Gene expression analysis in the roots of salt stressed wheat and the cytogenetic derivatives of wheat combined with the salt-tolerant wheatgrass, *Lophopyrum elongatum*

Zina Hussein, Ani Dryanova, Deborah Maret, Patrick J. Gulick[1]

Biology Department and Centre for Structural and Functional Genomics

Concordia University

7141 Sherbrooke W.

Montreal, Quebec, H4B 1R6

Canada

[1]Author to whom correspondence should be addressed:

Patrick Gulick

Telephone 514 848-2424 ext. 3407

FAX: 514- 848-2881

Email: patrick.gulick@concordia.ca

Abstract

Lophopyrum elongatum is among one of the most salt tolerant members of the Triticeae; important genetic stocks developed from crosses between wheat and *L. elongatum* provide a unique opportunity to compare gene expression in response to salt stress between these highly related species. The octaploid amphiploid contains the entire genome of *T. aestivum* and *L. elongatum*, and the wheat disomic substitution line DS3E(3A) has chromosome 3A of wheat replaced by chromosome 3E of *L. elongatum.* In this study, microarray analysis was used to characterize gene expression profiles in the roots of three genotypes, *Triticum aestivum*, the octaploid amphiploid, and the DS3E(3A) substitution line, in response to salt stress. We first examined changes in gene expression in wheat over a time course of three days of salt stress, and then compared changes in gene expression in wheat, the *T. aestivum* x *L. elongatum* amphiploid and in the DS3E(3A) substitution line after three days of salt stress. In the time course experiment, 237 genes had a 1.5 fold or greater change at least once out of three time points assayed in the experiment. The comparison between the three genotypes revealed 304 genes with significant differences in changes of expression between the genotypes. Forty two of these genes had at least a two-fold change in expression in response to salt treatment; 18 of these genes have signaling or regulatory function. Genes with significant differences in induction or repression between genotypes included transcription factors, protein kinases, ubiquitin ligases and genes related to phospholipid signaling.

Keywords: microarray analysis, gene expression profile, salt stress, octaploid amphiploid, DS3E(3A) disomic substitution line, induction, repression

Key Message: Using microarray analysis, we identified regulatory and signaling-related genes with differential expression in three genotypes with varying degrees of salt tolerance, *Triticum aestivum*, the amphiploid, and the wheat substitution line DS3E(3A).

Introduction

Salinity affects more than 6% of land area worldwide, and is one of the most severe abiotic stresses limiting crop plant productivity (Munns and Tester 2008). Salinity causes two major types of stresses affecting plant growth: osmotic stress caused by ions outside the root which lowers soil water potential, and ionic stress caused by ions (Na^+ or Cl^-) that enter the plant (Munns and Tester 2008). The response to osmotic stress is rapid and results in a significant decrease of shoot growth (Tavakkoli et al. 2010), while the response to ionic stress is slower and results in preferential death of older leaves (Munns and Tester 2008). There is a wide range of salt tolerance among plant species, with highly tolerant plants being able to survive in soils with a NaCl concentration of up to 10 g L^{-1}, and sensitive plants being able to withstand a salt concentration of up to 2.5 g L^{-1} (Dvorak and Ross 1986; Hasanuzzaman et al. 2013). There is also significant genetic diversity within crop species that can be used for comparative studies and as a source for genetic improvement for stress tolerance of crops (Witzel et al. 2009).

In order to survive in a saline environment, plants have evolved protective adaptations such as tolerance to osmotic stress, including Na^+ and Cl^- exclusion, as well as NaCl accumulation, and the capacity to sequester or compartmentalize Na^+ and Cl^- ions in the vacuoles of older tissues (Munns and Tester 2008; Rajendran et al. 2009). There is an increase in expression of biosynthetic enzymes in response to osmotic stress which leads to the synthesis and accumulation of low molecular weight organic osmotica (Munns and Tester 2008; Aghaei and Komatsu 2013). The two other mechanisms of salt tolerance focus on relieving ionic stress on the plant by decreasing the amount of Na^+ accumulating in the cytosol of cells (Hasanuzzaman et al. 2013). Na^+-exclusion from leaves (Moller and Tester 2007; Munns and

Tester 2008), and the compartmentalization of Na^+ in vacuoles or in specific cell types (Pardo et al. 2006; Munns and Tester 2008) involve changes in expression of specific ion transporters controlling the transport of Na^+ throughout the plant (Davenport 2007).

The plant's response to salinity is mediated via signal transduction pathways that include osmotic and ionic homeostasis signaling pathways, detoxification response pathways, and pathways for growth regulation. A number of genes reported to be up-regulated by salt stress in plants have also been shown to be upregulated by other types of abiotic stress, and it appears that the MAPK cascade may act as a point of convergence for cross-talk between different stress signaling responses (Saijo et al. 2000; Teige et al. 2004). Calcium signaling, reactive oxygen species, and abscisic acid (ABA) have been shown to be important signals in the response to salt stress (Huang et al. 2012). Although a number of protein kinases, transcription factors, and calcium-binding proteins have been implicated in the response to salinity stress, many elements of gene regulation remain poorly understood (Huang et al., 2012).

Microarray technology has been used to characterize the global transcriptional profiles of genes in response to salt stress (Taji et al. 2004; Ouyang et al. 2007; Rodriquez-Uribe et al. 2011). While some studies focused on gene expression in roots (Kawasaki et al. 2001; Kreps et al. 2002; Wang et al. 2003; Yao et al. 2011), others have studied RNA profiles from seedlings, cotyledons and shoot tips (Seki et al.2002; Chao et al. 2005; Zhou et al. 2007). Among the salt-stress-regulated genes identified in these studies were regulatory genes, genes encoding proteins involved in signal transduction such as protein kinases, protein phosphatases, calmodulin, as well as transcription factors such as EREBP, WRKY, bZIP, MADS box, Zinc finger and NAC gene-family members. The expression of genes encoding proteins involved in osmolyte synthesis, cell wall structure modification, ion transport, detoxification enzymes and key enzymes in metabolic

pathways of carbohydrates, amino acids and fatty acids were also found to be modulated in response to salinity.

