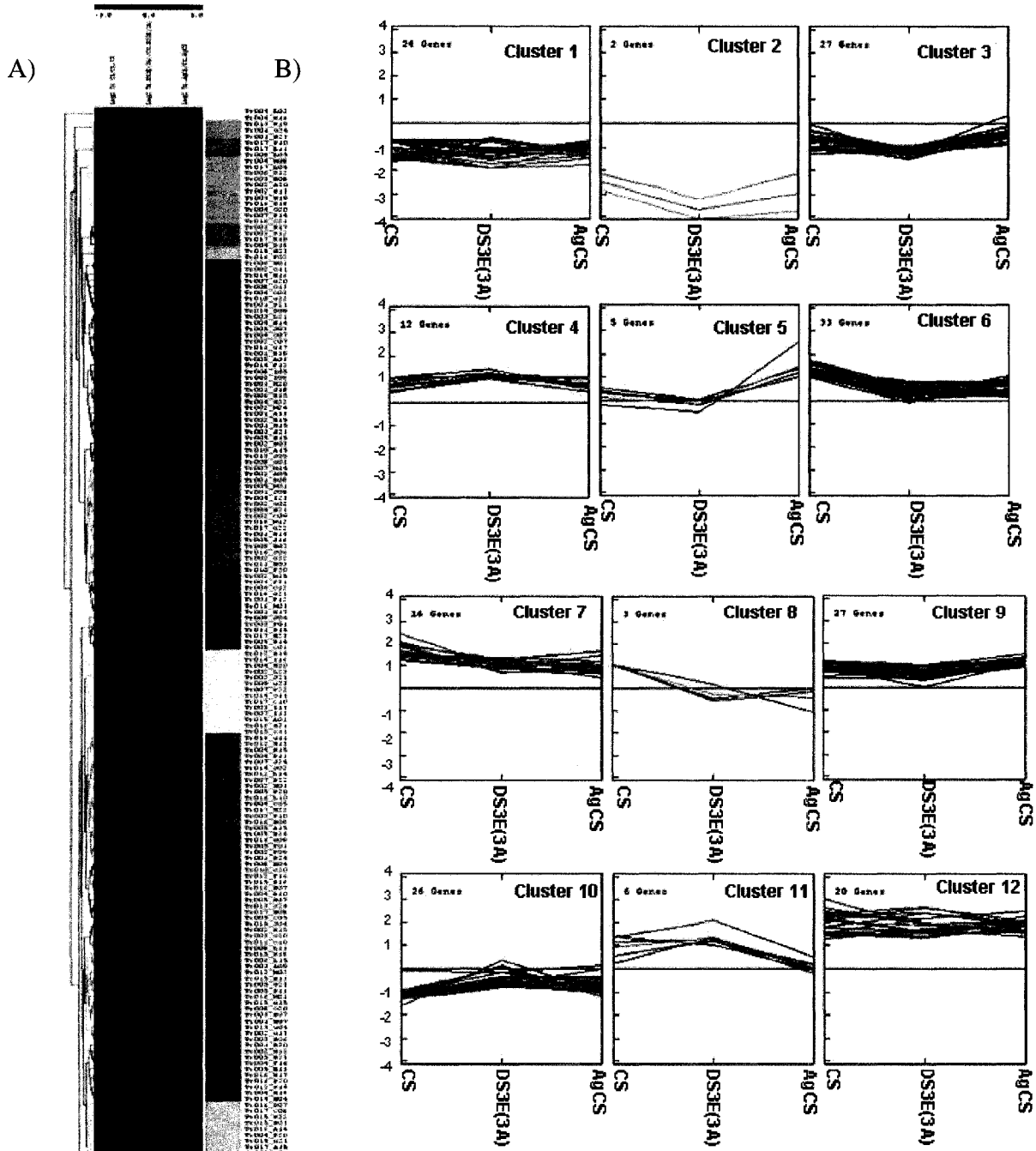

Figure 7. Clustering analysis of ANOVA significant genes that have a ≥2-fold change in expression in at least one genotype. Change in expression log2 values are used for analysis and shown on y axis. A) HCL clustering pattern, from left to right; Tr.CS/Ct.CS, Tr.DS3E(3A)/Ct. DS3E(3A), Tr.AgCS/ Ct.AgCS. B) KMC clustering pattern of change in expression in each genotype. Abbreviation used CS= Tr.CS/ Ct.CS, DS3E(3A)= Tr. DS3E(3A)/ Ct. DS3E(3A), AgCS= Tr.AgCS /Ct.AgCS

3.6 Genotype-Comparison Array; Clustering Analysis of mRNA Steady State Levels in Salt Treated and Non-Treated Plants between Each Genotype

To study specifically genes that have differently regulated transcripts in CS, DS3E(3A) and AgCS, two-way ANOVA was used to identified genes with genotype by treatment interaction significance ($P \leq 0.05$). There are 305 genes with genotype by treatment interaction significance, 34% of which can be classified has having regulatory and signal transduction functions. For genotype by treatment interaction significance ($P \leq 0.05$) no threshold of fold change was used. Significant differences between genotypes can be caused not only by gene induction and repression but also by significant differences in the steady state levels of mRNA in control and salt-stressed plants between each genotype. KMC analysis was applied to the control and salt treated plants using relative expression levels of each genotype, the values of the control and the treated plants relative to their common reference. The clustering results are depicted in Figure 8. The clustering results of the steady state mRNA levels reveal that none of significant genotype by treatment interaction genes has identical patterns of expression in all three genotypes. In addition, it is interesting to note that the majority of differences between the genotypes transcript levels are not as apparent under control conditions where most clusters' mean expression levels are similar but are evident in the genotypes under salt stress. This result concurs with physiological results in which AgCS has no growth advantage over CS under control growth conditions but greatly outperforms CS under salt-stress (Omeilian and Epstein, 1991).

