

1 **Gene expression analysis in the roots of salt stressed wheat and the**
2 **cytogenetic derivatives of wheat combined with the salt-tolerant wheatgrass,**
3 ***Lophopyrum elongatum***
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5 Zina Hussein, Ani Dryanova, Deborah Maret, Patrick J. Gulick¹

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7 Biology Department and Centre for Structural and Functional Genomics

8 Concordia University

9 7141 Sherbrooke W.

10 Montreal, Quebec, H4B 1R6

11 Canada

12

13 ¹Author to whom correspondence should be addressed:

14 Patrick Gulick

15 Telephone 514 848-2424 ext. 3407

16 FAX: 514- 848-2881

17 Email: patrick.gulick@concordia.ca

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22 **Abstract**

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

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

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

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65 **Introduction**

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

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

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

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

101 Microarray technology has been used to characterize the global transcriptional profiles of
102 genes in response to salt stress (Taji et al. 2004; Ouyang et al. 2007; Rodriguez-Uribe et al.
103 2011). While some studies focused on gene expression in roots (Kawasaki et al. 2001; Kreps et
104 al. 2002; Wang et al. 2003; Yao et al. 2011), others have studied RNA profiles from seedlings,
105 cotyledons and shoot tips (Seki et al. 2002; Chao et al. 2005; Zhou et al. 2007). Among the salt-
106 stress-regulated genes identified in these studies were regulatory genes, genes encoding proteins
107 involved in signal transduction such as protein kinases, protein phosphatases, calmodulin, as well
108 as transcription factors such as EREBP, WRKY, bZIP, MADS box, Zinc finger and NAC gene-
109 family members. The expression of genes encoding proteins involved in osmolyte synthesis, cell
110 wall structure modification, ion transport, detoxification enzymes and key enzymes in metabolic

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

113 Bread wheat (*Triticum aestivum*), one of the world's most important cereal crops, is
114 moderately salt-tolerant (Hasanuzzaman et al. 2013). There is a wide range of salt tolerance
115 among species within the Triticeae, and *Lophopyrum elongatum* (Host) Love, [syn *Agropyron*
116 *elongatum*, *Elytrigia elongata*, *Thinopyrum elongatum*], a close relative of wheat, is one of the
117 most salt-tolerant members of the tribe (Colmer et al., 2006). The octaploid amphiploid produced
118 from *L. elongatum* and *T. aestivum*, contains the genomes of both species ($2n=8x=56$, AA BB
119 DD EE) and is significantly more salt-tolerant than its wheat parent, although it does not have
120 the full tolerance of *L. elongatum* (Dvořák and Ross 1986; Dvořák et al. 1988; Schachtman et al.
121 1989; Omeilian et al.1991). In addition, disomic chromosome addition and substitution lines
122 derived from crosses between the amphiploid and *T. aestivum* have been shown to exhibit varied
123 degrees of salt-tolerance. Three of the E chromosome substitutions in five lines (2E(2D),
124 3E(3A), 7E(7A), 7E(7B) and 7E(7D)) showed significant levels of tolerance, and chromosome
125 3E had the largest effect on salt tolerance (Dvořák et al. 1988; Omeilian et al. 1991).

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

135 **Materials and Methods**

136

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

138 Seeds from the *T. aestivum* cultivar Norstar were germinated and transferred to
139 hydroponic tanks containing a modified Hoagland's solution (Gulick and Dvořák 1987). Plants
140 were grown with a light cycle of 11 h light and 13 h darkness, with day/night temperatures of
141 22°C and 15°C, respectively. The growth solution was replaced at day seven and 14; 18 days
142 after germination, the hydroponic solution was replaced with fresh growth solution supplemented
143 with 150 mM NaCl, as well as 15 mM CaCl₂ to mitigate the toxic effects of sodium ions.
144 Without adequate supplementation of Ca²⁺, the effects seen by salt treatments may be due to
145 impaired root membrane function (Munns 2005). Control plants were grown without addition of
146 NaCl and CaCl₂. After salt treatments of 6, 24 and 72 h, plants were harvested, the roots were
147 removed, frozen in liquid nitrogen and stored at -80°C. The treatments were initiated at staggered
148 times in order to harvest plants at the same time in the light cycle, after approximately 6 h of
149 light. Tissues were ground in liquid nitrogen and RNA was purified with TRIZOL reagent
150 (5ml/g) according to the manufacturer's protocol (Invitrogen, Burlington, Canada). Target cDNA
151 synthesis, labeling with Cy3 and Cy5 dyes, prehybridization and hybridizations were carried out
152 as described in Monroy et al. (2007). Three biological replicates, each comprised of ten plants,
153 were analyzed.

