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Dissecting salt stress pathways

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Abstract

Upon salt-stress treatment, *Arabidopsis* mobilizes a complex set of pathways that includes alterations in the regulation of gene expression and metabolic adjustments that attempt to establish a new energetic and developmental equilibrium. The responses share common elements with reactions to many other stresses, such as challenges by osmotic fluctuations, pathogens, mechanical interference, or cold stress. Also, hormones, such as ABA, ethylene, and jasmonic acid, play important roles in salt-stress signalling and adaptation. Publicly available and our own transcript profiling data are used here to dissect gene regulation under salt stress in *A. thaliana* Col-0. Applying the clustering method 'fuzzy k-means clustering' on 1500 strongly regulated genes, the salt-stress response could be categorized into distinct segments. Fewer than 25% of the regulated genes are salt stress-specific, while the majority also responded to other stresses and/or hormone treatments. Significantly, roots and shoots showed differences in hormone responsiveness, and early and late responses correlated with different signalling events. A network begins to emerge, revealing the basis of cross-talk between high salinity and other stresses.

Key words: *Arabidopsis thaliana* Col-0, cross-talk, fuzzy k-means clustering, salinity, transcript profiles.

Introduction

Decades of research into the effects of salinity on plant physiology and development have generated a wealth of information, among which the most advanced understanding is based on the detection and analysis of a signalling pathway (SOS) (Zhu, 2003) and engineering of sodium storage by cells (Apse *et al*., 1999; Blumwald, 2003). Other results also pointed to the importance of the plant hormone ABA, the calcium sensor, calcineurin B-like 1 (CBL1), potassium homeostasis, and MAPK and CDPK genes in salt-stress responses that lead to protection (Hasegawa *et al*., 2000; Xiong *et al*., 2002; Albrecht *et al*., 2003; Cheong *et al*., 2003; Kim *et al*., 2004). However, current knowledge is still largely restricted to individual genes and pathways, and the unifying picture remains hidden.

Plants have evolved complex signalling pathways in response to various stimuli, such as salt, drought, cold, wounding, or pathogen invasion, and have acquired plasticity in metabolic functions and developmental switches to cope with changing environmental conditions (Genoud and Metraux, 1999). Cross-talk connecting different pathways appears to be a common feature in plants, as exemplified by biotic defences involving ethylene, salicylic acid, and jasmonic acid (Dong, 1998; Kunkel and Brooks, 2002), or by the DREB/CBF pathway on which signals from several abiotic stress conditions converge (Chinnusamy *et al*., 2004; Shinozaki and Yamaguchi-Shinozaki, 2000). The understanding of salinity stress will be greatly enhanced by identifying the convergent and divergent pathways between salinity and other abiotic stress responses and the nodes of signalling convergence. Indeed, several studies have addressed cross-talk between abiotic stresses and hormone signalling (Cheong *et al*., 2002; Kreps *et al*., 2002; Seki *et al*., 2002).

Recently, public efforts have been directed to *Arabidopsis* global transcript profiling that monitored the response of the plant under different treatments. Large sets of data have been made publicly available through several databases, such as TAIR, NASC, and Genevestigator (Garcia-Hernandez *et al*., 2002; Craigon *et al*., 2004; Zimmermann *et al*., 2004).

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Especially useful has been the AtGenExpress consortium project which had generated standard Affymetrix microarray data for *Arabidopsis* (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). Different methods, among them electronic northern and co-regulation analysis tools, have been created to integrate these data (Steinhauser *et al*., 2004; Zimmermann *et al*., 2004; Persson *et al*., 2005; Toufighi *et al*., 2005).

Salt-stress response pathways in *Arabidopsis* are dissected using the publicly available AtGenExpress data. In addition, microarray data generated by long oligo (70-mer) glass-array slides monitoring salt-stressed plants are compared with those deposited in AtGenExpress. This analysis revealed a well-defined salt-stress response in *Arabidopsis* that could be contrasted against reactions in response to other stresses. From the datasets, 1500 salt-regulated genes have been extracted and analysed by the fuzzy k-means clustering method (Gasch and Eisen, 2002). This analysis provided a distinction between genes that responded only to salinity from those that also responded to biotic, osmotic, low temperature stress, and hormone treatments. By assigning specificity and identifying nodes of cross-talk, general patterns of gene regulation in *Arabidopsis* upon salinity stress can be identified.

Materials and methods

Affymetrix microarray data

The abiotic transcript profile data were downloaded from Weigel World (http://www.weigelworld.org/resources/microarray/AtGenExpress/), which has been processed via gcRMA (Wu *et al*., 2003). For biotic and hormone treatments, the CEL files for Affymetrix microarray data were downloaded from TAIR and processed into expression estimates using gcRMA implemented in R with default settings. For each experiment, the log2 intensities for individual probe sets were averaged across two replicates for treatment and control, and their differences were used as log2 of fold changes. Among the 12 salt-stress experiments (roots or shoots, six time points), the maximum and minimum regulation values were used to extract, for this pilot analysis, the top 1000 up-regulated and top 500 down-regulated genes, which were analysed using the fuzzy k-means clustering method (Gasch and Eisen, 2002), using the parameter k=30. The process generated 22 centroids with each gene linked to every centroid by a membership value. Then, 22 clusters were generated in a way that a gene was assigned to the cluster with which it had the highest membership value. A 0.2 membership cutoff was applied, which resulted in 1143 genes with clear patterns in these 22 clusters. Results were visualized by mapletree (http://rana.lbl.gov/EisenSoftware.htm) software.

Glass microarray data

A. thaliana (Col-0) plants were grown hydroponically in pots filled with isolite artificial soil (Sundine Enterprises, Arvada, CO), supplied with 0.5× Hoagland's nutrient solution with increased (4×) Fe amounts, at 24 °C (16/8 h light/dark; ~150 μmol photons m^{-2} s^{-1}). Four-week-old plants before bolting were irrigated with 150 mM NaCl at midday, and remained in the presence of NaCl solution. Control plants were irrigated with nutrient solution. After treatment for 3 h and 24 h, respectively, plants, at least 10 per sample, were frozen in liquid N_2. Two biological repeats, grown separately at different times, were used.