Figure 2. KMC Analysis of the Relative Levels of Genotype by Treatment Interaction Genes

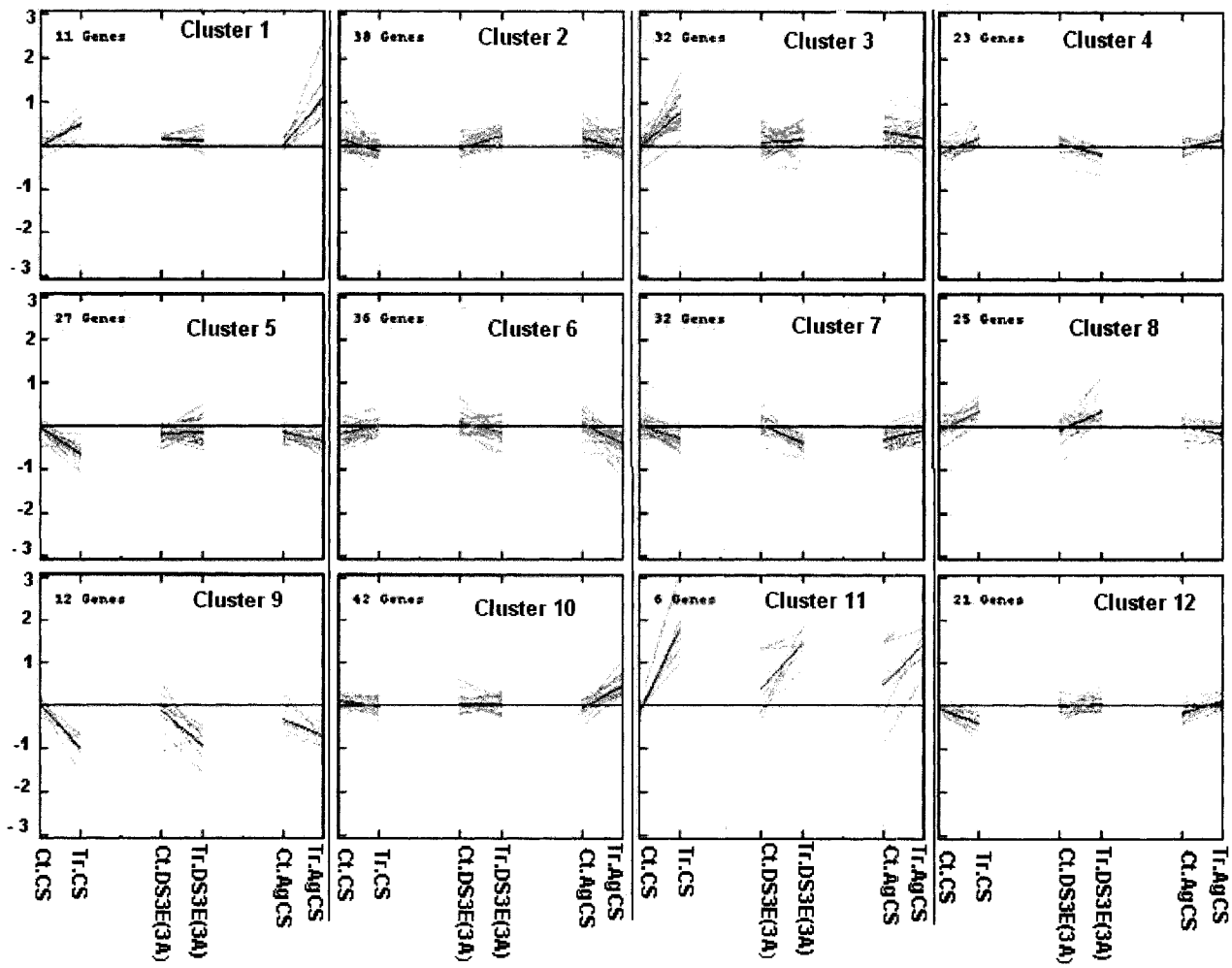

Figure 8. Cluster analysis using KMC analysis of log2 values of the relative levels of signal for each genotype under control and treatment conditions provides a means to visual the change in each genotype for a cluster of genes. Each grey line represents a gene at control and salt stress conditions, pink lines are the average in each genotype for that cluster of genes.

Clusters 3, 6, 7, 8, 10 and 12 (Figure 8) encompass 61% of genes that have significant genotype by treatment interaction, showing differential transcript regulation between AgCS and CS. Clusters 3 (32 genes), 6 (36 genes) and 8 (25 genes) have increases in mRNA levels in CS under salt stress while in AgCS there is down regulation of transcripts and may account for the increase salt tolerance found in AgCS compared to CS. In clusters 7 (32 genes), 10 (42 genes) and 12 (21 genes) there are decreases in CS mRNA levels and increases in AgCS. Clusters 1, 2, 4, 5, 9 and 11 have the same pattern of gene expression in CS and AgCS although for some clusters there are differences in the magnitude of change from control to salt-stress mRNA levels. In cluster 1(11 genes) there is up regulation in both CS and AgCS under salt stress but AgCS had a higher degree of induction. In clusters 9 (12 genes) and 11 (6 genes), AgCS has smaller changes between control and treated mRNA levels resulting in smaller changes in expression levels as compared to CS but under salt-stress, the mRNA levels in AgCS and CS for these genes are equalized. Genes in DS3E(3A) does not appear to follow the expression patterns solely of either CS or AgCS. DS3E(3A) has the same pattern of gene expression as CS in clusters 7, 8 and 9. In clusters 3, 6, and 12 the expression patterns reflect those of AgCS. These genes may be regulated by genes on chromosome 3E since it is the chromosome that is common between these two genotypes. In clusters 1, 2, 4, 5 the pattern of expression for CS and AgCS are the same either up or down regulated but in DS3E(3A) the pattern is opposing or essentially unchanged. There could be several possible reasons for why DS3E(3A) in some cases does not seem to follow the expression profile of either CS or AgCS; 1) The substitution of chromosome 3E for 3A may result in a loss of regulation of CS genes on other chromosomes ; 2) Regulation of genes on

chromosome 3E requires genes on other *L. elongatum* chromosomes to be present; 3) The substitution of only chromosome 3E may result in differences in the regulation of genes on other chromosomes in CS. Genes that have differences in clustering patterns between the most salt tolerant genotype AgCS and the most sensitive CS, are good candidates for further study and characterization of regulation of salt-tolerance genes, in addition, genes that are commonly regulated between AgCS and DS3E(3A) are the best candidates to understand the effects of chromosome 3E and increased salt tolerance.

3.7 Analysis of Differentially Expressed Genes by Functional Classes

To further analyze genes found to have altered expression in response to salt stress the genes are discussed in terms of the functional classes into which they belong in order to and how these genes may be involved in the regulation of salt tolerance.