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155 A second experiment compared the response to 150 mM NaCl + 15 mM CaCl₂ treatment
156 in three genotypes: the wheat cultivar Chinese Spring, the octaploid *T. aestivum* x *L. elongatum*
157 amphiploid, and the wheat disomic substitution line, DS3E(3A), which has the pair of 3A
158 chromosomes of wheat substituted with 3E chromosomes from *L. elongatum*. Chinese Spring

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

164

165 **Microarray construction**

166 The microarray, previously described by Monroy et al. (2007), consisted of 5728 printed
167 cDNA amplicons which included 1630 genes of regulatory or signaling function. The remaining
168 cDNA amplicons in the microarray were random clones from the Genome Canada wheat EST
169 program, Functional Genomics of Abiotic Stress (FGAS) (Houde et al. 2006), and from a
170 unigene set of the National Science Foundation (USA) wheat EST clone collections (Qi et al.
171 2004). The details for the construction of the microarray, annotation of the array and statistical
172 analysis are described in Monroy et al. (2007). The updated annotation for the microarray is
173 given in Supplemental Table S4. To determine the sequence similarity between *L. elongatum* and
174 *T. aestivum*, the nucleotide sequences of 89 *L. elongatum* ESTs were compared to the FGAS
175 wheat EST database by Blastn (Altschul et al. 1997).

176

177 **Experimental design**

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

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

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199 **Gene location**

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

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

208

209 **Results and discussion**

210 **cDNA sequence comparison between species**

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

219

220 **Time-course salt-treatment experiment**

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

229 There were 50, 33 and 57 genes repressed to 0.66 or less than of that of the control at 6,
230 24 and 72 h, respectively (Fig. 1). The biphasic pattern of expression in response to salt stress
231 has previously been reported for individual genes that were induced by salt stress in wheat

232 (Galvez et al. 1993), and Arabidopsis (Kreps et al. 2002). Only seven genes were induced at least
233 1.5-fold at all three time points, and no genes were induced more than two-fold at all three times;
234 three genes were identified that were repressed at all three time points to less than 0.66 of control
235 levels (Supplemental Table S1B).

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

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239 The patterns of gene expression in roots in response to salt stress were compared between
240 the salt-tolerant *T. aestivum* x *L. elongatum* amphiploid, the moderately salt-tolerant disomic
241 substitution line DS3E(3A), and the least salt-tolerant line, Chinese Spring wheat. A treatment of
242 72 h of salt stress, representative of the second phase of induction of gene expression, was
243 chosen for the comparison of the genotypes. The time course experiment described above
244 indicated that different sets of genes are up- and down-regulated over the time course of salt
245 treatment, and the longer exposure was hypothesized to reflect the acclimation to high salt
246 conditions in contrast to the genes responding to the initial shock of increased salt in the growth
247 medium. Genes with significant differences ($p \leq 0.05$) in expression due to treatment effect,
248 genotype effect, and genotype-by-treatment interaction effect were identified by two-way
249 ANOVA. Genes with a significant interaction effect were those that had changes in expression in
250 response to salt treatment but whose response was different among the three genotypes. The
251 analysis revealed that 775 genes had significant ($p \leq 0.05$) differences in expression for at least
252 one factor, and at least a 1.5-fold change in expression, and 214 of these had at least a two-fold
253 change in expression in at least one genotype; data is presented in Supplemental Table S2. There
254 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

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

279

280 **Genotype comparison by cluster analysis**

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

300 The genes in the clusters which show stronger induction in the salt tolerant genotypes are
301 candidates for further study; they give an insight into the basis of salt tolerance and the potential
302 to discover genes that are under common regulatory pathways. The common expression pattern
303 between the amphiploid and the DS3E(3A) for the genes in clusters 10 and 12 suggest that these
304 genes may lie on chromosome 3E or be regulated by genes on 3E. Twenty one genes in clusters
305 10 and 12 had strong sequence identity with EST clones that have been bin-mapped in wheat (Qi
306 et al. 2004); however, only 4 of these genes were mapped to chromosome group 3 (Supplemental
307 Table S3B). The 24 genes from clusters 10 and 12 that could not be localized with mapped wheat
308 ESTs were used to search for the chromosomal location of their rice homolog. Only four of the
309 24 had the most similar rice genes located on rice chromosome 1, the chromosome with the
310 highest degree of synteny with wheat chromosome group 3. Thus it appears that the majority of
311 the genes in clusters 10 and 12 are not located on chromosome 3 but instead are regulated by
312 genes on chromosome 3.