From these samples, total RNA was isolated (RNeasy; Qiagen, Carlsbad, CA). Glass microarray slides consisting of 70-mer oligonucleotide probes (http://ag.arizona.edu/microarray/) were used in hybridizations. RNA samples (70 μg each) for control and treatment conditions were reverse transcribed (SuperScript III; Invitrogen, Carlsbad, CA) and hybridization performed according to TIGR (http://atarrays.tigr.org/arabprotocols.shtml). For each time point in each biological repeat three hybridizations were carried out. To avoid bias in microarrays as a consequence of dye-related differences in labelling efficiency, dye labelling for each paired sample (stress/control) was swapped in one of three independent hybridizations. In total, 12 microarray hybridizations were carried out.

After hybridization, signal intensities for each array element were collected (GenePix 4000B; Axon Instruments, Union City, CA) and images analysed (GenePix Pro 4.0). Spots with intensities lower than local background or aberrant spot shape were flagged by the GenePix software, checked manually, and excluded. The resulting GPR files were analysed by TIGR-TM4 (http://www.tm4.org/) (Saeed *et al*., 2003). Total intensity normalization, Lowess (Locfit) normalization, standard deviation regulation, and intensity filtering were done for each slide with TIGR-MIDAS, version 2.18. Then, using 'Multiple Experiment Viewer' (MEV, a tool in TM4), version 3.0.3, a class t test (P=0.05, permutation=64) was applied to pick up the significantly regulated genes. Adjusted Bonferroni P-value correction was used at the same time to reduce FDR (false discovery rate). The t test output was then compared with salt stress microarray data from the AtGenExpress consortium projects.

Comparison of results with Affymetrix and glass microarray slides

The list of differentially regulated genes using 70-mer olionucleotide glass slides was compared with the list of genes identified by AtGenExpression as regulated. For this comparison, only the trend of regulation was considered. If the log2 ratio value was less than 0, the gene was considered repressed, otherwise induced.

Results

Arabidopsis *oligonucleotide-based microarrays*

Based on results from previous studies (Kreps *et al*., 2002; Seki *et al*., 2002; Taji *et al*., 2004), a shock treatment of 150 mM NaCl for 3 h and 24 h, respectively, was chosen. This concentration and times represent the maximum tolerable for a specific response without inducing pathological reactions. The gene expression levels were compared with those of untreated controls (see Materials and methods). Each experimental condition was represented by six slides from two biological repeats, including cy3/cy5 dye-swaps with a microarray platform that included 70-mer oligonucleotides, selected to reduce or abolish cross-hybridization, for approximately 26 000 genes.

Normalization and statistical analysis (P <0.05) resulted in 2419 genes expressed differentially in the 3 h salt-stress experiment compared with the control, and 3930 genes at 24 h. These data were compared to those from AtGenExpress salt-stress experiments, which had been carried out using the Affymetrix ATH1 GeneChip platform. 2109 genes (out of 2419) were found in 3 h experiments in

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both types of slides, and 3415 genes (out of 3930) at the 24 h time point. While whole plants were used in these experiments, AtGenExpress experiments were done separately for roots and shoots. Considering this, only those genes regulated in the same direction in both roots and shoots were compared with our data. This resulted in 79% of the genes sharing the same trend in the 3 h data, and 84% in the 24 h data (Table 1). The numbers of genes regulated in the opposite direction in roots and shoots are also listed.

A comparison of data for genes represented on both platforms indicated a highly similar trend in gene regulation and significant superimposition in all functional categories (categories not shown). Considering the differences between the tools, technical differences, biological sampling and preparation, it seems reassuring to confirm that salt stress generated stable regulation patterns in *Arabidopsis* wild-type plants that can be replicated, independent of the platform used.

The Affymetrix data collection: Advantages of Affymetrix transcript analysis slides are the inclusion of a standard probe set and well-defined hybridization protocols. With the generous contribution of the AtGenExpress projects, the public databases now include a variety of microarray experiments conducted after different treatments of the plants. The focus was on stress-relevant and hormone-specific AtGenExpress data to harness the high reproducibility of this hybridization platform in comparisons of different treatments. Raw average data were also used without statistical filtering as an acceptable strategy because general trends are the point of interest.

Table 1. *Comparing glass array slide to Affymetrix GeneChips*

The trends of regulation in both array platforms were compared with each other. Glass arrays used material from root and shoot tissues combined: for the Affymetrix chip experiments these tissues had been separated. At the 3 h time point, 1016 genes (475+541) out of 1284 genes were regulated in the same direction. At 24 h, 1841 genes (692+1,149) out of 2187 were regulated in the same direction. All genes with a negative value were considered down-regulated, and all genes with a positive value were considered up-regulated, irrespective of the degree of regulation.

Time point	Glass array	Affymetrix shoots	Affymetrix roots	Number of genes
3 h	Up	Up	Up	475
	Down	Down	Down	541
	Up	Down	Down	136
	Down	Up	Up	132
	Up	Up	Down	242
	Up	Down	Up	175
	Down	Up	Down	216
	Down	Down	Up	192
24 h	Up	Up	Up	692
	Down	Down	Down	1149
	Up	Down	Down	164
	Down	Up	Up	182
	Up	Up	Down	238
	Up	Down	Up	237
	Down	Up	Down	458
	Down	Down	Up	295

The overall pattern: After extraction of all data from the AtGenExpress database, the analysis focused on 1496 genes, which represent the 1000 most highly up-regulated and 500 most strongly down-regulated salt-responsive genes in *Arabidopsis* Col-0 (see supplementary Table 1 at JXB online). Fuzzy k-means clustering (Gasch and Eisen, 2002) placed 1143 genes into 22 clusters (Fig. 1) (see supplementary Table 2 at JXB online). A total number of 353 genes was removed from further analysis based on their low membership values (see Materials and methods).