3.7.1 Osmoprotectants

Osmoprotectants are small organic solutes that can accumulate to high concentrations within the cell with no toxic effects on metabolism; such compounds are generally hydrophilic and have a neutral pH (McNeil et al., 1999; Tester and Davenport, 2003; Munns, 2005). They are important for the maintenance of osmotic potential during salt stress by providing a counter balance to Na^+ ions sequestered in the vacuoles when plants are exposed to higher external concentrations of NaCl. The lowered internal water potential created by the accumulation of solutes allows water uptake when salt concentrations in the soil are high. In addition, small organic solutes are thought to have protective functions by stabilizing proteins and membranes (McNeil et al., 1999;

Hasegawa et al., 2000; Yokoi et al., 2002; Tester and Davenport, 2003; Munns, 2005). The rise of internal Na^+ and Cl^- concentrations of wheat roots plateaus when the external concentration of NaCl reaches 50mM, beyond this point increases in internal levels are not proportional to external concentrations. This infers that maintenance of turgor and water intake occurs through osmolyte synthesis (Munns et al., 2006). There are several classes of compounds that play osmotic and protective role in plants including sugars (e.g. sucrose and trehlose), polyols (e.g. mannitol) and N-containing solutes (e.g. glycine betaine, proline), (McNeil et al., 1999; Hasegawa et al., 2000; Tester and Davenport, 2003; Munns, 2005). Both microarray experiments detected a few genes that could encode proteins involved in osmolyte transport or synthesis which were induced by salt stress. In the Norstar time-course array, potential genes involved in osmolyte biosynthesis are an aldehyde dehydrogenase (Tr005_A21) which is only induced early on at 6 hrs of salt treatment then normalizes, a glycosyl hydrolase family 1/β-glucosidase (Tr002_J13) which is induced both at 6 hrs and 72 hrs of salt stress and lastly a thaumatin-like protein (Tr004_I01) which shows later induction at 24 hrs and 72 hrs. The alterations of gene expression for these genes at different time points during an applied salt stress demonstrate complex regulation.

In the genotype-type comparison array, potential genes for increasing osmolytes were an aldehyde dehydrogenase (Tr004_D18), an O-methyltransferase (Tr017_P10) and a hexose transporter (Tr002_H03), no genes were found to have genotype by treatment interaction significance which indicates that none of these genes have altered expression patterns between genotype under salt-stress.

3.7.2 Dehydrins

Dehydrins are related to the *Lea* protein family and many are expressed only after an osmotic stress, they have chaperone-like properties and are thought to have protective functions by acting to stabilize proteins in plants during water loss (Borovskii et al., 2002; Munns, 2005). Dehydrins have previously been shown to have increased expression in the roots of CS and AgCS under salt stress (Galvez et al., 1993).

In CS, DS3E(3A) and AgCS, four genes encoding for dehydrins, Tr007_G16, Tr003_A11, Tr016_F17 and Tr016_L07, had pronounced up-regulation during salt stress. While other genes belonging to this family (Tr002_F09, Tr009_116, Tr010_E22, and Tr016_P20) had variable levels of induction in each of the genotypes (Table 3)and all have a $\geq$ 2-fold induction in at least one genotype. None of the dehydrins had significant treatment by genotype effects. Galvez *et al.* (1993) reported that after 6 hours of 250 mM NaCl treatment the mRNA expression of two dehydrins tested in *L. elongatum* and AgCS was significantly higher than in CS and after 24 hours of treatment the expression profiles of the dehydrins in *L. elongatum*, CS and AgCS were all similar. It is therefore plausible that there are significant differences under salt stress between the genotypes but at earlier than 72 hours that was tested.

In the Norstar time-course array the same dehydrins genes as those in the genotype-comparison array with exception of Tr007_G16 were seen to be up regulated and all had significant expression differences between time-points. At 6 hours of salt-

treatment the greatest induction occurred with a decrease at 24 hours and by 72 hours only slight differences between the control and treated samples were seen.

3.7.3 Photosynthetic genes

Transcripts of photosynthetic genes in roots has been previously been reported in many cDNA libraries and EST projects (Wang et al., 2003). Transient induction of photosynthetic genes were found in microarray experiment with *Zea mays* (maize) roots under salt stress (Wang et al., 2003)). The function of these genes in roots is yet to be characterized. In the Norstar time-course array 8 genes encoding the large subunit of rubisco and six genes encoding the small subunit were all induced at 72 hrs of salt treatment. In addition, a gene encoding a photosystem I subunit N was induced at 72 hr, a photosystem-1 F subunit precursor was repressed at 6 hrs and a chlorophyll a/b binding protein gene was repressed at 6 hrs and 24 hrs of salt stress. Seven other genes in Norstar had significant differences between time points without being significantly regulated under salt stress at any one time-point.

In the genotype-comparison array two genes encoding components of the photosynthetic apparatus were induced in the genotype-comparison array. One encoding for a ribulose-1, 5-bisphosphate carboxylase/ oxygenase (rubisco) small subunit (Tr016_F02) and a photosystem II type 1 chlorophyll a/b binding protein (Tr016_L10) neither had genotype by treatment significance indicating that there were no significant difference in expression under salt stress between genotypes.

3.7.4 Genes regulated by Cold Acclimation

Many genes that are regulated by salt stress have been reported to be regulated by other environmental stresses, especially cold and drought. All of these stresses share an element of osmotic stress. In the genotype comparison array, seven genes annotated as cold acclimation or cold regulated (Tr016_D03, Tr016_E07, Tr017_C08, Tr017_N08, Tr012_F16, Tr016_B08, Tr002_M20) were significantly induced under salt stress and one was repressed (Tr016_C22). None of the genes had genotype by treatment interaction indicating that their regulation was similar in all three genotypes and does not likely account for difference in salt regulation found between each genotype.

In the Norstar-time course experiment five cold acclimation/ regulated genes (Tr016_D03, Tr016_E07, Tr016_B09, Tr012_F16, Tr002_M20) were induced at 6 hrs, one additionally had induction at 24 hrs and one (Tr002_N07) was only induced after 72 hrs of salt stress. The result from both array experiments indicates there is an overlap in cold and salt responsive genes.

3.7.5 Signal Transducers: Receptor Kinases

Receptor kinases play a central role in detecting external factors and transmitting the information intercellularly by activating signal transduction pathways. Receptor kinases are composed of three domains a ligand-binding extracellular domain, a transmembrane domain and a cytoplasmic kinase domain. The extracellular domains of

these kinases are diverse indicating their interaction with a large array of external ligands, while the kinase domains are highly conserved (Shiu and Bleecker, 2001; Haffani et al., 2004). The microarray analysis for genotype-comparison identified nine receptor kinases with significant salt treatment effects in at least one of the genotypes; three receptor kinases had no significant treatment effects but had significant genotype by treatment interaction effects indicating they are regulated differently between genotypes and two receptor kinases had significant genotype by treatment interaction effects. Homologues of the protein kinase receptor ARK3 (Tr003_E06) and PERK1 (Proline Extensin-like Receptor Kinase 1) -like receptor kinase (Tr002_N12) were found to be induced in all three genotypes. The ARK3 homologue had the strongest induction in AgCS perhaps contributing to its much higher increased salt tolerance and the Perk1-like receptor kinase being most strongly upregulated in DS3E(3A). Evidence suggests both ARK3 and PERK1 have roles in plant defense (Pastuglia et al., 2002; Silva and Goring, 2002). Two genes annotated as receptor protein kinases (Tr003_G20, Tr001_B17) and two annotated as receptor-like protein kinases (Tr012_M02, Tr001_J18) had differing degrees of induction and repression. Three of these genes (Tr003_G20, Tr001_B17 and Tr012_M02) had genotype by treatment interaction effects with largest changes in regulation occurring in AgCS inferring that these genes are strong candidates for further study of salt tolerance in AgCS.