Bread wheat (*Triticum aestivum*), one of the world's most important cereal crops, is moderately salt-tolerant (Hasanuzzaman et al. 2013). There is a wide range of salt tolerance among species within the Triticeae, and *Lophopyrum elongatum* (Host) Love, [syn *Agropyron elongatum, Elytrigia elongata, Thinopyrum elongatum*], a close relative of wheat, is one of the most salt-tolerant members of the tribe (Colmer et al., 2006). The octaploid amphiploid produced from *L. elongatum* and *T.* aestivum, contains the genomes of both species (2n=8x=56, AA BB DD EE) and is significantly more salt-tolerant than its wheat parent, although it does not have the full tolerance of *L. elongatum* (Dvořák and Ross 1986; Dvořák et al. 1988; Schachtman et al. 1989; Omeilian et al.1991). In addition, disomic chromosome addition and substitution lines derived from crosses between the amphiploid and *T. aestivum* have been shown to exhibit varied degrees of salt-tolerance. Three of the E chromosome substitutions in five lines (2E(2D), 3E(3A), 7E(7A), 7E(7B) and 7E(7D)) showed significant levels of tolerance, and chromosome 3E had the largest effect on salt tolerance (Dvořák et al. 1988; Omeilian et al. 1991).

This work was carried out to study the gene expression profiles in the roots of wheat subjected to salt stress for up to three days, and to compare changes in gene expression in response to salt stress in the roots of three genotypes with differing degrees of salt tolerance: wheat (*T. aestivum),* the octaploid *T. aestivum* x *L. elongatum* amphiploid, and the wheat disomic substitution line DS3E(3A), in which the chromosome pair 3A of wheat has been replaced by chromosome pair 3E of *L. elongatum*. The experiments were conducted using a microarray constructed with 5728 cDNA amplicons from wheat, which was enriched for genes involved in signal transduction and gene regulation.

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Materials and Methods

Plant material, growth conditions, salt stress treatment and RNA extraction

Seeds from the *T. aestivum* cultivar Norstar were germinated and transferred to hydroponic tanks containing a modified Hoagland's solution (Gulick and Dvořák 1987). Plants were grown with a light cycle of 11 h light and 13 h darkness, with day/night temperatures of 22°C and 15°C, respectively. The growth solution was replaced at day seven and 14; 18 days after germination, the hydroponic solution was replaced with fresh growth solution supplemented with 150 mM NaCl, as well as 15 mM $CaCl_2$ to mitigate the toxic effects of sodium ions. Without adequate supplementation of Ca^{2+}, the effects seen by salt treatments may be due to impaired root membrane function (Munns 2005). Control plants were grown without addition of NaCl and $CaCl_2$. After salt treatments of 6, 24 and 72 h, plants were harvested, the roots were removed, frozen in liquid nitrogen and stored at -80°C. The treatments were initiated at staggered times in order to harvest plants at the same time in the light cycle, after approximately 6 h of light. Tissues were ground in liquid nitrogen and RNA was purified with TRIZOL reagent (5ml/g) according to the manufacturer's protocol (Invitrogen, Burlington, Canada). Target cDNA synthesis, labeling with Cy3 and Cy5 dyes, prehybridization and hybridizations were carried out as described in Monroy et al. (2007). Three biological replicates, each comprised of ten plants, were analyzed.

A second experiment compared the response to 150 mM NaCl + 15 mM $CaCl_2$ treatment in three genotypes: the wheat cultivar Chinese Spring, the octaploid *T. aestivum* x *L. elongatum* amphiploid, and the wheat disomic substitution line, DS3E(3A), which has the pair of 3A chromosomes of wheat substituted with 3E chromosomes from *L. elongatum*. Chinese Spring

wheat was used in this comparison since it is the wheat genetic background for the amphiploid and the DS3E(3A) line. Plants were grown hydroponically as described above and root samples were taken after 72 h of salt treatment. Roots from three biological replicates of 10 plants each were harvested, frozen in liquid nitrogen and stored at -80°C. RNA was extracted and labeled as described above.

Microarray construction

The microarray, previously described by Monroy et al. (2007), consisted of 5728 printed cDNA amplicons which included 1630 genes of regulatory or signaling function. The remaining cDNA amplicons in the microarray were random clones from the Genome Canada wheat EST program, Functional Genomics of Abiotic Stress (FGAS) (Houde et al. 2006), and from a unigene set of the National Science Foundation (USA) wheat EST clone collections (Qi et al. 2004). The details for the construction of the microarray, annotation of the array and statistical analysis are described in Monroy et al. (2007). The updated annotation for the microarray is given in Supplemental Table S4. To determine the sequence similarity between *L. elongatum* and *T. aestivum*, the nucleotide sequences of 89 *L. elongatum* ESTs were compared to the FGAS wheat EST database by Blastn (Altschul et al. 1997).

Experimental design

A common reference design was used in each of the two experiments. In the time course experiment, each experimental sample, including non-stressed controls and samples from salt-stressed plants, was compared to the common reference sample that consisted of pooled RNA from the three replicates of control non-NaCl treated plants. In the comparison between wheat,

the amphiploid, and DS3E(3A), all the samples from each genotype were compared to a common reference sample consisting of a pool of RNA from the three control samples from wheat. All hybridizations were carried out with three biological replicates. Image analysis, normalization and quantification were carried out as described in Monroy et al. (2007). Changes in gene expression were calculated as (salt-treated sample / common reference) / (control sample / common reference). The measure for gene induction and repression relative to control levels was expressed as $\log_2$ values for all statistical and clustering analysis. The time course experiment of salt stress treatments was analyzed by one-way ANOVA. The gene expression comparison of the three genetic lines was analyzed by two-way ANOVA. The criteria for fold-change threshold selection was $\geq$ 1.5-fold change of expression, and in some cases $\geq$ two-fold change of expression. The cutoff for the p-value was ≤ 0.05, and in some cases ≤ 0.01. The combination of fold-change and p-value for each analysis is indicated in the respective figure legend or table footnote. Analysis by *k*-mean clustering (KMC) was performed for the genotype comparison using the K means function from the bioconductor project (Gentleman et al. 2004). Clustering was carried out for genes that were found to have significant gene expression in at least one genotype, and the total number of clusters parameter was set at 12.