Of the remaining 1143 genes 82% assembled into 10 major clusters, which distinguished responses under a selection of experimental conditions that included biotic interactions (viral, bacterial, and fungal), cold, osmotic, salinity, drought, oxidative, and wounding stress treatments, as well as different hormone treatments (clusters C0 through C9; Fig. 1). Approximately 18% of the genes were placed into the small clusters 10 through 21, which will not be discussed. Among the large groupings, clusters 0, 2, 4, 6, 8, and 9 include salt-stress up-regulated genes, and clusters 1, 3, 5, and 7 include the down-regulated genes. Interestingly, genes in cluster 0 and 8 were also up-regulated by elicitor treatments, genes in clusters 4 and 6 by ABA treatment, and cluster 9 united salt-responsive and methyl-jasmonate (MeJA)-induced genes. By contrast, the genes in cluster 2 were up-regulated only by salt-stress and only in roots. Notably, only a small portion of the genes was directly induced by more than one of the treatments by elicitors, ABA, and MeJA. A further distinction emerged in the timing of the response and in hormone-specific correlations that were different in space and time. The remaining clusters 1 and 3 included genes that were down-regulated in both abiotic and biotic stresses, while clusters 5 and 6 included genes down-regulated only by abiotic stresses. In the following sections, an analysis of the functionally annotated genes in each centroid will be presented. This provides a basis for dissecting the *Arabidopsis* salt-stress pathways, and also presents pointers that can guide future analyses into the function of currently unknown genes that appeared in each cluster.

C8: immediate responses: Genes in cluster 8 (141 in total, C8; Fig. 1) showed immediate regulation changes and retained up-regulation in salt-stressed roots, but in shoots the changes were insignificant. Strong up-regulation of this group of genes was also observed following osmotic stress, cold stress, and a variety of biotic stress treatments. Transient induction was seen in drought-stressed roots and shoots, and in wounded shoots. Interestingly, genes in C8 were only minimally induced by exogenous ABA. One-third of the genes are functionally unknown, while the rest could be categorized. Ethylene appeared to be the dominant hormone here, suggested by the presence of At-ERFs 1, 5, 6, and 11; and the ACC synthase, ACS6. Various calcium-dependent signalling pathways seemed to be involved,

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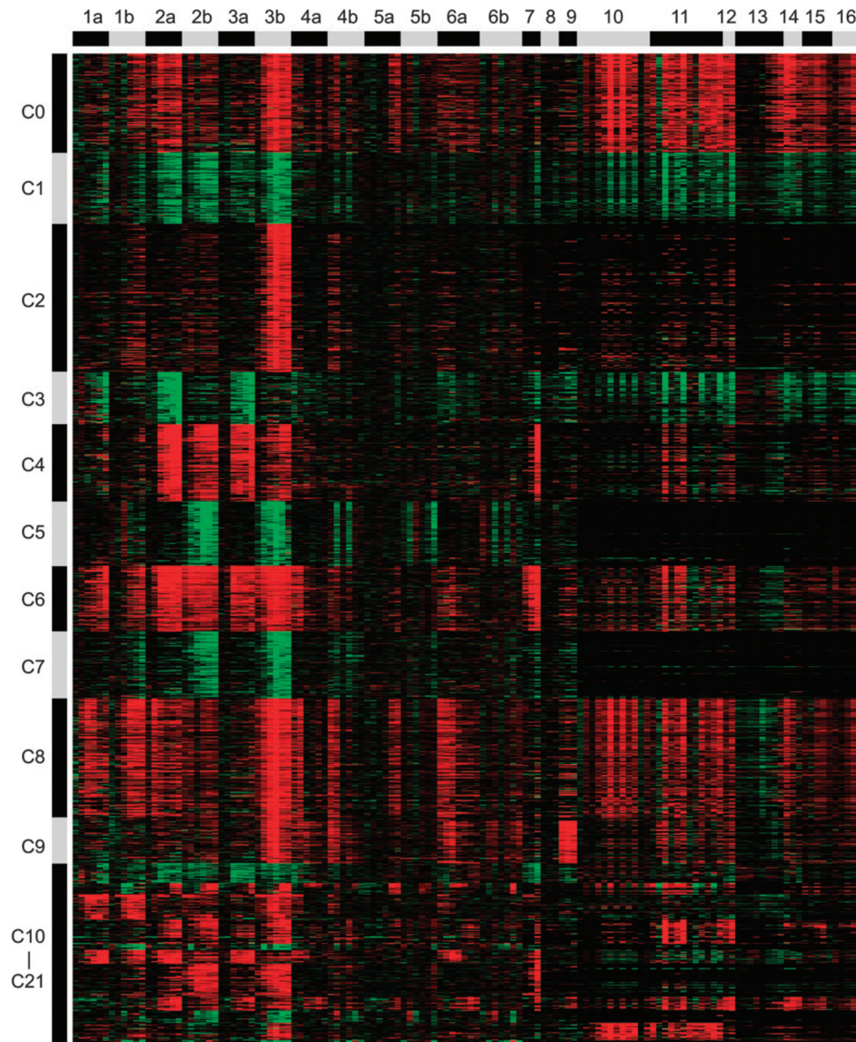

Fig. 1. Clustering of 1143 salt-regulated genes. Each row represents a gene, while each column represents an experiment. The code for the experiments are: 1, cold stress; 2, osmotic stress; 3, salt stress; 4, drought stress; 5, oxidative stress; 6 wounding stress. For the experiments 1 to 6, a represent shoots, while b identifies roots. For 1a to 5b, the time points are, from left to right, 0.5, 1, 3, 6, 12, 24 h, while for 6a and 6b, time points are 0.25, 0.5, 1, 3, 6, 12, 24 h. The numbers 7, 8, 9 represent experiments with ABA, ACC, and MeJA treatments, respectively, for 0.5, 1, and 3 h in each case. Number 10: bacteria-derived elicitors treatment, which are $MgCl_2$+$CaCl_2$, GST, Harpin Z, GST-necrosis-inducing *Phytophthora* protein 1, flagellin and lipopolysaccaride, for 1 h and 4 h, respectively. Number 11: *Pseudomonas syringae* pv. *tomato* (Pst) DC3000, Pst avrRpm1, Pst DC3000 hrcC- and *Pseudomonas syringae* pv. *phaseolicola*, for 2, 6, and 24 h. Number 12: *Botrytis cinerae* treatment for 18 h and 48 h. Number 13: *Erysiphe orontii* treatment for 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, and 5 d. Number 14: *Phytophthora infestans* treatment for 6, 12, and 24 h. Number 15: *Pseudomonas syringae* ES4325 avrRpt2 treatment for 4, 8, 16, 24, and 48 h. Number 16: *Pseudomonas syringae* ES4325 treatment for 4, 8, 16, 24, and 48 h. C0 through C21 identify clusters 0 through 21 after fuzzy k-means analysis.

exemplified by many calcium-binding proteins, calmodulins, calmodulin-binding proteins, including TCH3, calcineurin CBL1, and calcium-transporting ATPases. The transcription factors found in centroid C8 were mainly zinc finger and WRKY transcription factors such as ZAT10, ZAT12, WRKY 22, and WRKY 53. Finally, included were several disease-resistance protein genes, genes functioning in post-translational modification and protein degradation, and a few MAPKs (MPK 3, 5, and 11).