Leucine-rich repeats (LRR) are a common motif in the extracellular domain of receptor kinases and are thought to mediate protein-protein interactions (Shiu and Bleecker, 2001). LRR-receptor kinases have previously been shown to be up regulated

by cold, salt stress, dehydration and ABA treatments (Hong et al., 1997; Haffani et al., 2004). In the genotype-comparison array one LRR-receptor kinase (Tr002_A03) was highly induced in all three genotypes with a more marked induction in AgCS followed by DS3E(3A) indicating that increased regulation of DS3E(3A) may be due to chromosome 3E. Two other LRR-receptor kinases (Tr005_J03, Tr001_B19) were repressed in CS, with lesser down-regulation occurring in the AgCS and DS3E(3A) once again implying that these genes in DS3E(3A) are likely being regulated by chromosome 3E . There were two LRR-receptor kinases (Tr016_I14 and Tr012_G03) and a receptor protein kinase (Tr013_N12) that did not have large changes in gene expression but did have subtle difference between mRNA levels indicated by genotype by treatment interaction significance (Supp. Table 2). The results from the genotype-comparison array show that there are receptor kinases regulated by salt treatment with differences in expression in the different genotypes suggesting altered perception and signal transduction under salt stress in each of the genotypes.

In Norstar the time-course array, four receptor kinases have significant time-dependent regulation. Two LRR-receptor kinases (Tr013_J14, Tr002_A03) showed early induced levels at 6 hrs of treatment, one continued to have induction but with decreasing levels for 24 hrs and 72 hr, the other showed the same trend but by 72 hrs transcript levels had returned to near control levels. A receptor–like protein kinase (Tr013_C20) was induced at 6 hrs and 24 hrs while another receptor kinase (Tr013_O02) was repressed at 72 hrs. S-receptor kinases are likely involved in plant defense (Pastuglia et al., 2002). One S-receptor kinase (Tr005_G07) was induced at 72 hrs of salt stress, two

other S-receptor kinases (Tr013_F02, Tr001_J23) were repressed, one at 6 hrs and the other at both 24 hrs and 72 hrs. These results show a temporal pattern of early induction under salt stress for the genes of the protein receptor kinases and suggest their role in early signaling transduction under salt stress.

3.7.6 Signaling Molecules: Phospholipids

During osmotic stress phospholipids may play important roles in the production of secondary signaling molecules (Xiong et al., 2002). In animal systems phosphotidylinositol 4,5-bisphophate (PIP_2) is hydrolyzed by activated phospholipase C (PI-PLC) into the secondary messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which trigger Ca^{2+} release from cellular stores and activation of protein kinase C, respectively (Mueller-Roeber and Pical, 2002; Xiong et al., 2002). In plants, IP_3 has been shown to be involved in the release Ca^{2+} from internal stores but the role of DAG is less understood due to the absence of a protein kinase C homologue (Alexandra et al., 1990; Sanders et al., 2002). DAG can be converted to phosphatidic acid (PA) either via phospholipase D or through DAG kinase and PA can be further phosphorylated into diacylglycerol pyrophosphate (DGPP) which rapidly accumulates in Arabidopsis cells after salt stress, both PA and DGPP act as secondary messengers in the ABA signaling pathway (Pical et al., 1999; Zalejski et al., 2005). The first committed step in the production of PIP2 is the phosphorylation of phosphatidylinositol (PI) by PI 4-Kinase to form PI-4-P and is potentially a crucial point of regulation in PI-dependent pathways (Stevenson et al., 1998; Mueller-Roeber and Pical, 2002). In the Norstar time-course experiment there are two PI-4 kinases (Tr015_O24 and Tr016_P09) and a gene

encoding PI-PLC (Tr013_L08) which had significant regulation between salt treated time-points. One PI-4 kinase and the phospholipase C had decreasing levels of transcript through the time-course while the other PI-4 kinase has significant induction at 72 hours of NaCl treatment. This indicates that there might be transient expression of the secondary messengers IP_3 and DAG that leads to a second wave of signaling responses with different members involved at various stages of the salt stress response.

In the genotype-comparison array two genes that encode for PI 4-kinases (Tr016_M03, Tr017_K19) were significantly regulated under salt-stress, Tr016_M03 is down regulated in all three genotypes. The other PI 4-kinase (Tr017_K19) has genotype by treatment interaction and is repressed in AgCS but has no change in DS3(3E) indicating that the regulation of this gene is not inherent to chromosome 3E but elsewhere in the E genome , the difference in regulation may also lead to genotypic differences of secondary messengers levels and cellular responses to salinity stress in the genotypes. Two other significant genes involved in PI-pathway were significantly down regulated, a phospholipase in all three genotypes and a DAG kinase in AgCS (the only data available). Taken as a whole the genes identified may correspond to a PI-pathway in which there is down regulation of secondary signaling molecules in these genotypes at 3 days of NaCl stress and differences in regulation between genotypes. Further investigation of these genes and the roles they play in salinity stress is deserved

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3.7.7 Ca^{2+}as a Secondary Signaling Molecule and Signal Transduction via Kinases and Calcium Binding Proteins

Early response to abiotic stress, including salinity involves a transient increase in cytosolic calcium through the release from an internal store or from an external source. As previously described, IP_3 is one candidate signal molecule for triggering the release of Ca^{2+} from internal stores (Sanders et al., 2002). Changes in calcium levels are perceived by two important calcium sensors calmodulins (CaMs) and calcineurin B-like proteins (CBLs) that bind Ca^{2+} and undergo conformational changes which is relayed to an interacting target protein (Sanders et al., 2002; Cheong et al., 2003). CaMs' targets include enzymes, structural proteins and transporters; CBL specifically interacts with CBL-interacting kinases (Sanders et al., 2002; Cheong et al., 2003). Another group of sensors is the calcium-dependent protein kinases (CDPKs) which have a serine/threonine kinase domain and a C-terminal calmodulin domain which directly binds to calcium resulting in activation of the kinase domain and propagating the signal to downstream targets (Sanders et al., 2002; Ludwig et al., 2004). In the Norstar time-course experiment two CaM genes (Tr013_M22, Tr012_J20) had significant differences in transcript levels under salt stress between time-points, additionally one had significant induction at 24 hours and 72 hours of treatment relative to control plants. Two CBL-interacting protein kinases (Tr013_L09, Tr014_N18) had induction at 6 hours of NaCl treatment but then returned to near control transcript levels at 24 hrs and one CDPK (Tr013_F13) was induced at 72 hours. The result of calcium sensing encoding genes indicates that there is a temporal pattern of transcription and perhaps signaling during the course of salt stress.