Gene location

Selected features of the microarray were checked for possible location on wheat/ *Lophopyrum* chromosome 3 by a Blastn search in the Grain Genes database for wheat cDNA clones (http://wheat.pw.usda.gov/GG2/blast.shtml) that have been mapped to chromosomal bins in partial chromosome deletion lines in *T. aestivum* (Munkvold et al. 2004). In addition, the rice homologs for microarray features were identified by a Blastx search of the rice genomic

sequence database at NCBI (http://blast.ncbi.nlm.nih.gov/). The top scoring hit was taken as the probable ortholog. If the Blastx score for the top rice sequence match was weak, the Blastx search was repeated without the low complexity filter.

Results and discussion

cDNA sequence comparison between species

The sequence similarity between *L .elongatum* and *T. aestivum* averaged 94.16%. The high sequence conservation between these two species is not surprising since they are both members of the tribe Triticeae, and indicates that mRNA derived from *L. elongatum* is expected to readily hybridize with *T. aestivum* cDNA amplicons on a microarray. Previous work has shown that cDNA probes from *L. elongatum* readily hybridize to northern blots of *T. aestivum* RNA (Galvez et al. 1993) and sequence identity between orthologous genes in other members of the Triticeae are between 95 and 97% identical (Ridha Farajalla and Gulick 2007), and commonly cross-hybridize with nucleic acid probes.

Time-course salt-treatment experiment

During the time-course salt treatment experiment with *T. aestivum,* 237 genes had at least a 1.5-fold change ($p \leq 0.05$), and 62 of these genes had a two-fold or more change in expression (Supplemental Table S1; genes with $p \leq 0.01$ and at least two-fold change are listed in Table 1)**.** The number of genes with a significant induction showed a biphasic pattern of gene expression. The expression of 71 genes were significantly up-regulated 1.5-fold or more after six h of salt stress and only 35 genes were significantly induced after 24 h of treatment, whereas after three days of exposure to NaCl, 72 genes were induced more than 1.5-fold (Supplemental Table S1, Fig. 1).

There were 50, 33 and 57 genes repressed to 0.66 or less than of that of the control at 6, 24 and 72 h, respectively (Fig. 1). The biphasic pattern of expression in response to salt stress has previously been reported for individual genes that were induced by salt stress in wheat

(Galvez et al. 1993), and Arabidopsis (Kreps et al. 2002). Only seven genes were induced at least 1.5-fold at all three time points, and no genes were induced more than two-fold at all three times; three genes were identified that were repressed at all three time points to less than 0.66 of control levels (Supplemental Table S1B).

Gene expression profiles of *T. aestivum*, the amphiploid and DS3E(3A)

The patterns of gene expression in roots in response to salt stress were compared between the salt-tolerant *T. aestivum x L. elongatum* amphiploid, the moderately salt-tolerant disomic substitution line DS3E(3A), and the least salt-tolerant line, Chinese Spring wheat. A treatment of 72 h of salt stress, representative of the second phase of induction of gene expression, was chosen for the comparison of the genotypes. The time course experiment described above indicated that different sets of genes are up- and down-regulated over the time course of salt treatment, and the longer exposure was hypothesized to reflect the acclimation to high salt conditions in contrast to the genes responding to the initial shock of increased salt in the growth medium. Genes with significant differences ($p \leq 0.05$) in expression due to treatment effect, genotype effect, and genotype-by-treatment interaction effect were identified by two-way ANOVA. Genes with a significant interaction effect were those that had changes in expression in response to salt treatment but whose response was different among the three genotypes. The analysis revealed that 775 genes had significant ($p \leq 0.05$) differences in expression for at least one factor, and at least a 1.5-fold change in expression, and 214 of these had at least a two-fold change in expression in at least one genotype; data is presented in Supplemental Table S2. There were 42 genes with at least a two-fold change in expression and a significant genotype-by-

255 treatment interaction effect ($p \leq 0.05$). Among these were 11 transcription factors, five protein
256 kinases, and three genes which belong to other classes of regulatory genes (Table 2). Other
257 studies comparing the response of different genotypes under conditions of salt stress have also
258 found transcription factors (NAC family, EREBP family, and zinc finger family transcription
259 factors) and protein kinases among the stress-induced genes that were differentially expressed
260 between tolerant and sensitive genotypes (Chao et al. 2005; Ouyang et al. 2007). The comparison
261 of changes in gene expression among the three genotypes showed that *T. aestivum* had the largest
262 number of genes with at least two-fold changes in expression. *T. aestivum* also had the greatest
263 number of salt-stress-regulated genes above the 2X induction threshold that are unique to one
264 genotype. Though many of these genes had significant changes in expression in the other
265 genotypes, the change was less than two-fold. There were 24 genes that were induced two-fold
266 or more in all three genotypes. Most of the genes with significant induction in all three genotypes
267 were strongly induced; with an average level of induction of 3.5-fold. These genes included
268 many previously-characterized classes of stress-inducible genes such as dehydrins, CORE
269 proteins, catalases and disease resistance genes (Munns 2005). All but one of these did not have
270 significant genotype-by-treatment interaction effects in the two-way ANOVA. Some of the
271 strongly-induced genes such as aldehyde dehydrogenases and an O-methyl transferase have
272 been previously shown to be protective under stress conditions (Rodrigues et al. 2006; Ahn et al.
273 2011). However, the lack of significant differences between genetic lines in this analysis
274 indicates they do not account for the differences in salt tolerance among these lines. The group of
275 highly-induced genes also included candidates for regulatory genes and genes involved in cell
276 signaling pathways, including one NAC and one WRKY transcription factor, NAC-4

(Tr003_C04) and WRKY-71-like (Tr003_K07), respectively, which were both found to be upregulated in all three genotypes (Supplemental Table S2).