C6: early responses: Cluster 6 included 76 genes (C6; Fig. 1) that were highly induced by salinity and osmotic stress treatments, early in roots and 1 h later in shoots, by cold after 6 h, and by drought early in roots. These genes were also early and strongly induced by ABA. Several genes in C6 have been established as key regulators in abiotic stress responses, such as RD29A and DREB2A (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu *et al.*, 1998). Also included were RD20 and KIN1. Not surprisingly, genes functioning in ABA synthesis and signal transduction appeared, including NCED3, ABF3, ABI1, ABI2, and other PP2Cs. A third large group included transcription factors, especially MYBs and NACs. Several have been studied for their involvement in abiotic stress responses,

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including ATAF1, ATHB12, NAP, AZF2, HSF2, and ATERF4. Finally, a few genes involved in cell wall biosynthesis and LEAs appeared in centroid C6. Overall, most genes are clearly involved in abiotic stresses, and have been characterized before, in the ABA-dependent or ABA-enhanced early response cascade of abiotic stress.

C4: delayed responses in roots: The 89 genes in C4 (C4, Fig. 1) were strongly up-regulated by salt and osmotic stresses in roots after only a 3 h treatment, and also induced 3 h after ABA treatment, while in shoots up-regulation was observed earlier. Many of the C4-genes identified diverse metabolic pathways, including lipid, for example, LTP3 and LTP4, and carbohydrate metabolism, for example, a sucrose synthase isoform and APL3 and APL4 that are involved in starch biosynthesis.

C0: defence genes shared with biotic stress conditions: Genes in cluster 0 (114 in total, C0; Fig. 1) were strongly induced in roots starting after 1 h of salt stress, but showed no significant change in shoots. Unambiguous induction could also be seen in osmotically stressed shoots, oxidatively stressed shoots, and in cold-treated roots. These genes were also greatly induced by various biotic stress treatments. Significantly, these genes showed only minor fluctuations following ABA, 1-aminocyclopropane-1-carboxylic acid (ACC), or MeJA treatment, and, hence, could not be identified as responsive to the typically invoked stress hormones. Enriched in this cluster were genes involved in redox homeostasis control and post-translational modification, including many GSTs, FAD-linked oxidoreductases, protein kinases and PP2Cs, and oxidoreductin AERO1. A significant number of genes were receptor-like protein kinases, suggesting the existence and involvement of dynamic intercellular signalling events. Defence genes abounded: cell wall proteins including AGP2 and AGP5, lignin synthesis genes including CCR2, P450 genes including PAD3 (phytoalexin biosynthesis), the calcium-transporting ATPase ACA12 and ABC transporters, and disease resistance proteins of various classes appeared in C0. Several WRKY transcription factors (At1g62300, At4g18170, At5g24110, At5g49520), and the ethylene biosynthesis gene ACS2 may be considered as defence-related as well.

C2: the salt- and root-specific response: Cluster 2 included 171 genes (C2; Fig. 1; Table 2) that were only or most strongly up-regulated in salt-stressed roots. Some of these genes showed a moderate induction in osmotic or drought stresses, but no clear pattern could be seen, while ABA seemed to have no impact on their expression levels. Among the genes with functional annotations in C2, several categories emerged. Similar to C6, many ethylene synthesis and signalling genes were observed, including ERF1 and ACS8. More than 10 genes in C2 identified so-called disease resistance proteins (labelled as biotic stress responsive) and an equal number of receptor-like kinases. Also, genes with functions in post-translational modification and protein degradation were included. Surprisingly, nearly 20% of the genes in C2 were transcription factors. In addition to AP2 genes, that were otherwise almost exclusively found in C2, and a few Mybs and WRKYs, the group included a number of unknown, putative transcription factors, which should become important new targets in salt-stress studies. Finally, approximately 60 genes with unknown functions were C2-specific.

C9: The cluster related to MeJA: C9 comprises a small cluster with 49 genes (C9, Fig. 1) that showed strong induction only in salt-stressed roots, drought-stressed roots and shoots, wounded roots and shoots, and by MeJA. Most annotated members of this centroid are involved in the biosynthesis of various secondary metabolites. Among these, all major JA synthesis genes (AOS, AOC1, and OPR3), amidohydrolase ILL6 (for auxin homeostasis) and a 2-oxoglutarate-dependent dioxygenase (for ethylene synthesis), an anthocyanidin synthase, and two P450s were identified. The remaining genes included the well-known ATMYC2/JIN1 and two other bHLHs, and two annexins, ANNAT3 and ANNAT4.

The down-regulated genes: clusters C1 and C3: Genes in clusters 1 and 3 (142 in total, C1, C3; Fig. 1) were down-regulated by salinity and osmotic stress treatment. Compared with roots, shoots showed higher (C3) or similar (C1) but slightly delayed repression that became obvious after the 3 h time point. These genes were also repressed in various biotic stress treatments, and by ABA treatment mainly at the same 3 h time point. Moderate down-regulation was observed in almost all other treatments with a slight bias towards a response in the shoots. An unusually large proportion, approximately 40%, of the genes in these two clusters is annotated as functionally unknown. Most of the remaining genes identified function in growth. Many belonged to transcription factor families such as bHLH, bZIP, and Myb. Also HAT1 and MYC1 were included here. The second group was made up of auxin-responsive genes including SAUR-AC1. A third group, finally, included cell wall modification genes and genes of related function, for instance the GDSL lipases, XTH9 and PEM3.