In the analysis of the genotype-comparison experiment, a gene encoding a CDPK (Tr002_C09) was found to be down regulated under salt stress in all three genotypes and a CaM (Tr013_G11) was down-regulated in CS. None had genotype by treatment interaction and they most likely represent a similar signaling method in three genotypes. It is also interesting to note that the down regulation coincides with the genes involved in the PI-pathway, described above, and suggest that these Ca^{2+} regulated genes may be target genes in the PI-pathway. In addition, to being stimulated by Ca^{2+}, CDPK activity has also been shown to be activated by phospholipids. In Arabidopsis, AtCPK1 is stimulated by a crude lipid extract, phosphatidylinositol (PI), and lysophosphatidylcholine (LysoPC) (Harper et al., 1993), in *Zea mays* (maize), ZmCPK11 autophosphorylation is stimulated by phosphatidic acid (PA) and phosphatidylserine (PS) (Szczegielniak et al., 2005). This may indicate a dual regulation method of the identified CDPK via internal Ca^{2+} rises triggered by IP_3 and also activation by phospholipids. Two other CaMs (Tr003_H23, Tr012_I01), a calcium-binding protein (Tr012_G15), three CDPKS (Tr012_M08, Tr013_A23, Tr013_B17) and two CBL-interaction proteins (Tr013_C04, Tr013_E11) did not have significant regulation in any of the genotypes under salt stress however all had genotype by treatment interaction (Supp. Table 2) suggesting the products of these genes may elicit variations in the signaling pathway of CS, DS3E(3A) and AgCS.

In the genotype-comparison array four genes simply annotated as protein kinases and two serine/threonine protein kinases had changes in gene expression under salt stress in CS, DS3E(3A) and AgCS. Two protein kinases (Tr013_G04, Tr013_K19) had high

expression in AgCS under salt stress (2.09 fold and 5.85 fold respectively). In addition, Tr013_K19 had genotype by treatment interaction and having a 5.85 fold level of induction in AgCS whereas in CS and DS3E(3A) it is slightly down regulated (< 2 fold); this suggests that the regulation seen for this gene does not lie on chromosome 3E but elsewhere in the E genome as DS3E(3A) did not show the same pattern of expression AgCS. Both of these protein kinases are good candidate for further study into the increase salt tolerance of AgCS.

In CS, two other protein kinases (Tr003_A09, Tr014_011) had high induction with Tr014_011 also having genotype by treatment interaction. In both AgCS and DS3E(3A) there is little change suggesting that this gene is under the regulation of chromosome 3E and may account for the increase salt tolerance found in both these genotypes as compared to CS. The serine/threonine protein kinases (Tr014_N16, Tr016_K18) were both down regulated, one having the greatest repression in CS and the other in DS3E(3A). There are also eight other protein kinases (Tr012_A03, Tr012_J22, Tr012_K15, Tr012_M10 , Tr013_J04, Tr013_N03, Tr014_I12 and Tr014_M14) and two serine/threonine protein kinases (Tr002_B14 and Tr002_H16) that have significant genotype by treatment interaction but have no significant changes in gene expression compared to their relative controls (Supp. Table 2). The variations in gene expression amidst the genotypes indicate that there are regulatory differences in transcription of these genes and the investigation of what roles they may play during signaling may provide insight into differences in the levels of salt tolerance seen in each of the genotypes.

In Norstar there are six protein kinases (Tr001_l01, Tr013_I17, Tr003_E22, Tr006_E19, Tr016_F24, Tr012_I10) that all have changes in gene expression at 72 hrs of salt stress. Four are down regulated and two are up regulated, with one up regulated and one down regulated kinase also having similar significant levels at the 6 hrs time point. Moreover, three protein kinases (Tr001_L11, Tr003_G1, Tr013_D13) do not have changes in genes expression compared to the control but have significant difference between time-points indicating subtle changes in gene expression at different times. One serine/threonine protein kinase was found to be induced at 6 hrs with a return to near control levels at 24 hrs and 72 hrs. A well documented component of signaling, a MAPK (mitogen- activated protein kinase) (Tr001_N15) was also induced early on at 6 hrs of salt treatment. The induction of MAPK has been characterized under numerous stresses including salt stress (Mizoguchi et al., 1996; Matsuoka et al., 2002; Agrawal et al., 2003). Among all the kinases there is an absence of a transcriptional activity at 24 hours. Further study of the roles these kinases play in response to salt stress is deserved.

3.7.8 Effector Genes: Transcription Factors

In the comparison of genotypes experiment, 25 genes encoding transcription factors were identified as being significantly transcriptionally regulated and 10 of these had significant genotype by treatment interaction. Additionally, there are 34 transcription factors that have genotype by treatment interaction but do not have a ≥ 2 fold change in gene expression in any of the genotypes under salt stress (Supplemental table 2). Significantly regulated members of the AP2/EREBP (2 genes), MYB (2 genes), WRKY

(4 genes) and Zinc-finger (2 genes) transcription families were all induced in at least one genotype. Members of the ARF-AUX/IAA transcription factor family (2 genes) and the bHLH family (3 genes) were repressed in at least one genotype. Other transcription factor families were variably expressed having both up and down regulated gene family members under salt stress in the genotypes.

Members of the AP2/EREBP and WRKY transcription factor families have roles in physiological processes and responses to biotic and abiotic stresses (Eulgem et al., 2000; Feng et al., 2005; Nakano et al., 2006). In the genotype-comparison experiment two AP2 transcription factors were significantly induced. An AP2 transcription factor gene (Tr011_D14) had significant genotype by treatment interaction with significant induction seen only in DS3E(3A). The other AP2/EREBP transcription factor (Tr014_J02) was induced in CS with slight induction in AgCS and virtually no change in DS(3E)3A. Seven other AP2/EREBP genes were significant only for genotype by treatment interaction in the ANOVA analysis which is indicative of variations in transcript levels between the genotypes under salt stress. Four WRKY gene family members (Tr003_F01, Tr003_I13, Tr003_K07, Tr002_K15) were all induced in at least one genotype, they all have differences in the magnitude of induction in a given genotype, as well as the genotype in which the largest level of induction occurs, this is reflected in the significant genotype by treatment interaction effect in ANOVA analysis. Moreover, 5 WRKY (Tr003_C23, Tr013_N16, Tr013_019 and Tr002_I11) transcription factors had genotype by treatment interaction significance, in all cases AgCS was slightly repressed (<2.0 fold) under salt stress while CS was slightly induced (<2.0 fold).