Genotype comparison by cluster analysis

Cluster analysis was conducted to compare the change in gene expression in *T. aestivum*, the amphiploid and DS3E(3A) genotypes, using *K*-means clustering (KMC) with Euclidian distances. Clustering was applied to 201 genes that had at least a two-fold change in expression and significant ($p \leq 0.05$) effect of treatment without consideration of the genotype x treatment effect. Only genes with detectable expression in all three genotypes were included in the analysis. Genes with higher induction or repression in the amphiploid, the most tolerant genotype, are the best candidates for genes that contribute to salt tolerance. Genes with similar patterns of expression in the amphiploid and the DS(3A)3E line are good candidates for salt tolerance genes and are candidates for genes located on chromosome 3E or regulated by genes on 3E. The results of cluster analysis are shown in Fig. 2 and the identification of the genes in each cluster is listed in Supplemental Table S3. Genes in cluster 8 had a moderately higher induction in the amphiploid than in *T. aestivum* and DS(3A)3E, and are candidates for genes that may contribute to the exceptional salt tolerance of the amphiploid. Genes in this cluster included transcription factors and protein kinases, such as zinc finger protein ZAT10-like, homeobox-leucine zipper protein HOX19, WRKY transcription factor 23, LRR receptor-like serine/threonine protein kinase, TAK14-like, and U-box domain-containing protein 34-like. Genes found in clusters 2 and 7 had changes in gene expression which were similar in all three genotypes; thus they represented a common response to salt stress in all the genotypes.

The genes in the clusters which show stronger induction in the salt tolerant genotypes are candidates for further study; they give an insight into the basis of salt tolerance and the potential to discover genes that are under common regulatory pathways. The common expression pattern between the amphiploid and the DS3E(3A) for the genes in clusters 10 and 12 suggest that these genes may lie on chromosome 3E or be regulated by genes on 3E. Twenty one genes in clusters 10 and 12 had strong sequence identity with EST clones that have been bin-mapped in wheat (Qi et al. 2004); however, only 4 of these genes were mapped to chromosome group 3 (Supplemental Table S3B). The 24 genes from clusters 10 and 12 that could not be localized with mapped wheat ESTs were used to search for the chromosomal location of their rice homolog. Only four of the 24 had the most similar rice genes located on rice chromosome 1, the chromosome with the highest degree of synteny with wheat chromosome group 3. Thus it appears that the majority of the genes in clusters 10 and 12 are not located on chromosome 3 but instead are regulated by genes on chromosome 3.

There was a negative correlation between gene induction levels and salt tolerance in genes in clusters 6 and 10. Genes in these clusters had higher levels of induction in *T. aestivum* than the other two genotypes. These genes are candidates for reporter genes for salt sensitivity, and low levels of induction could be used as indicators for salt tolerance.

Functional classes of differentially expressed genes

A number of genes that belong to functional classes involved in signaling and regulation have been identified in this study as being salt stress regulated, and in several cases, the pattern of induction in the three genotypes is parallel to their degree of salt tolerance. These include transcription factors, protein kinases, phospholipases and proteins involved in the ubiquitin protein degradation pathway. These classes of proteins have previously been implicated in the stress responses; however, they are encoded by members of large gene families, and microarray analysis offers an important approach to identify members of the gene family which are responsive to salt stress, as well to characterize their temporal patterns of expression. Each of these classes of genes were represented by multiple members of their respective gene families, and the identification of specific members of these gene families that are salt stress regulated in the three genotypes is summarized.

Transcription factors

Eight transcription factors had at least a two-fold change in expression in the roots of salt-stressed plants, and there were significant differences in expression in the three genotypes indicated by significant genotype-by-treatment interaction effects (Table 2)**.** The array contained 186 probes for AP2 transcription factors. One AP2/EREBP transcription factor gene, RAP2-3-like (Tr011_D14), had induction only in DS3E(3A) (Table 2). The expression of other AP2/EREBP transcription factor genes showed a significant genotype-by-treatment effect, but had less than a two-fold change in gene expression levels. The AP2/EREBP transcription factor RAP2-7-like (Tr014_F19), showed a decrease in expression in the amphiploid and in DS3E(3A) due to salt stress but showed no significant change in expression in wheat (Supplemental Table S2). AP2/EREBP transcription factor genes BIERF-3-like (Tr001_G03), CRT/DRE-9

(Tr014_L14), and C repeat-binding factor 2-like (Tr012_M01), showed an increase in expression in the amphiploid, and very little or no regulation in the DS3E(3A) line or in wheat (Supplemental Table S2). The array contained 122 probes for AP2/EREBP genes that did not show significant changes in gene expression under the conditions studied.

WRKY transcription factors were represented by 86 probes in the array. Among these, the WRKY-79-like transcription factor (Tr002_K15) was more strongly induced in the amphiploid than in the other genotypes and the WRKY-99-like transcription factor (Tr003_I13) was more strongly induced in Chinese Spring wheat (Supplemental Table S2); they are candidates for further characterization. Expression of the WRKY-74-like transcription factor (Tr013_O19) was induced in wheat with less than a two-fold change, but showed very little regulation of expression in the amphiploid or the DS3E(3A) line. In contrast, the WRKY-2-like transcription factor (Tr009_D02) was induced in the amphiploid with less than a two-fold change, but showed slight repression in DS3E(3A) and wheat (Supplemental Table S2). Seki et al. (2002) and Ma et al. (2006) reported that members of the WRKY gene family in *Arabidopsis* had a highly-altered level of expression in response to environmental stresses. WRKY transcription factors were also reported to be induced by environmental stress in sunflower (Giacomelli et al. 2010). The array contained 56 probes for WRKY genes that did not show significant changes in gene expression under the conditions studied.