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

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321 **Functional classes of differentially expressed genes**

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

334

335 **Transcription factors**

336 Eight transcription factors had at least a two-fold change in expression in the roots of
337 salt-stressed plants, and there were significant differences in expression in the three genotypes
338 indicated by significant genotype-by-treatment interaction effects (Table 2). The array contained
339 186 probes for AP2 transcription factors. One AP2/EREBP transcription factor gene, RAP2-3-
340 like (Tr011_D14), had induction only in DS3E(3A) (Table 2). The expression of other
341 AP2/EREBP transcription factor genes showed a significant genotype-by-treatment effect, but
342 had less than a two-fold change in gene expression levels. The AP2/EREBP transcription factor
343 RAP2-7-like (Tr014_F19), showed a decrease in expression in the amphiploid and in DS3E(3A)
344 due to salt stress but showed no significant change in expression in wheat (Supplemental Table
345 S2). AP2/EREBP transcription factor genes BIERF-3-like (Tr001_G03), CRT/DRE-9

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

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

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

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

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

390

391 In the time course experiment, which monitored gene expression after 6, 24 and 72 h of
392 salt treatment, several members of the AP2 transcription factor gene family had significant
393 changes in gene expression in the roots of salt-stressed wheat plants. One AP2 transcription
394 factor, DRFL1b, (Tr014_F03) was repressed to 0.5, the level of the control at 6 h of stress (Table
395 1). A second AP2 family member, RAP2-3-like, (Tr011_D14) was induced 1.5-fold at 24 h, and
396 16 other AP2 genes were moderately repressed ($> .66$) (Supplemental Table S1). In *Arabidopsis*,
397 DREB2 and members of the AP2/EREPB transcription factor family have been reported to be
398 induced by dehydration and salinity (Nakashima et al. 2000). In contrast, the wheat DREB2
399 showed repression rather than induction over the time course of salt stress (Table 1 and
400 Supplemental Table S1); it is unlikely that any of the DREB2 genes reported here are orthologs
401 of the DREB2 *Arabidopsis* genes. Orthology is difficult to establish in such distantly related
402 species, and the patterns of expression were quite different. The ERF genes are a subgroup of the
403 AP2/EREPB family and have been noted to be induced by high-salinity stress in *Arabidopsis*
404 (Hsieh et al. 2013; Seki et al. 2004). During the time course experiment, homologs of ERF1
405 (Tr011_F12), ERF3 (Tr014_N15) and ERF4 like genes (ethylene-responsive transcription factor
406 1-like, Tr014_J11; ethylene-responsive element binding protein 2-like, Tr011_B24; ERF071-like
407 , Tr014_J12), all had significant repression at 72 h in wheat (Supplemental Table S1). One NAC
408 transcription factor, NAC-2A (Tr003_J21), was significantly repressed at 72 h (Supplemental
409 Table S1). One member of the MYB transcription factor family, myb-related protein LTR1-like,
410 (Tr001_J09) was induced at 72 h of salt stress and four members of the MYB gene family, myb-
411 related protein 306-like (Tr001_I02), myb-15-like (Tr012_G20), myb13-1-like (Tr001_N23),
412 and R2R3-myb-like, (Tr001_D09) were repressed at 6 h (Supplemental Table S1).
413

414 **Protein kinases**

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

432

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

437 induced in all three genotypes by salt stress (Supplemental Table S2). ARK1 was most strongly
438 induced in the amphiploid and the PERK1-like receptor kinase was most strongly upregulated in
439 DS3E(3A). Both ARK1 and PERK1 have roles in plant defense (Pastuglia et al. 2002; Silva and
440 Goring 2002). The LRR-receptor kinase, protein kinase Xa21-like (Tr002_A03) was highly
441 induced in all three genotypes with a more marked induction in the amphiploid and intermediate
442 induction in DS3E(3A) (Supplemental Table S2). Two other LRR-receptor kinases, protein
443 kinase PERK8-like (Tr005_J03) and phyto-sulfokine receptor 2-like (Tr001_B19), were repressed
444 in *T. aestivum* and were also repressed in the amphiploid and DS3E(3A), albeit to a lesser degree
445 (Supplemental Table S2). LRR-receptor kinases have previously been shown to be up-regulated
446 by cold, salt stress, dehydration and ABA treatments (Hong et al. 1997; Haffani et al. 2004, de
447 Lorenzo et al. 2009 and Ouyang et al. 2010).