Differences in gene expression among the CS, DS3E(3A) and AgCS genotypes indicate that they are good candidates for further characterization under salt stress.

Homeobox (HB) transcription factors have a role in cell fate and development (Riechmann and Ratcliffe, 2000). In the genotype-comparison experiment, three homeobox transcription factors were significantly regulated. One was repressed in CS (Tr002_O11), one was induced in AgCS (Tr002_G13) and the other (Tr008_l05) was induced in CS and repressed in AgCS with significant genotype by treatment interaction effect. All three genes in DS3E(3A) have a change in gene expression that lies between CS and AgCS in correlation with the differing degrees of salt tolerance found in each genotype. In Helianthus annuus (sunflower), a Homeodomain-leucine zipper (HD-ZIP) *Hahb-4* was found to be induced under drought and ABA, playing a role in developmental responses and conferring drought tolerance (Dezar et al., 2005). In Arabidopsis an HD-ZIP gene, Athb-2 was found to regulate Na^+ exclusion in yeast and its transcription in Arabidopsis was found to be induced under NaCl (Shin et al., 2004). The roles of the identified homeobox transcription factors warrant further investigation as they may give insight into the differences of salt tolerance between the genotypes.

Four NAC family transcription factor including two annotated as NAM transcription factors were significantly regulated in the genotype-comparison array and an additional two have significant genotype by treatment interaction. NAC factors are important to plant growth and development (Riechmann and Ratcliffe, 2000; He et al., 2005) and one NAM transcription factor has been implicated in a critical role in the

positioning of meristems and primordial tissues (He et al., 2005). Recently, He *et al* (2005) found AtNAC5 functions in lateral root development and it has increased expression under salt stress that is dependent on ethylene and auxin signaling pathways. In this array, one NAC transcription factor (Tr003_J21) is significantly down regulated in CS and DS3E(3A) and has significant genotype by treatment interaction effects; since its regulation in DS3E(3A) is similar to CS, it is unlikely regulated by genes on chromosome 3E. Three other genes, one annotated as a NAC transcription factor (Tr012_K14) and two as NAM transcription factors (Tr003_C04, Tr003_C06) were induced to some degree in all three genotypes but have not genotype treatment interaction indicating that they likely do not contribute to the increased salt tolerance found in DS3E(3A) nor AgCS. There are three NAC transcription factors with significant genotype by treatment interaction e (Tr001_I09, Tr003_J21, Tr012_L04), Tr003_J21 is the only one that has significant change in gene expression found only in DS3E(3A) and not similar to either CS nor AgCS. The other two NAC transcription factors do not show changes in the expression levels under salt stress (relative treated/relative control), they do however have differences in the inherent levels of mRNA for these genes in the different genotypes, with AgCS showing the most changes under salt stress making them good candidates for further study. The mRNA used in the comparisons is from root and the roles of these NAC transcription factors may be related to the development of roots under salt stress.

Two MADS transcription factors were found to be significantly regulated and an additional three had significant genotype by treatment interactions without ≥ 2.0 fold

change in any genotype under salt stress once again indicating difference in the inherent mRNA levels in between genotypes. One of the significantly regulated MADS (Tr014_F12) had genotype by treatment interaction with high repression in CS and little change in AgCS, the other TaVRT-1 (Tr014_HO4) was induced to some degree in all three genotypes with highest induction in DS3E(3A). The TaVRT-1 gene from wheat is involved in the transition from vegetative to reproductive growth and its accumulation is negatively associated with freezing tolerance (Danyluk et al., 2003), its expression has not previously been characterized in salt stressed wheat and its role in plant roots during salt stress deserves further investigation.

Auxin/indole acetic acid response transcription factors (*Aux/IAA)* genes encode short-lived nuclear proteins that are up-regulated early in the auxin responsive pathway; under low auxin concentrations many AUX/IAA proteins repress transcription of genes with auxin-responsive promoter elements (AuxREs) by interacting with promoter bound auxin responsive (ARFs) transcription factors (Tiwari et al., 2004). Auxin leads to the transcription of AuxRE genes by stimulating the interaction between the conserved domain II of AUX/IAA proteins and TIR1. TIR1 is a component of the SCF^{TIR1} E3 ubiquitin ligase complex and mediates the addition of ubiqutin to AUX/IAA proteins leading to their subsequent degradation by 26S proteosome, as AUX/IAA proteins repress AuxRE protein through the interaction with ARF transcription factors, the degradation of AUX/IAA proteins release ARF transcription factors which are free to transcribe target genes(Ward and Estelle, 2001) . In the genotype-comparison experiment, four genes, two ARF-Aux/IAA transcription factors (Tr001_H15 and

Tr002_M15) and two IAA1 proteins (Tr001_D21 and Tr003_L21), had a significant treatment effect and are down regulated in all three genotypes. The IAA1 proteins have significant genotype-by treatment interaction effect with CS having a more marked repression as compared to DS3E(3A) and AgCS, which may lead to a higher expression of genes under the control of AuxREs in CS.

MYB transcription factors have diverse functional roles in plants and have been implicated in the regulation of secondary metabolism, hormonal responses, control of cell shape and cell cycle, and in responses to biotic and abiotic stresses (Kranz et al., 1998; Yoo et al., 2005). In the genotype comparison array a MYB24 transcription factor (Tr005_P20) had significant induction in CS with elevated levels in DS3E(3A) and AgCS under salt stress, another MYB transcription factor (Tr001_G24) only had induction in AgCS and is significant for genotype by treatment interaction. Three other MYB transcription factors (Tr001_K03, Tr003_H15, Tr012_O19) only have genotype by treatment interaction significance demonstrating that there are difference in the regulation of these genes under salt stress in CS, DS3E(3A) and AgCS. Recent studies by Yoo *et al.* (2005) have demonstrated that MYB2 from Arabidopsis has a Ca^{2+} -dependent CaM binding domain that binds specific isoforms of CaM enhancing its activity and resulting in the induction of drought and salt responsive genes, *P5CS1* (Δ^1-pyrroline-5-carboxylate synthetase-1), *ADH1 (*alcohol dehydrogenase 1), and *rd22* (responsive to dehydration 22*)*. This result integrates Ca^{2+} signaling into the regulation of salt tolerance genes and it also provides insight to specific interactions between signaling molecules, sensors and effectors. In addition, MYB2 and MYC2 a bHLH transcription factor were identified in

invitro studies to function as cis-acting activators of rd22 (Abe et al., 1997). In transgenic studies overexpression MYC2 and MYB2 together lead to higher induction of both ADH1 and rd22 transcripts when treated with ABA (Abe et al., 2003). Our array detected three bHLH transcription factors (Tr002_A05, Tr001_A15, Tr003_N19) that are significantly repressed in at least one genotype. As the bHLH transcription factors have an opposing expression pattern to the detected MYB genes it is unlikely that they function as cis-acting factors in gene regulation.