NAC and NAM transcription factors were represented by 25 members of their gene family. The NAC-2-like transcription factor (Tr003_J21) had significant genotype-by-treatment interaction effects and was significantly down-regulated in *T. aestivum* and DS3E(3A) (Table 2). The NAC-8-like transcription factor (Tr012_L04) also had significant genotype-by-treatment

effects and was upregulated, though less than two-fold change, in the amphiploid, and to a much lesser extent in wheat, but was not regulated in DS3E(3A) (Supplemental Table S2). In an earlier report, the *Arabidopsis* NAC transcription factor ANAC 092 was noted to be up-regulated by salt stress (Balazadeh et al. 2010). The array contained probes for seven paralogous members of the NAC gene family (specifically, NAC-12, -39, -37, -7, -9, -21, and -16) that did not show significant changes in gene expression under the conditions studied.

MYB transcription factors were represented by 67 probes in the array. One MYB transcription factor, MYB-related protein MYBAS2 (Tr001_G24), was induced only in the amphiploid (Supplemental Table S2). Seki et al. (2002) observed that a MYB transcription factor had highly-altered levels of expression under salt stress in *Arabidopsis* and Rahaie et al. (2010) also reported that three MYB genes were up-regulated under long-term salt stress in *T. aestivum.* There were 40 probes representing bHLH transcription factors in the array; only one of these, a bHLH transcription factor, bHLH 20-like (Tr001_A13), showed genotype-by-treatment interaction effects and was down-regulated in DS3E(3A), and to a similar extent in *T. aestivum* (Table 2). The array contained 30 probes for bHLH genes that did not show significant changes in gene expression under the conditions studied. Transcriptomic analysis of salt stress in the roots of *Medicago truncatula* genotypes revealed that a bHLH-type transcription factor was differentially regulated between the two genotypes studied, and overexpression of the bHLH-type transcription factor increased root growth under salt stress (Zahaf et al. 2012). An earlier study also identified 29 bHLH transcription factors that were regulated due to salt stress in the roots of *Arabidopsis* (Jiang and Deyholos 2006).

In the time course experiment, which monitored gene expression after 6, 24 and 72 h of salt treatment, several members of the AP2 transcription factor gene family had significant changes in gene expression in the roots of salt-stressed wheat plants. One AP2 transcription factor, DRFL1b, (Tr014_F03) was repressed to 0.5, the level of the control at 6 h of stress (Table 1). A second AP2 family member, RAP2-3-like, (Tr011_D14) was induced 1.5-fold at 24 h, and 16 other AP2 genes were moderately repressed (> .66) (Supplemental Table S1). In *Arabidopsis,* DREB2 and members of the AP2/EREPB transcription factor family have been reported to be induced by dehydration and salinity (Nakashima et al. 2000). In contrast, the wheat DREB2 showed repression rather than induction over the time course of salt stress (Table 1 and Supplemental Table S1); it is unlikely that any of the DREB2 genes reported here are orthologs of the DREB2 *Arabidopsis* genes. Orthology is difficult to establish in such distantly related species, and the patterns of expression were quite different. The ERF genes are a subgroup of the AP2/EREPB family and have been noted to be induced by high-salinity stress in *Arabidopsis* (Hsieh et al. 2013; Seki et al. 2004). During the time course experiment, homologs of ERF1 (Tr011_F12), ERF3 (Tr014_N15) and ERF4 like genes (ethylene-responsive transcription factor 1-like, Tr014_J11; ethylene-responsive element binding protein 2-like, Tr011_B24; ERF071-like , Tr014_J12), all had significant repression at 72 h in wheat (Supplemental Table S1). One NAC transcription factor, NAC-2A (Tr003_J21), was significantly repressed at 72 h (Supplemental Table S1). One member of the MYB transcription factor family, myb-related protein LTR1-like, (Tr001_J09) was induced at 72 h of salt stress and four members of the MYB gene family, myb-related protein 306-like (Tr001_I02), myb-15-like (Tr012_G20), myb13-1-like (Tr001_N23), and R2R3-myb-like, (Tr001_D09) were repressed at 6 h (Supplemental Table S1).

Protein kinases

Protein kinases, including receptor protein kinases, were represented by 396 probes in the array. Two protein kinases had more than a two-fold induction in the roots in response to salt treatments and had significant differences in induction among the three genotypes (Table 2)**.** The protein kinase, U-box protein 34-like (Tr013_K19), had a 5.85-fold induction in the amphiploid under salt stress but was slightly down-regulated in *T. aestivum* and DS3E(3A). In contrast, the protein kinase, NPKL3-like (Tr014_O11), had high induction in *T. aestivum* but had little change in both the amphiploid and DS3E(3A), which suggests that its induction is related to salt sensitivity. The LRR receptor-like serine/threonine-protein kinase (Tr003_G20) was more strongly induced in the amphiploid than in the other two genotypes. Another receptor kinase, cysteine-rich receptor-like protein kinase-10, (Tr001_B17) was strongly downregulated in the amphiploid (Table 2). The time course experiment with *T. aestivum* that monitored gene expression after 6 h, 1 and 3 days of salt treatment, revealed PERK9-like protein kinase (Tr001_L01), calcium-dependent protein kinase 5-like (Tr013_F13), and cysteine-rich receptor-like protein kinase 23 (Tr013_L09), with two-fold or greater changes in expression in response to salt treatment, and two LRR receptor kinases (protein kinase Xa21-like (Tr002_A03), and phytosulfokine receptor2 (Tr013_J14), with strong increases in expression during the time course of salt treatment (Table 1).

The analysis detected additional protein kinases whose expression was changed by salt treatment but whose expression did not show a significant genotype-by-treatment effect or whose induction or repression levels were less than two-fold. Homologs of the receptor kinase, ARK1 (Tr003_E06) and PERK1 (Proline Extensin-like Receptor Kinase, Tr002_N12) were found to be

induced in all three genotypes by salt stress (Supplemental Table S2). ARK1 was most strongly induced in the amphiploid and the PERK1-like receptor kinase was most strongly upregulated in DS3E(3A). Both ARK1 and PERK1 have roles in plant defense (Pastuglia et al. 2002; Silva and Goring 2002). The LRR-receptor kinase, protein kinase Xa21-like (Tr002_A03) was highly induced in all three genotypes with a more marked induction in the amphiploid and intermediate induction in DS3E(3A) (Supplemental Table S2). Two other LRR-receptor kinases, protein kinase PERK8-like (Tr005_J03) and phytosulfokine receptor 2-like (Tr001_B19), were repressed in *T. aestivum* and were also repressed in the amphiploid and DS3E(3A), albeit to a lesser degree (Supplemental Table S2). 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, de Lorenzo et al. 2009 and Ouyang et al. 2010).