The down-regulated genes: clusters 5 and 7: In contrast to the genes in clusters 1 and 3, clusters 5 and 7 (151 genes in total, C5, C7; Fig. 1) showed a root-specific pattern of down-regulation, initiated immediately after salt, osmotic, drought, and oxidative stress treatments. ABA moderately repressed their expression as well. However, biotic stress treatments have no effects on the expression of these genes. Unique to these two clusters were a group of peroxidases, metal transporters, and several aquaporins. Similar to C1 and C3 genes, a large number of genes were involved in cell wall modification, including several AGPs, FUT5, and PRP3; and the GDSL lipases and LTPs. A few AP2 transcription factors, bHLH and Mybs were also identified, together with genes involved in development. Finally,

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Table 2. *Genes exclusively up-regulated by salt stress in roots (cluster C2)*

Gene ID	Annotation (acc. to TAIR)	Gene ontology (Mapman)[a]	Membership value
At4g18990	Xyloglucan endotransglycosylase, putative	Cell wall, modification	0.475528
At1g61290	Syntaxin SYP124	Cell, vesicle transport	0.865652
At4g24170	Kinesin motor family protein	Cell, organization	0.570721
At4g30430	Senescence-associated protein homologue	Development, unspecified	0.311292
At5g40260	Nodulin MtN3 family protein	Development, unspecified	0.312566
At2g36640	Late embryogenesis abundant protein (AtECP63)	Development, unspecified	0.316648
At1g19025	DNA cross-link repair protein-related	DNA, repair	0.236476
At1g20390	Hypothetical protein	DNA, gypsy-like retrotransposon	0.34032
At2g18180	Putative phosphatidylinositol/phophatidylcholine transfer protein	Transporter activity	0.61379
At2g14960	Putative auxin-regulated protein	Hormone metabolism, auxin, regulated	0.334001
At3g62100	Auxin-induced protein homologue	Hormone metabolism, auxin, regulated	0.344991
At1g05670	Putative indole-3-acetate β-glucosyltransferase	Hormone metabolism, auxin, synthesis/degradation	0.323077
At2g44840	Ethylene response element binding protein (EREBP)	Hormone metabolism, ethylene, signal transduction	0.254441
At3g23240	Ethylene response factor 1 (ERF1)	Hormone metabolism, ethylene, signal transduction	0.292626
At3g23230	Ethylene responsive element binding protein, putative	Hormone metabolism, ethylene, signal transduction	0.356867
At3g23220	Ethylene responsive element binding protein, putative	Hormone metabolism, ethylene, signal transduction	0.512815
At5g43450	1-aminocyclopropane-1-carboxylate oxidase	Hormone metabolism, ethylene, synthesis/degradation	0.243084
At4g37770	1-aminocyclopropane-1-carboxylate synthase-like	Hormone metabolism, ethylene, synthesis/degradation	0.392426
At1g44090	Gibberellin 20-oxidase, putative	Hormone metabolism, gibberellin, synthesis/degradation	0.723534
At4g31780	Monogalactosyldiacylglycerol synthase-like protein	Lipid metabolism, galactolipid synthesis	0.358765
At1g21530	Amp-binding protein, putative	Lipid metabolism, FA synthesis/elongation, acyl CoA ligase	0.474401
At1g30370	Lipase class 3 family protein, similar to DEFECTIVE IN ANTHER DEHISCENCE1	Lipid metabolism, lipid degradation, lipases	0.234672
At2g31690	Putative triacylglycerol lipase	Lipid metabolism, lipid degradation, lipases	0.29695
At4g16820	Lipase class 3 family protein, similar to DEFECTIVE IN ANTHER DEHISCENCE1	Lipid metabolism, lipid degradation, lipases	0.453779
At3g20520	Glycerophosphoryl diester phosphodiesterase family protein	Lipid metabolism, lipid degradation, lysophospholipases	0.210923
At3g26190	Cytochrome p450 family	Misc. cytochrome P450	0.296921
At4g37360	Cytochrome p450 family	Misc. cytochrome P450	0.313713
At5g52400	Cytochrome p450 family	Misc. cytochrome P450	0.73239
At3g14225	GDSL-motif lipase/hydrolase family protein, EMB1474	Misc. GDSL-motif lipase	0.340436
At5g24540	Glycosyl hydrolase family 1	Misc. gluco-, galacto- and mannosidases	0.293076
At1g14550	Anionic peroxidase, putative	Misc. glutathione *S*-transferases	0.625433
At5g60310	Lectin protein kinase, similar to receptor lectin kinase 3	Misc. myrosinases-lectin-jacalin	0.494127
At1g70130	Receptor-like kinase, putative	Misc. myrosinases-lectin-jacalin	0.771136
At3g51680	Short-chain alcohol dehydrogenase-like protein	Misc. short chain dehydrogenase/reductase (SDR)	0.25172
At3g22360	Alternative oxidase 1b precursor	Mitochondrial electron transport/alternative oxidase	0.259509
At2g20800	Putative NADH-ubiquinone oxireductase	Mitochondrial electron transport/NADH-DH, type II	0.57336
At3g62380	Putative protein	Not assigned, no ontology	0.202694
At3g06433	Hypothetical protein	Not assigned, no ontology	0.210247
At4g39640	Putative γ-glutamyltransferase	Not assigned, no ontology	0.232161
At5g40880	Putative protein	Not assigned, no ontology	0.298378
At3g51810	Embryonic abundant protein AtEm1	Not assigned, no ontology	0.385323
At5g66640	LIM domain-containing protein-related	Not assigned, no ontology	0.40961
At2g32020	Putative alanine acetyl transferase	Not assigned, no ontology	0.416065
At2g38830	Unknown protein	Not assigned, no ontology	0.419731
At1g69150	DC1 domain-containing protein	Not assigned, no ontology	0.439032
At2g28820	Unknown protein	Not assigned, no ontology	0.44632
At1g08860	Hypothetical protein	Not assigned, no ontology	0.45734
At4g37710	Putative protein	Not assigned, no ontology	0.473267
At1g61280	Hypothetical protein	Not assigned, no ontology	0.489152
At1g51915	Hypothetical protein	Not assigned, no ontology	0.841651
At1g21850	Pectinesterase (pectin methylesterase), putative	Not assigned, no ontology	0.846657
At5g58680	Putative protein	Not assigned, no ontology, armadillo/β-catenin repeat	0.376546
At1g42980	Hypothetical protein	Not assigned, no ontology, formin homology 2 domain	0.86166
At4g37900	Putative protein	Not assigned, no ontology, glycine-rich proteins	0.290615
At4g33930	Putative protein	Not assigned, no ontology, glycine-rich proteins	0.738637
At2g20720	Hypothetical protein	Not assigned, no ontology, pentatricopeptide (PPR) repeat	0.217173
At1g72240	Hypothetical protein	Not assigned, unknown	0.201859
At2g28305	Expressed protein	Not assigned, unknown	0.210502
At5g40180	Putative protein	Not assigned, unknown	0.230106
At1g12030	Hypothetical protein	Not assigned, unknown	0.236047
At2g46640	Hypothetical protein	Not assigned, unknown	0.243837
At5g24600	Putative protein	Not assigned, unknown	0.260495

