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Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress

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Abstract Barley (*Hordeum vulgare* L.) is a salt-tolerant crop species with considerable economic importance in salinity-affected arid and semiarid regions of the world. In this work, barley cultivar Morex was used for transcriptional profiling during salinity stress using a microarray containing ~22,750 probe sets. The experiment was designed to target the early responses of genes to a salinity stress at seedling stage. We found a comparable number of probe sets up-regulated and down-regulated in response to salinity. The differentially expressed genes were broadly characterized using gene ontology and through expression-based hierarchical clustering to identify interesting features in the data. A prominent feature of the response to salinity was the induction of genes involved in jasmonic acid biosynthesis and genes known to respond to jasmonic acid treatment. A large number of abiotic stress (heat, drought, and low temperature) related genes were also found to be responsive to salinity stress. Our results also indicate osmoprotection to be an early response of barley under salinity stress. Additionally, we compared the results of our studies with two other reports characterizing gene expression of barley under salinity stress and found very few genes in common.

Keywords Barley · Salinity stress · Microarray · Jasmonic acid

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Introduction

Salinity is one of the major obstacles to increasing crop productivity. Some of the most severe problems in soil salinity occur in arid and semiarid regions of the world. Besides these regions, salinity also affects agriculture in coastal regions and areas affected by low-quality irrigation water.

Barley (*Hordeum vulgare* L.) is widely grown in the arid and semiarid regions of the Mediterranean for forage purposes and as a grain crop (Al-Karaki 2001). It is rated as a moderately tolerant forage crop and a highly tolerant grain crop (Mass 1984; Francois and Mass 1999). Salt tolerance of most species including barley varies with plant growth stage. Barley is most sensitive to salinity at germination and young seedling stage, and exhibits increased tolerance with age. Salt stress for barley at seedling stage has been mainly attributed to ionic effects rather than to osmotic effects (Storey and Wyn Jones 1978). This is different from the germination stage, where osmotic effect is the primary stress component (Mano et al. 1996). No correlation was observed between salt tolerance at seedling vs germination stage in barley (Mano and Takeda 1995). Salinity tolerance at germination and seedling stages determines the stand density in the field under saline conditions. Most cereals including wheat (Mass and Poss 1989), rice (Heenan et al. 1988), and barley (Greenway 1965) are reported to be more salt-tolerant at germination than at seedling stage. Therefore seedling stage is relatively the most sensitive growth stage determining the plant stand density, which affects the final yield.

Salt tolerance of barley has been of interest for a long time and has resulted in a considerable body of data from studies using physiological (Cramer et al. 1990; Munns and

Rawson 1999), genetic (Mano and Takeda 1997; Ellis et al. 2002), and cytogenetic approaches (Forster et al. 1997). Recent molecular characterization of salinity stress in plants has indicated the involvement of multiple genes responsive to salinity. Large-scale approaches including microarrays (Ozturk et al. 2002; Ueda et al. 2004; Kawasaki et al. 2001; Seki et al. 2001) and differential display (Ueda et al. 2002) have been employed to identify genes responding to salinity stress in plants. Different microarray platforms have been used such as cDNA and oligonucleotide arrays (Duggan et al. 1999; Lipshutz et al. 1999). Two microarrays composed of spotted cDNAs that include drought- and salt-responsive clones have been used to investigate the expression of salt stress-induced genes in barley (Ozturk et al. 2002; Ueda et al. 2004). An oligonucleotide array with 22,495 probe sets became publicly available in 2003 (Close et al. 2004). The Barley1 GeneChip contains probe sets that were designed from expressed sequence tags (ESTs) generated from 84 cDNA libraries representing various developmental stages as well as biotic and abiotic stress treatments.

In the current work, we report the transcriptional response of barley cv. Morex to a gradually imposed salinity stress at the salt-sensitive seedling stage of the plant using Barley1 GeneChip. The experiment was designed to focus on the ion-specific effects of salinity on gene expression as opposed to osmotic shock effects.