Phospholipid signaling

There were 19 probes in the array representing genes involved in phospholipid signaling. The analysis of gene expression in the roots of *T. aestivum* over the time course of salt treatment for three days revealed a gene encoding a phospholipase-C, phosphoinositide-specific phospholipase C1-like (Tr013_L08), which had significant differences in expression at different time-points (Supplemental Table S1). The phospholipase-C had decreasing levels of transcript throughout the time course. Although there were changes in gene expression in genes related to phospholipid signaling detected in the three genotypes, comparison of the expression pattern in the three genetic lines did not identify a gene in this class that had a significant genotype-by-treatment interaction effect. Phospholipid signaling has been observed to play an important role in the production of secondary signaling molecules in response to abiotic stress in plants (Xiong

et al. 2002). Wang et al. (2007) reported that the signaling compounds phosphatidic acid and phosphoinositides, which are generated by phospholipases, play important roles in plants' response to drought and salinity. In rice, the levels of phosphatidic acid, phosphatidylinositol bisphosphate and diacylglycerolpyrophosphate, which are phospholipase reaction products, have been shown to increase in response to salt stress, though the increase in levels did not parallel the degree of salt tolerance when different genotypes were compared (Darwish et al. 2009). Since phospholipase C and phospholipase D are members of multigene families, the expression and localization of different gene families may affect different signaling pathways. Measurement of changes in global levels of signaling molecules released by phospholipases may overlook differential distribution in different cell types that result from the action of different gene family members. The microarray results indeed show differential expression of different phospholipase C gene family members, though the lack of a correlation between phospholipase induction and salt tolerance suggests that a wider survey of gene family members may be necessary to detect key signaling components in the salt stress response.

Protein degradation and the ubiquitin proteosome pathway

In plants, adaptation in response to abiotic stresses can be achieved through ubiquitination and degradation of specific proteins related to stress signaling. Only a small number of E3 ligases related to abiotic stress signaling have been studied and further characterization of the biological roles of newly identified E3 ligases and their related substrates is essential in order to clarify the functional relationship between abiotic stress and E3 ligases (Lee and Kim 2011). There were 53 probes in the array representing ubiquitin ligases. In this study, one Kelch repeat E3 ubiquitin ligase, kr1-like (Tr003_E08) was significantly repressed in

DS3E(3A) and found to have a significant genotype-by-treatment interaction effect (Table 2). Three other ubiquitin ligases, SGT1-1-like (Tr014_A07), SKP1-like protein 1B (Tr003_J03), and SKP1-like protein 4 (Tr003_D03), had significant genotype-by-treatment interaction effects with stronger induction in the amphiploid than in the other genotypes; they were induced between 1.5- and two-fold (Supplemental Table S2).

Conclusions

Microarray experiments identified a large number of salt-stress-regulated genes in the roots of wheat, and a number of genes with differential regulation in between three genotypes with different levels of salt tolerance. Microarray analysis has the advantage of measuring the change in gene expression in a quantitative and sensitive manner. Though the differentially regulated genes with large changes in expression are readily detected and have the highest level of statistical significance, the most promising new candidate genes are those with relatively modest changes in expression and often have subtle differences in expression in different genotypes. The genes most strongly-induced by salt stress include members of several well-known gene families of stress-related proteins including dehydrins, CORE proteins, oxylate oxidase, and chitinase. However, these genes did not show significant genotype-by-treatment interactions, i.e., their changes in expression were not significantly different in the three genotypes and they are not strong candidates to explain the differences in stress tolerance in the genetic lines compared in this study. Genes with significant genotype-by-treatment interaction had more modest degrees of induction and this class of genes included many genes with regulatory functions. Regulatory genes and genes involved in signal transduction may affect

many downstream targets and subtle changes in expression may have compound effects mediated by the genes that they regulate. As well, changes in regulatory gene expression may have subtle effects on plant metabolism and promote protection from the osmotic and ionic effects of NaCl. The genes with regulatory function with significant differences in expression between genotypes listed in Table 2 are the most promising candidates for further study found in this analysis.

Classical statistical analysis such as ANOVA offers useful criteria for determining if changes in gene expression are statistically significant. However, the threshold values for both statistical significance and degree of change in gene expression can limit the detection of genes with important regulatory functions. Among genes with similar levels of induction or repression, some may be designated as significantly different whereas others may not simply because they do not meet the cut-off threshold. KMC cluster analysis is an additional tool to use in genotype comparison to recognize groups of genes whose pattern of expression parallels the degree of stress tolerance among genotypes. In these experiments, genes with stronger induction in the amphiploid than in the other two genotypes, as we observed for genes in cluster 8 (Fig. 2), are promising candidates for further study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1. The number of genes induced and repressed at least 1.5-fold in the roots of *T. aestivum*, with a significant p-value ≤ 0.01 after 6, 24 and 72 h of treatment with NaCl.

Fig. 2. *K*-Means Cluster analysis of genes that have ≥ two-fold change in expression in at least one genotype and significant treatment effect ($p \leq 0.05$) in two-way ANOVA. Wh-wheat, DS-DS3E(3A), Am-Amphiploid.