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Table 2. (*Continued*)

Gene ID	Annotation (acc. to TAIR)	Gene ontology (Mapman)[a]	Membership value
At5g22540	Putative protein	Not assigned, unknown	0.262908
At1g70630	Hypothetical protein	Not assigned, unknown	0.270149
At2g23270	Expressed protein	Not assigned, unknown	0.271949
At4g17410	Hypothetical protein	Not assigned, unknown	0.272911
At5g57510	Unknown protein	Not assigned, unknown	0.28043
At1g68330	Hypothetical protein	Not assigned, unknown	0.292341
At2g41730	Hypothetical protein	Not assigned, unknown	0.297847
At1g05060	Expressed protein	Not assigned, unknown	0.314235
At2g05000	Hypothetical protein	Not assigned, unknown	0.319508
At5g47440	Putative protein	Not assigned, unknown	0.338392
At1g68765	IDA, loss of function mutations are defective in ethylene independent floral organ abscission	Not assigned, unknown	0.365902
At1g13310	Expressed protein	Not assigned, unknown	0.382558
At3g04620	Unknown protein	Not assigned, unknown	0.399807
At1g10880	Hypothetical protein	Not assigned, unknown	0.402775
At5g66670	At14a, putative	Not assigned, unknown	0.429151
At2g36650	Hypothetical protein	Not assigned, unknown	0.431538
At2g20625	Hypothetical protein	Not assigned, unknown	0.438484
At5g03270	Lysine decarboxylase-like protein	Not assigned, unknown	0.444432
At4g25330	Hypothetical protein	Not assigned, unknown	0.482217
At5g38310	Hypothetical protein	Not assigned, unknown	0.528565
At4g40020	Putative protein	Not assigned, unknown	0.541454
At5g64450	Putative protein	Not assigned, unknown	0.552543
At3g25655	Expressed protein	Not assigned, unknown	0.55716
At1g07860	Hypothetical protein	Not assigned, unknown	0.578333
At3g54520	Hypothetical protein	Not assigned, unknown	0.580195
At5g60350	Putative protein	Not assigned, unknown	0.589964
At4g27580	Expressed protein	Not assigned, unknown	0.619288
At1g76210	Hypothetical protein	Not assigned, unknown	0.634611
At1g48980	Hypothetical protein	Not assigned, unknown	0.660787
At3g53450	Putative protein	Not assigned, unknown	0.679878
At2g36440	Hypothetical protein	Not assigned, unknown	0.796049
At1g74870	Hypothetical protein	Not assigned, unknown	0.816778
At2g37880	Expressed protein	Not assigned, unknown	0.821758
At3g10830	Hypothetical protein	Not assigned, unknown	0.867859
At1g09800	tRNA pseudouridine synthase family protein	Nucleotide metabolism, deoxynucleotide metabolism	0.341889
At4g15100	Hydroxynitrile lyase-like protein	Protein, degradation	0.29554
At2g31860	Putative poly(ADP-ribose) glycohydrolase	Protein, degradation	0.5312
At3g28600	AAA-type ATPase family protein	Protein degradation, AAA type	0.203079
At3g50940	BCS1 protein-like protein	Protein degradation, AAA type	0.342178
At3g28610	AAA-type ATPase family protein	Protein degradation, AAA type	0.683502
At2g18190	Putative AAA-type ATPase	Protein degradation, AAA type	0.790586
At1g32970	Subtilase, putative	Protein degradation, subtilases	0.253225
At3g08750	F-box family protein	Protein degradation, ubiquitin, E3, SCF, FBOX	0.729372
At1g67000	Protein kinase family protein	Protein, post-translational modification	0.324713
At5g55090	MAPKKK15	Protein, post-translational modification	0.335295
At1g16160	WAK-like kinase (WLK)	Protein, post-translational modification	0.388435
At5g47850	Receptor kinase-like protein	Protein, post-translational modification	0.456693
At4g26890	MAPKKK16	Protein, post-translational modification	0.548531
At1g61460	Receptor kinase, putative	Protein, post-translational modification	0.56006
At1g71530	Protein kinase family protein	Protein, post-translational modification	0.673873
At2g24130	Leucine-rich repeat transmembrane protein kinase, putative	Protein, post-translational modification	0.742576
At2g44070	Putative translation initiation factor eIF-2B delta subunit	Protein, synthesis, initiation	0.46894
At2g05720	Putative U4/U6 small nuclear ribonucleoprotein	RNA processing	0.355508
At4g16680	RNA helicase	RNA processing	0.604112
At1g74930	AP2 domain-containing protein, putative	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.206856
At1g77640	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.227997
At2g33710	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.261666
At1g19210	AP2 domain-transcription factor, putative	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.29746

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Table 2. (*Continued*)