448

449 **Phospholipid signaling**

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

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

474

475 **Protein degradation and the ubiquitin proteasome pathway**

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

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

488

489

490 **Conclusions**

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

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

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

522

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527 3E(3A) chromosome substitution line.

528

529 **Conflict of interest**

530 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 \geq 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 \geq two-fold.^a

Microarray ID	GenBank ID	Annotation	Change of expression			ANOVA P-value
			6 hr	24 hr	72 hr	
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)	<i>T.aestivum</i>	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 \geq 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 \geq 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*.

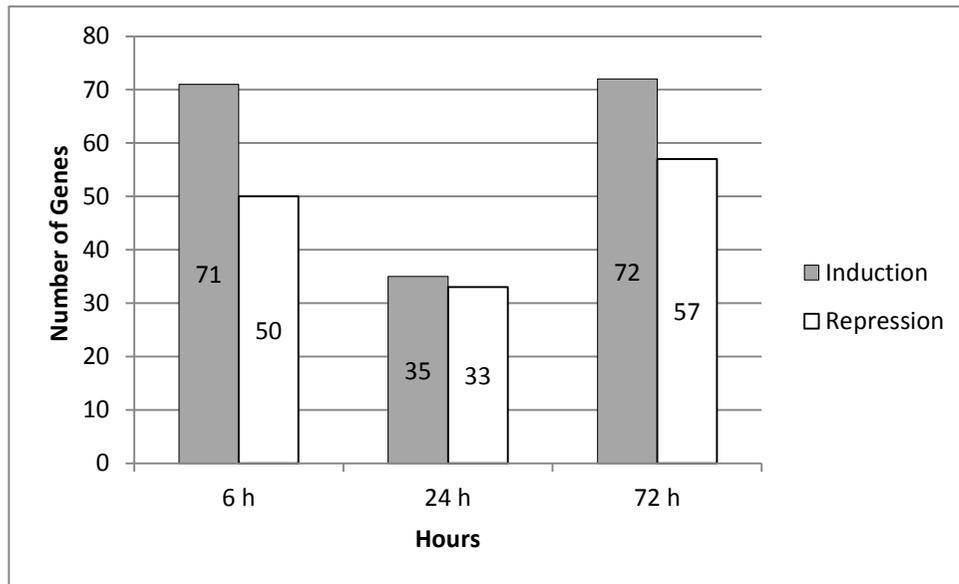


Fig. 1. The number of genes induced and repressed at least 1.5-fold in the roots of *T. aestivum*, with a significant p-value ≤ 0.01 after 6, 24 and 72 h of treatment with NaCl.

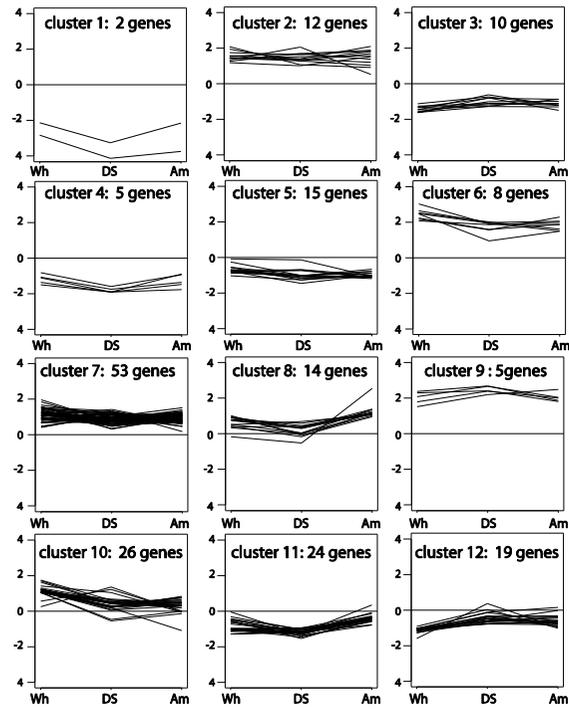


Fig. 2. *K*-Means Cluster analysis of genes that have \geq two-fold change in expression in at least one genotype and significant treatment effect ($p \leq 0.05$) in two-way ANOVA.

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