Table 1. Salt treatment of *Triticum aestivum*. Genes with change of expression ≥ two-fold.[a]

Microarray ID	GenBank ID	Annotation	Change of expression			ANOVA
			6 hr	24 hr	72 hr	P-value
Tr010_E22	BF145792	dehydrin 5	**4.85**	**2.72**	1.21	0
Tr002_A03	CK161386	LRR receptor kinase Xa21-like	**4.43**	**3.88**	1.15	0
Tr016_D07	DY741922	membrane protein	**4.23**	**2.02**	1.48	0
Tr002_I18	CK204453	steroid sulfotransferase	**3.70**	**2.04**	1.42	0
Tr013_C11	DR741682	COR39 protein	**3.21**	**2.06**	0.88	0
Tr003_A11	CK161186	dehydrin 3	**2.38**	**2.25**	1.03	0
Tr013_J14	DR741812	Phytosulfokine receptor 2-like	**2.03**	**2.53**	**1.79**	0
Tr017_A19	DY742217	membrane protein	**3.60**	**1.96**	**1.50**	0
Tr012_F16	DR741434	cold regulated protein	**2.64**	1.38	1.11	0
Tr016_L07	DY741974	dehydrin WZY1-1	**2.61**	**1.74**	1.08	0
Tr005_M06	BE404371	ADH glutamate dehydrogenase	**2.34**	1.20	1.29	0
Tr016_B09	DY741910	cold acclimation protein WCOR80	**2.34**	1.31	0.97	0.001
Tr017_C10	DY741736	senescence-associated protein-like	**2.29**	**1.59**	1.11	0.006
Tr016_D03	DY741919	cold acclimation protein	**2.19**	1.20	1.17	0.001
Tr001_L01	CK202855	Protein kinase PERK9-like	**2.18**	1.27	**2.43**	0.005
Tr016_J14	DY741777	late embryogenesis abundant LEA14A	**2.15**	1.46	**1.62**	0.006
Tr013_L09	DR741830	Cysteine-rich receptor protein kinase23-like	**2.12**	1.21	0.91	0
Tr005_L20	BE518273	cysteine proteinase inhibitor	**2.09**	1.13	1.08	0
Tr017_L02	DY741731	delta-COP	**2.01**	**1.51**	**2.21**	0.006
Tr015_K22	DY742113	membrane protein	**2.01**	1.39	1.14	0
Tr005_H08	BE517736	p68 RNA helicase	**1.92**	**2.42**	**1.88**	0.001
Tr008_N04	BF292996	aluminum induced protein wali 3	1.38	**2.25**	**1.81**	0.001
Tr004_I01	BE423905	thaumatin-like protein	1.06	**2.12**	**1.95**	0
Tr017_P02	CK207698	caffeic acid O-methyltransferase	**1.69**	1.15	**3.01**	0
Tr012_F04	CV782373	proteinase inhibitor-protein bsi1	**1.57**	**1.99**	**2.22**	0.002
Tr013_J24	DR741820	RNA-binding glycine-rich protein	**1.53**	**1.99**	**2.23**	0
Tr017_P17	DY742581	RuBisCO/ssu	1.46	1.22	**2.75**	0.005
Tr017_L03	DY742643	No Blast Hit	1.42	1.42	**6.84**	0
Tr017_C17	DY742228	chitinase II precursor	1.41	**1.72**	**3.09**	0.007
Tr013_F13	CV774621	Calcium-dependent protein kinase5-like	1.22	1.19	**2.00**	0.005
Tr016_P05	DY741999	RNA polymerase II, 28841-29486	1.17	1.37	**2.31**	0
Tr016_B18	DY742019	ribulosebiphosphate carboxylase	1.14	1.01	**2.25**	0
Tr017_P06	CK162825	o-methyltransferase ZRP4	1.01	1.20	**6.49**	0
Tr016_F11	DY741936	cytochrome	0.99	1.19	**2.41**	0
Tr016_J23	DY741969	RuBisCO/LS	0.94	1.18	**3.22**	0
Tr017_I07	DY742262	promoter-binding factor-like protein	0.88	1.27	**3.46**	0
Tr016_D13	DY741925	RuBisCO/SS	0.86	1.23	**2.05**	0
Tr017_N03	DY742554	potassium transporter	0.85	1.39	**4.83**	0
Tr001_I02	CK199342	MYB transcription factor	**0.22**	**0.60**	**0.13**	0.001
Tr014_F03	DY761221	AP2 transcription factor DRFL1b-like	**0.49**	1.02	**0.60**	0
Tr001_N14	DY741748	lipid transfer protein	0.75	0.78	**0.46**	0.004
Tr013_N14	DR741898	NAC transcription factor	0.72	0.77	**0.46**	0.005
Tr012_P09	DR741616	ARF-Aux/IAA transcription factor	0.68	0.79	**0.48**	0.001

[a] Table 1 includes genes that were significantly ($p \leq 0.01$) induced or repressed in roots of *T. aestivum* by NaCl treatment, with at least a two-fold change at one or more time points. Data is ordered by genes induced at 6 h, 24 h, and 72 h, followed by genes repressed at the same time points. Changes in expression two-fold or greater are in bold. RuBisCo ; ribulose-1,5-bisphosphate carboxylase.

Table 2. Regulatory genes in *T. aestivum*, the amphiploid and DS3E(3A) with significant differences in induction between genotypes.[a]