Gene ID	Annotation (acc. to TAIR)	Gene ontology (Mapman)[a]	Membership value
At1g44830	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.306712
At2g46310	Putative AP2 domain transcription factor	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.390719
At1g22810	TINY-like transcription factor	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.504744
At5g07310	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.593625
At1g71450	Transcription factor TINY, putative	RNA, regulation of transcription, AP2/EREBP, APETALA2/ Ethylene-responsive element binding protein family	0.59929
At1g31290	PAZ domain-containing protein/piwi domain-containing protein	RNA, regulation of transcription, Argonaute-like	0.788571
At5g35900	LOB domain family protein	RNA, regulation of transcription, AS2, Lateral Organ Boundaries Gene Family-Class I	0.501222
At4g34400	Transcriptional factor B3 family protein	RNA, regulation of transcription, B3 transcription factor	0.646999
At2g22760	bHLH protein	RNA, regulation of transcription, bHLH,	0.210422
At1g51700	Dof zinc finger protein	RNA, regulation of transcription, C2C2(Zn) DOF zinc finger	0.208299
At2g32930	Zinc finger (CCCH-type) family protein, ZFN2	RNA, regulation of transcription, C2H2 zinc finger family	0.464091
At3g53600	Zinc finger-like protein	RNA, regulation of transcription, C2H2 zinc finger family	0.494576
At5g59450	Scarecrow-like transcription factor 11 (SCL11)	RNA, regulation of transcription, GRAS transcription factor	0.215366
At2g44910	Homeobox-leucine zipper protein, Athb-4	RNA, regulation of transcription, HB, Homeobox transcription factor family	0.301105
At3g50060	Myb DNA-binding protein (MYB77)	RNA, regulation of transcription, MYB domain transcription factor family	0.280587
At4g17785	Myb family transcription factor (MYB39)	RNA, regulation of transcription, MYB domain transcription factor family	0.296121
At3g62610	Myb family transcription factor	RNA, regulation of transcription, MYB domain transcription factor family	0.355075
At4g37780	Myb DNA-binding protein (AtMYB87)	RNA, regulation of transcription, MYB domain transcription factor family	0.466435
At1g10170	NF-X1 type zinc finger family protein	RNA, regulation of transcription, putative DNA-binding protein	0.294611
At5g27310	Expressed protein	RNA, regulation of transcription, putative DNA-binding protein	0.516112
At5g01380	Transcription factor GT-3a	RNA, regulation of transcription, Trihelix, Triple-Helix transcription factor family	0.301192
At4g25380	Zinc finger (AN1-like) family protein	RNA, regulation of transcription, unclassified	0.201293
At5g63740	Zinc finger protein-related	RNA, regulation of transcription, unclassified	0.276842
At1g04500	Zinc finger CONSTANS-related	RNA, regulation of transcription, unclassified	0.76629
At2g37810	CHP-rich zinc finger protein, putative	RNA, regulation of transcription, unclassified	0.822544
At2g21900	WRKY family transcription factor, WRKY59	RNA, regulation of transcription, WRKY domain transcription factor family	0.246233
At1g66550	WRKY family transcription factor, WRKY67	RNA, regulation of transcription, WRKY domain transcription factor family	0.383213
At1g29860	WRKY family transcription factor, WRKY71	RNA, regulation of transcription, WRKY domain transcription factor family	0.510944
At4g22710	Cytochrome p450 family, CYP706A2	Secondary metabolism, flavonoids, dihydroflavonols	0.217096
At3g59740	Receptor lectin kinase 3	Signalling, receptor kinases	0.255826
At4g11470	Serine/threonine kinase-like protein	Signalling, receptor kinases	0.577696
At4g11480	Serine/threonine kinase-like protein	Signalling, receptor kinases	0.587375
At3g63350	Heat shock transcription factor-like protein	Stress, abiotic, heat	0.67223
At4g11170	Disease resistance protein (TIR-NBS-LRR class), putative	Stress, biotic	0.220672
At3g04220	Disease resistance protein (TIR-NBS-LRR class), putative	Stress, biotic	0.232285
At1g59620	Disease resistance protein (CC-NBS class), putative	Stress, biotic	0.287561
At5g51630	Disease resistance protein (TIR-NBS-LRR class), putative	Stress, biotic	0.327981
At3g44630	Disease resistance protein RPP1-WsB-like (TIR-NBS-LRR class)	Stress, biotic	0.368207

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Table 2. (*Continued*)

Gene ID	Annotation (acc. to TAIR)	Gene ontology (Mapman)[a]	Membership value
At2g26390	Serpin, putative/serine protease inhibitor, putative	Stress, biotic	0.516773
At5g41550	Disease resistance protein (TIR-NBS-LRR class)	Stress, biotic	0.616406
At1g02530	Multidrug resistance P-glycoprotein, putative	Stress, biotic	0.623524
At2g26380	Disease resistance protien-related (LRR)	Stress, biotic	0.711293
At4g14370	Disease resistance protein (TIR-NBS-LRR class)	Stress, biotic	0.824501
At5g14740	Carbonic anhydrase 2	TCA, carbonic anhydrases	0.278928
At2g04070	MATE efflux family protein	Transport misc.	0.45519
At2g04050	MATE efflux family protein	Transport misc.	0.507313
At1g12950	MATE efflux family protein	Transport misc.	0.702004
At3g17690	Cyclic nucleotide-binding transporter 2/CNBT2 (CNGC19)	Transport, cyclic nucleotide or calcium-regulated channels	0.296605
At4g11730	H^+-transporting ATPase-like protein	Transport, p- and v-ATPases	0.285986
At1g09930	Oligopeptide transporter OPT family protein, ATOPT2	Transport, peptides and oligopeptides	0.879645
At5g46480	Disease resistance protein (TIR class), putative	N/A	0.262639

[a] Ontology based on Mapman program (Usadel *et al*., 2005).

genes with a function in the biosynthesis of amino acids and secondary metabolites (terpene, glucosinolate, cytokinin, gibberellin) were also down-regulated.

Discussion

Among the *Arabidopsis* transcript profiling platforms, the most complete set includes approximately 26 000 DNA elements for known and hypothetical coding regions. It is based on 70-mer oligonucleotides. In several constantly improving versions this array has become a reliable tool (http://www.ag.arizona.edu/microarray) in the hands of skilled experimenters. The Affymetrix GeneChip platform with a slightly lower complexity, approximately 22 000 genes, has become a standard because it represents a closed system, shows ease of use, and includes customized analysis software. This comparison of data for genes represented on both platforms indicated a highly similar trend in gene regulation, where approximately 80% of the transcripts behaved similarly when analysed by the two platforms. In essence, both platforms provide comparable results.

Clustering methods have been widely used to analyse large gene-expression datasets. The most commonly used methods included hierarchical clustering, k-means clustering, and SOM (self-organization map) (Eisen *et al*., 1998; Sherlock, 2000; Toronen *et al*., 1999). Here, fuzzy k-means clustering, in combination with principal component analysis (PCA) (Gasch and Eisen, 2002), was used to analyse the publicly available Affymetrix *Arabidopsis* gene-chip data on abiotic stress, biotic stress, and hormone treatments. Using this clustering method, the most informative expression patterns were captured as centroids. Instead of following the fuzzy k-means protocol where genes belong to multiple clusters, each gene was assigned to the cluster to which it had the highest membership value, because the focus was on the overall regulation pattern instead of the behaviour of individual genes, while discarding genes without significant membership to any cluster in order to reduce chance or false assignments. An important consideration in fuzzy k-means clustering is the selection of the cluster number k. By choosing a higher k, higher distinction is possible. For this study, increasing k from 30 to 120 generated a large number of clusters with very few genes, while the large clusters chosen here split into 2 or 3 smaller clusters (data not shown). Overall, this clustering method was found especially useful when dealing with large microarray data set with multiple time points.