Materials and methods

Plant culture and stress treatments

Barley cv. Morex was obtained from Dr. Patrick Hayes (Oregon State University, Corvallis) and propagated at the University of California (Riverside). Seeds were surface-sterilized by using bleach and Tween 20 (Sigma), washed several times with deionized water, and germinated on moistened filter paper in glass crystallization dishes for 2 days in darkness. The plants were grown in a greenhouse at George E. Brown Jr. Salinity Laboratory, USDA-ARS, at Riverside, CA, USA (33°58′24″N latitude, 117°19′12″W longitude) in the months of June and July 2003. Germinated seeds were transferred onto two circular plastic grids with cheese cloth sandwiched between the grids. The grids were placed on plastic tanks (volume, 25 l) containing aerated half-strength Hoagland's solution, with double iron (50 g l^{-1}). The pH of the solution was maintained within the range of 5–6.5 using concentrated sulfuric acid. Uniform plant density was maintained by transplanting seedlings from a replacement tank. Electrical conductivity, pH, and solution temperature were monitored daily. Tanks were topped daily with deionized water to replace losses due to evapotranspiration. Reflective insulation material was used to keep the nutrient solution temperature between 21 and 25°C.

Gradual salt stress was imposed starting on day 14 after transplanting until day 17 (Fig. 1). NaCl concentrations were brought up to 100 mM by increments of 25 mM NaCl per day. The NaCl concentration of 100 mM was chosen based on the electrical conductivity (EC_w) of San Joaquin Valley drainage water. $CaCl_2$ was added with NaCl to maintain a Na^+/Ca^{2+} concentration ratio of 10:1 on a molar basis. A final EC in the range of 14.5–15.5 dSm^{-1} was reached. Addition of NaCl in treatments and deionized water in controls was followed by stirring the solution in the tanks. The plants were harvested at 3, 8, and 27 h after reaching a final concentration of 100 mM NaCl. Seedlings were gently pulled out from the grids and then shoots were snap frozen and stored at −80°C. A total of 12 plants were

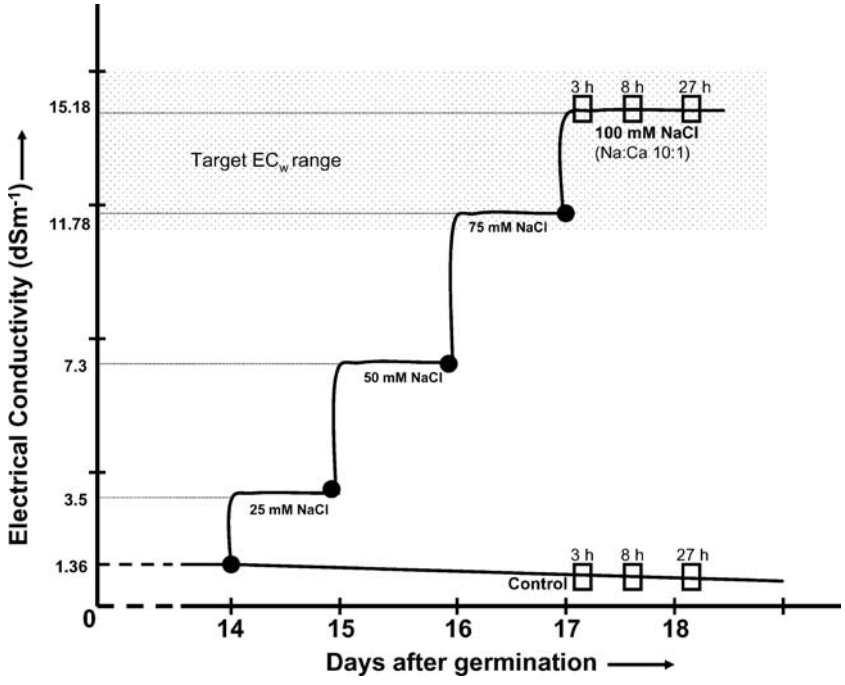

Fig. 1 Experimental set-up and imposition of salinity treatment of barley seedlings. Stress was imposed on 14-day-old plants in a stepwise manner. *Solid circles* on the graph represent the points of salt addition. The level of salinity after each addition of NaCl and $CaCl_2$ is shown in *y*-axis. The target salinity range found in highly challenged agricultural systems is *shaded*. The tissue harvest points for control and salt-stressed plants are indicated by *rectangular boxes*. The harvest for ion analysis was done 18 days after germination. Visual symptoms of salt damage were observed about 7 days after the initiation of salinity treatment

harvested per tank per time point to constitute a single biological replicate. The tissue from 12 plants was used for RNA extraction. The total RNA from three biological replicates was not pooled for array hybridization.

Plant ion measurement

Five plants per treatment were harvested for tissue ion analysis. Plants were washed with deionized water, dried in a forced air oven (70°C), then ground into fine powder. Shoot and root Na^+ concentrations were determined on nitric–perchloric acid digests by inductively coupled plasma optical emission spectrometry (ICP, Perkin-Elmer Co., Norwalk, CT, USA).