The Norstar time-course experiment had 54 transcriptions factors representing 17% of all Norstar genes that had significant regulation in at least one time-point under salt stress and/or significant regulatory differences between time points. Transcription factors belonging to AP2/ EREBP (25 genes), bHLH (4 genes), bZIP (3 genes), MYB (8 genes) and NAC (4 genes) transcription factors all had differential regulation among their members. ARF-Aux/IAA transcription factors (7 genes) had repression in all members, the only member of the WRKY transcription factor family was repressed and one MADS transcription factor was also repressed (only expression change data available). The most abundant family of regulated transcription factors belonged to the AP2 family with 25 members. Sixteen AP2 transcription factors were significantly regulated under salt stress, all have significant difference in mRNA steady state levels between time-points and an additional 9 are without significant regulation compared to control levels but have significant differences between time-points. Notably only one AP2 transcription factor (Tr011_D14) is induced while 16 AP2 genes are repressed. In Arabidopsis there are 147 members in the AP2/ EREBP family, 122 genes fall into the EREBP subfamily with 57

genes that belong to the DREB subgroup and 65 to the ERF subgroup (Feng et al., 2005). Five genes encoding for the DREB2 (Tr014_F03, Tr011_H04, TR011_H06, Tr014_F05, Tr014_D21) class of AP2 transcription factors are all repressed at 6 hrs of treatment with no change compared to controls at 24 hrs and all but one had repression again at 72 hrs of salt treatment. DREB transcription factors bind to dehydration-responsive-element/ C-repeat (CRT/DRE), in Arabidopsis DREB2 genes are induced by dehydration and salinity but not ABA (Nakashima et al., 2000). As the DREB2 genes seen in the Norstar time-course array shows repression rather than induction under salt stress it is unlikely that any of the DREB2 Norstar genes reported here are orthologs of the DREB2 Arabidopsis genes. The Norstar time-course experiment detected homologues of ERF1 (Tr011_F12), ERF3 (Tr014_N15) and ERF4 (Tr014_J11, Tr011_B24, Tr014_J12) that all had significant repression at 72 hrs. The ERF domain of ERF transcription factors binds to a GCC-box, ERF1 is an transcriptional activator of GCC-box genes while ERF3 and ERF4 both act as active transcriptional repressors (Fujimoto et al., 2000). The ERF3 and ERF4 genes identified in the Norstar array have an opposing regulation to those observed by Fujimoto *et al.* (2000) in Arabidopsis, which are induced under high salinity stress. This signifies that the ERF3 and ERF4 genes identified in Norstar are not exact orthologs of Arabidopsis genes. The role that these AP2 transcription factors play under salt stress in wheat deserves further study.

Under salt stress in Norstar, two NAC annotated transcription factors (Tr013_N14, Tr003_J21) were significantly repressed at 72 hrs. One NAM transcription factor (Tr013_D21) was induced at 6 hrs with near control levels of expression at 24 hrs

and 72 hrs. All three NAC transcription factors had significant differences in expressed levels between at least two time-points and an additional NAM transcription factor (Tr013_G12) was not significantly regulated as compared to the control but did have significant regulation between time-points. The results indicate that there is temporal change in levels of NAC transcription factor under salt stress in Norstar wheat and as NAC factors have roles in development may lead to physiological changes in roots.

bZIP transcription factors have diverse roles in plant development , defense responses and flowering (Riechmann and Ratcliffe, 2000). Two bZIP transcription factors (Tr012_D01, Tr011_H15) were significantly repressed at 6 hrs and 7hrs of salt stress in Norstar. *CONSTANS* is known to promote flowering under long day photoperiods (Onouchi et al., 2000) . A *CONSTANS* like zinc-finger transcription factor (Tr012_F11) had significant regulation at 24 hrs compared to 6 hrs and 72 hrs salt treated time-points suggesting it may act to integrate flowering time under salt stress. Further study of what roles these transcription factors play in wheat under salt stress is warranted.

In the Norstar time-course arrays, 7 genes annotated as ARF-AUX/IAA transcription factors were significantly down regulated under salt stress. One ARF-AUX/IAA transcription factor gene (Tr002_E15) had repression at 6 hrs of salt stress, four (Tr012_P09, Tr001_H15, Tr001_H21, Tr013_023) were repressed at 72 hrs and two (Tr003_F23, Tr002_G09) were repressed at both 6 hrs and 72 hrs. Two IAA1 proteins (Tr003_L21, Tr016_B15) were significantly repressed at 72 hrs of salt stress. These results indicate a temporal pattern of repression of genes involved in the regulation of

AuxRE promoter genes with the greatest repression occurring at 72 hrs of salt stress. Repression of genes encoding for IAA1 and ARF-Aux/IAA transcription factors coincides with results reported in the genotype-comparison array. The down regulation of both IAA1 proteins and ARF-Aux/IAA transcription factors is curious as the repression of IAA1 proteins would suggest that ARF-Aux/IAA transcriptions factors are free to transcribe genes under the control of AuxRE promoters but the added down regulation of ARF-Aux/IAA transcription factors suggest the down regulation of AuxRE genes. Further investigation of these genes and their targets is deserved.

Eight MYB genes were detected as having significance in the Norstar time-course array. Six have significant changes in gene expression and seven are significantly regulated between time-points. Four MYB genes (Tr001_I02, Tr012_G20, Tr001_N23, Tr001_D09) are repressed at 6 hrs, one is additionally repressed at 24 hrs and 72 hrs and another at 72 hrs of salt stress. Two other MYB transcription factors (Tr019_P19, Tr001_J09) are induced at 72 hrs of salt stress. The differential regulation of the MYB genes indicated that they more than likely have different roles in salt stressed Norstar. Four bHLH transcription factors were significantly regulated in Norstar. Two were repressed, one (Tr013_E19) at 6 hrs and 72 hrs, and another (Tr013_O04) at only 72 hrs. Two other bHLH transcription factors were up regulated one at both 6 hrs and 72 hrs and the other at 72 hrs of salt stress. As these bHLH transcription factors have overlapping expression patterns with MYB transcription factors it is plausible that they may function as cis-acting factors in the transcription of salt regulated genes and are therefore strong candidates for further characterization of the roles they elicit in salt stressed wheat.