Microarray ID	GenBank ID	Annotation	Change in Expression			Two-Way ANOVA P-Value		
			amphiploid	DS3E(3A)	T.aestivum	Genotype	Treatment	Interaction
Tr013_K19	DR741838	Protein kinase U-box 34-like	**5.85**	0.70	0.89	**0.001**	**0.002**	**0**
Tr002_K15	CK209561	WRKY transcription factor	**2.02**	1.00	1.26	0.078	**0.008**	**0.038**
Tr003_G20	CK209476	LRR receptor protein kinase	**2.59**	1.04	1.46	0.059	**0.01**	**0.047**
Tr012_M02	DR741557	receptor-like kinase	**2.22**	1.25	1.96	0.116	**0**	**0.032**
Tr003_F01	CK213748	WRKY transcription factor	0.89	**2.33**	ND	0.075	**0.008**	**0.011**
Tr011_D14	DY761119	AP2 transcription factor RAP2-3-like	0.96	**2.55**	1.19	0.117	**0.01**	**0.042**
Tr003_I13	CK163412	WRKY transcription factor	1.30	1.17	**2.21**	**0.023**	**0**	**0.003**
Tr012_E14	CV760924	transcriptional activator	1.24	1.03	**2.15**	**0**	**0.002**	**0.013**
Tr014_I16	CV777913	transducin/WD-40 repeat protein	1.43	1.06	**2.36**	0.11	**0.002**	**0.018**
Tr014_O11	DR734156	Protein kinase NPKL3-like	1.39	1.34	**2.36**	**0.011**	**0**	**0.021**
Tr008_L05	BG313851	Homeodomain leucine zipper protein	**0.47**	1.14	**2.11**	0.159	0.914	**0.043**
Tr002_I08	CK198628	Sulfotransferase family	0.74	**0.45**	**0.41**	0.067	**0**	**0.006**
Tr001_B17	CK201221	Cysteine-rich receptor protein kinase 10-like	**0.49**	1.02	0.53	**0.012**	**0.007**	**0.026**
Tr003_J21	CK217339	NAC transcription factor 2-like	0.72	**0.49**	0.51	**0.001**	**0**	**0.008**
Tr003_E08	CK208672	Kelch Ubiquitin ligase kr1-like	0.76	**0.34**	0.69	0.172	**0**	**0.023**
Tr001_A13	CK193354	bHLH transcription factor 20-like	0.81	**0.49**	0.51	0.062	**0**	**0.05**
Tr001_E23	CK195236	Kelch repeats-actin binding protein	1.27	**0.42**	0.65	0.19	**0.018**	**0.019**
Tr003_L21	CV781294	IAA1 inducible transcription factor	0.76	0.66	**0.45**	0.132	**0**	**0.039**

[a] Table 2 includes genes with a ≥ two-fold change in expression in one or more genotype and a p-value ≤0.01 in two-way ANOVA for the genotype-by-treatment interaction effect. Induction or repression ≥ two-fold and p-values ≤ 0.05 are in bold. Data is ordered by genes that were induced in the amphiploid, DS3A(3E), and *T. aestivum*, followed by genes that were repressed in the amphiploid, DS3A(3E), and *T. aestivum*.

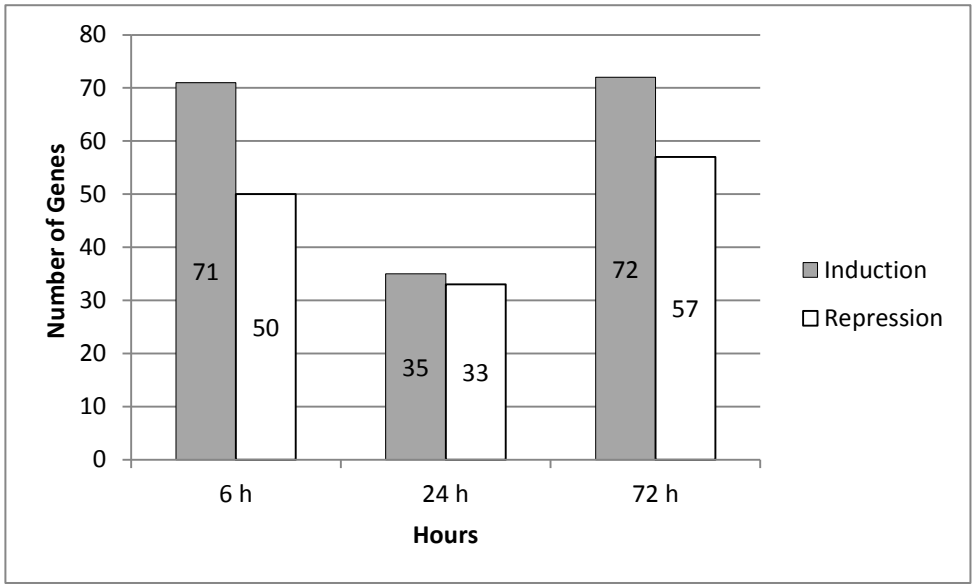

Fig. 1. The number of genes induced and repressed at least 1.5-fold in the roots of *T. aestivum*, with a significant p-value ≤ 0.01 after 6, 24 and 72 h of treatment with NaCl.

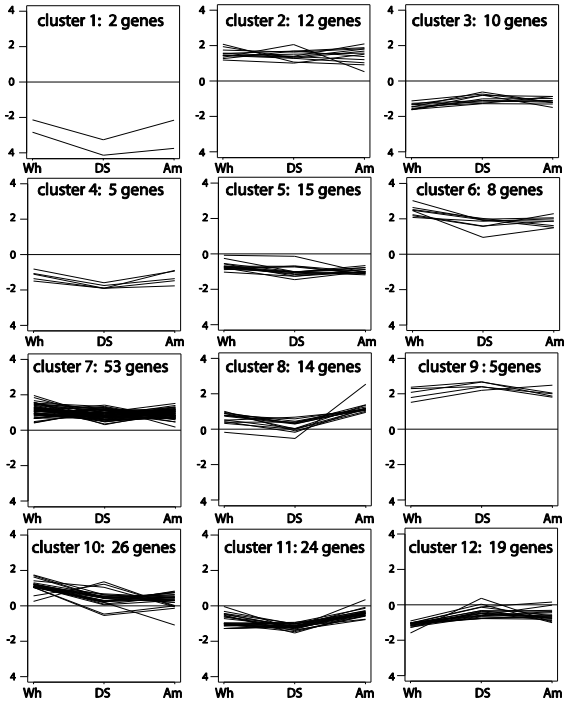
cluster 1: 2 genes
cluster 2: 12 genes
cluster 3: 10 genes
cluster 4: 5 genes
cluster 5: 15 genes
cluster 6: 8 genes
cluster 7: 53 genes
cluster 8: 14 genes
cluster 9 : 5genes
cluster 10: 26 genes
cluster 11: 24 genes
cluster 12: 19 genes
Wh
DS
Am

Fig. 2. *K*-Means Cluster analysis of genes that have ≥ two-fold change in expression in at least one genotype and significant treatment effect ($p \leq 0.05$) in two-way ANOVA.

Wh-wheat, DS-DS3E(3A), Am-Amphiploid.