After comparisons across both array platforms, analyses were focused on the Affymetrix data generated by the AtGenExpress consortium. The standardized protocol and data format, together with the strict experimental procedure employed by the consortium team, made it possible to integrate the whole dataset. Using the data without filtering genes with low expression was possible because the expression pattern over multiple treatments with multiple time points, for most of the genes, revealed trends of regulation at all time points that were consistent and without fluctuations within specific treatments (Fig. 1; see supplementary Table 1 at JXB online).

The results, for the ~1500 most strongly salt-regulated genes, revealed an unexpectedly complex interaction network between *Arabidopsis* stress-signalling pathways. Of 680 salt-induced genes, fewer than 25% (171, C2) were strictly salt-specific. Strikingly, most of the remaining genes were also induced by at least two different biotic stress treatments (C0, C6, C8, C9) and, in addition, shared common regulation with other abiotic stresses. Based on this co-induction pattern, the salt-induced signalling pathways in *Arabidopsis* may be divided into four categories (Fig. 2). One cluster includes salt-responsive genes that are also induced by elicitors (C0 and C8). Then, salt and ABA treatment (C4 and C6), and salt and MeJA exposure (C9) form distinct groupings of genes. Only cluster C2 contains genes that specifically respond to the ionic component of salt stress.

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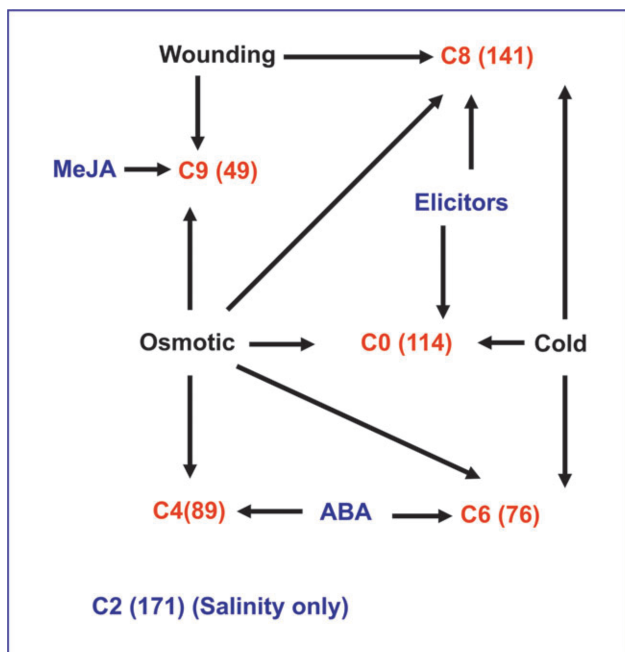

Fig. 2. Clusters of salt induced genes. The salt-induced genes could be divided into six clusters, C0, C2, C4, C6, C8, and C9 with the number of genes affected listed in parentheses. Represented are cross-talks and connections between high salinity and other factors that can represent stress. Induced expression is indicated by arrows connecting treatment and cluster.

The structure of the clusters, the types of genes within each cluster, and their appearance early or late in the time-courses identify functions that unite as well as distinguish different stresses. In cluster 8, for example, calcium signalling-related genes (e.g. *TCH3*) and ethylene-related *ATERF*s may represent early sensing and signalling components (Sistrunk *et al*., 1994; Fujimoto *et al*., 2000), as is the case for gene *CBL1*, which has been shown to mediate stress signalling without affecting ABA-related pathways (Cheong *et al*., 2003). Furthermore, cluster 6 includes ABA biosynthesis and signalling pathways, with high probability representing the general signal transduction chain related to osmotic adjustments. ABA has been recognized as a key regulator in abiotic stress responses (Gazzarrini and McCourt, 2001; Zhu, 2003; Sharp *et al*., 2004). A MPSS (massively parallel signature sequencing) study identified the ABA up-regulated genes in *Arabidopsis* (Hoth *et al*., 2002). Not surprising, the majority of the overlapping genes between the MPSS results and this analysis fell into clusters 2 and 4, the only two clusters that included ABA-responsive genes. Cluster 9 salt-induced genes are also highly induced by MeJA. Apart from the significant involvement of MeJA in biotic stresses, this hormone has also been reported to play a role during potassium starvation, which would make it an additional specific mediator of abiotic stress responses (Armengaud *et al*., 2004).

Of the 171 genes placed into cluster C2 most were induced only in roots, and they were specifically induced only by salt stress. This set of transcripts had not been observed before; it may constitute the ionic stress component of the *Arabidopsis* transcriptome. The reasons for this exclusivity might be that leaves, compared with roots, have a larger sodium storage capacity, or it may be a consequence of the relative higher concentration of sodium ions in the roots, as it has been reported in the wild type (Volkov *et al*., 2004), while *sos1* mutants deposit more sodium into the shoot system (Shi *et al*., 2002).

The SOS system, which has been established as an important defence mechanism potentially leading to salt tolerance (Zhu, 2003), is not represented among the strongly responding genes, because the SOS pathway seems to operate mainly at the protein modification and not the transcript level. However, among the early induced, ionic stress-specific genes in clusters C2 are most likely the components that, upstream of SOS, lead to the initiation and engagement of the SOS pathway. For example, 11 protein kinases of unknown function in this cluster represent a category that could make them candidates of early sensing or signalling.

In summary, it was demonstrated that large-scale microarray data can be used to recognize the cross-talk between different signalling pathways, providing information that will be useful in elucidating unknown signalling networks. Comparisons across different high-throughput transcript profiling platforms are possible and indicate the relative maturity of the procedures, in particular, of the statistical analyses and data representation tools. The general salt-stress signalling and response pattern, the multiple input elements, and a reliable, across-platform, identification of the many functionally unknown components, revealed by the analysis can provide guidance for forward genetic analysis of salt stress.

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