3.7.9 Transcriptional and Post-transcriptional Regulators: RNA Binding Proteins

RNA binding proteins (RBPs) contain RNA recognition motifs (RRM) and are able to regulate genes at the transcriptional level and post-transcriptionally either through direct interactions or by the modulation of other regulatory factors (Kwak et al., 2005). Post–transcriptional regulation includes RNA processing, mRNA trafficking, degradation and translation controls (Simpson et al., 2004). GR-RBPs are induced under a number of environmental stresses in plants and are implicated in responses to those stresses (Kwak et al., 2005). Under high salinity the study of eight GR-RBPs in Arabidopsis showed they had differential patterns of expression with one being up regulated and four down-regulated, further transgenic studies with *GR-RBP4* showed that under salinity the repression coincided with retardation in germination (Kwak et al., 2005).

In the Norstar time-course array 10 RBPs are significantly induced and all have significant regulation between time-points and two more are only significant between time-points. Nine of the significant RBPs are annotated as GR-RBP. There is a marked induction at 6 hrs hours of treatment with nine RBPs (Tr014_P20, Tr012_D05, Tr012_H21, Tr012_O08, Tr013_A11, Tr013_J24, Tr013_K17, Tr001_D02, Tr013_N19) being up regulated. One RBP (Tr013_J24) is additionally induced at 24 hrs and 72 hrs with increasing expression over time. One other RBP (Tr012_N13) only has induction at 24 hrs. Two others (Tr017_M20, Tr012_N09) only have significant regulation in transcription levels between time-points but have higher level of transcripts at 6 hrs of salt treatment. The high proportion of early-induced RBPs signifies that there is likely

early transcriptional and post-transcriptional regulation of salt responsive genes by RBPs gene products.

In the genotype-comparison experiment 12 RBPs were significant and 9 were annotated as RNA-binding glycine-rich proteins (GR-RBPs). Three RBPs (Tr012_N13, Tr013_J24, Tr001_B20) were induced in CS and AgCS with more marked induction in CS, one had no induction in DS3E(3A) and had genotype by treatment interaction significance likely not due to differences between AgCS and CS but rather its lack of induction in DS3E(3A) which may indicate loss of regulation under salt stress of this gene by the substitution . One RBP (Tr001_N17) was repressed in all three genotypes. Eight other RNA-binding proteins are not significantly regulated in any genotype compared to their respective controls but all have genotype by treatment interaction significance indicating genotypic differences in regulation of these genes and likely their targets under salt stress.

3.7.10 Protein Degradation: Ubiquitin-Proteosome Pathway

The ubiquitin-mediated degradation of proteins plays an important regulatory role in a variety of plant processes that include transcription, signal transduction, cell cycle control and endocytosis (Hershko and Ciechanover, 1998; Ward and Estelle, 2001). Proteins are targeted for degradation by the addition of multiple units of ubiquitin that are recognized by the 26S proteosome (Ward and Estelle 2001). The addition of ubiquitin to a protein is mediated by the sequential activities of an ubiqutin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and finally a ubiqutin protein ligase (E3). E3 ubiquitin ligase is responsible for ubquitination protein specificity and can be composed

of a single or multi-subunit complex (Ward and Estelle 2001). In the Norstar time-course experiment, an F-box E2 ubiquitin ligase (Tr012_D19) and two E3 ubiquitin conjugating enzymes (Tr014_M08, Tr017_B11) were induced at 6 hrs of treatment. Another E3 ubiquitin conjugating enzyme (Tr014_C15) was found to be repressed at 24 hrs of salt treatment and may indicate that it has a different target protein(s).

In the genotype-comparison experiment four Kelch repeat E3 ubiquitin ligases (Tr002_A20, Tr002_K23, Tr001_E23, Tr003_E08) were significantly repressed in DS3E(3A) with three having moderate down regulation in CS and AgCS and one (Tr001_E23) slight differential expression in CS and AgCS, two have genotype by treatment interaction significance due to the different pattern expression found in DS3E(3A). In the SCF^{TIR1} E3 ubiqutin ligase complex the F-box protein gene TIR1 interacts with domain II of Aux/IAA proteins leading to their consequent ubquitination and degradation (Ward and Estelle, 2001). In this array a TIR1 E3 ubiquitin ligase was found to be significantly induced in CS, with lesser but elevated levels also in DS3E(3A) and AgCS, interestingly this result correlates with the repression of two genes encoding IAA1 proteins (Tr001_D21, Tr003_L21). As SCF^{TIR1} acts on proteins rather than transcription it may indicate that there is a coordinated mechanism for transcriptional and post-translational regulation of the IAA1 genes and their products. Five E2 ubiquitin-conjugating enzymes, three E3 ubiquitin ligases and a cullin subunit of SCF (Ward and Estelle, 2001), all have genotype by treatment interaction indicating that there are genotypic differences between their transcriptional regulation in CS, DS3E(3A) and

AgCS under salt stress and suggests there are differences in the post-transcriptional regulation of their targets.

4.0 Conclusions

Microarray experiments to study expression profiles of three genotypes with varying levels of salt tolerance identified multiple differences in transcriptional regulation under salt stress. Through the study of transcriptional profiles of CS, DS(3E)3A and AgCS, 212 genes are identified as being significantly regulated under salt stress. In addition, the comparison of the mRNA expression profiles in each of the genotypes found 305 genes to have significant transcriptional regulation in between the genotypes with clustering analysis revealing that there are not only differences between each genotype's response to salt stress but also inherent differences in control and salt stressed root mRNA levels. Multiple classes of families were found to have genotypic differences which included members of signaling transduction pathways, transcription factors and post-transcriptional regulators. Differences in the transcriptional profiles taken with the increased salt-tolerance of AgCS and DS3E(3A) as compared to CS, identified genes that deserve further characterization to gain insight into the genetic mechanisms of salt tolerance in wheat and other plant species.

The global expression profile of Nst wheat had a temporal pattern of transcription during the application of salt stress. There is an early accumulation of mRNA at 6 hours of treatment, with a subsequent decline at 24 hours, followed by at secondary increase at 72 hours. There is little overlap of changes in gene expression at different time-points with 69% of the genes being significantly regulated only at one time-point and 4% at all three time-points. In addition, multiple subtle changes between salt treated time-points

were detected that were not necessarily significantly regulated at one time-point between treated and control plants indicating that there is a progression of dynamic alterations in mRNA levels over time to adapt to salt stress.

The use of microarray analysis has proved to be an extremely useful tool in identifying genes that may a play role in salt stress and has enabled the identification of genes that may lead to increased salt tolerance of DS3E(3A) and AgCS. Further characterization of these genes is required to obtain a comprehensive insight in how they function and the roles they play in conferring salt tolerance to wheat.

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