Target preparation/processing for GeneChip analysis

RNA samples were processed as recommended by Affymetrix, Inc. (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix, Inc., Santa Clara, CA, USA) at the DNA and Protein Microarray Facility at University of California, Irvine. Total RNA was initially isolated from frozen shoot tissue using TRIzol Reagent. RNA was purified by passage through an RNAeasy spin column (Qiagen, Chatsworth, CA) and on column DNase1 treatment. Eluted total RNAs were quantified with a portion of the recovered total RNA and adjusted to a final concentration of 1 μg μl^{-1}. All starting total RNA samples were quality-assessed prior to beginning target preparation/processing steps by running out a small amount of each sample (typically 25–250 ng $well^{-1}$) onto an RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA, USA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Single-stranded, then double-stranded, cDNA was synthesized from the poly(A)+ mRNA present in the isolated total RNA (10 μg total RNA starting material each sample reaction) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA, USA) and poly(T)-nucleotide primers containing a sequence recognized by T7 RNA polymerase. A portion of the resulting ds cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction (IVT), using the BioArray High-Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics, Inc., Farmingdale, NY, USA). Fifteen micrograms of the resulting biotin-tagged cRNA was fragmented to strands 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) to probe sets present on an Affymetrix Barley1 array (Close et al. 2004). The GeneChip arrays were washed and then stained (SAPE, streptavidin–phycoerythrin) on an Affymetrix Fluidics Station 400, followed by scanning on a Hewlett-Packard GeneArray scanner.

Data analysis

The scanned images were examined for any visible defects and then checked for the fitness of gritting. Chip images from four samples were not satisfactory, so these samples were hybridized to fresh chips. Satisfactory image files were analyzed to generate raw data files saved as .CEL files using default settings of GeneChip Operating Software (GCOS 1.2) (Affymetrix). We used a global scaling factor of 500, a normalization value of 1, and default parameter settings. The detection calls (present, absent, or marginal) for the genes were made by GCOS 1.2. Further analysis was performed using DChip software (Li and Wong 2001). DChip was set to import GCOS signals. The normalization of the chips was performed using an invariant set approach. For calculating the expression index of probe sets, we used the PM/MM model and opted for truncating the low expression values to the 10th percentile of the probe set signals called absent. The expression values were $\log_2$ transformed after calculating the expression index. We pooled the data from three biological replicate samples and enabled the measurement error feature in DChip while averaging the replicates. Use of the measurement error feature while pooling replicate arrays down-weighs unreliable expression values with large standard errors. DChip was used for comparative analysis of samples from salt stress and control treated plants. We considered a fold change of at least 1.5 ($\log_2$ 1.5=0.585) as an indication of significant change of gene expression for up-regulation or down-regulation.

To perform hierarchical clustering, we used the list of probe sets up-regulated at any of the three time points. Expression levels of the probe sets in the up-regulated list were standardized to have a mean of 0 and a standard deviation of 1 in all samples, and were then used to calculate the correlations between genes. The distance between two genes is defined as $1-r$, where r is the rank correlation coefficient between the standardized values of the genes across samples. The P value threshold of 0.001 was used for determining significant gene clusters.

Probe set annotations and gene ontology analyses

The probe sets which showed significant differential expression under salt stress treatment were annotated using HarvEST:Barley (Version 1.34) assembly 21 (harvest.ucr.edu). The output from HarvEST includes the best BLAST hit from the nonredundant (nr) database of NCBI http://www.ncbi.nlm.nih.gov), and best BLAST hits from rice (version 3) and *Arabidopsis* TIGR databases http://www.tigr.org). Besides a description of the best hit, output also includes the genome location (chromosome and basepair position) of the best BLAST hit gene models in rice and *Arabidopsis*. The software also provided information on which unigenes were represented by a particular probe set and which probe sets represented a given unigene.

For gene ontology (GO), we used the *Arabidopsis* gene model for each probe set. From the list of differentially expressed probe sets, we filtered the list of *Arabidopsis* gene models to remove redundancy and splice variants. These filtered gene model lists were then used to obtain ontologies from MIPS MatDB (mips.gsf.de/proj/thal/db) using the functional categorization feature along with *P* values for each category present in the input list.

Expression validation by semiquantitative RT-PCR

The expression profiles obtained from chip hybridizations were further validated by RT-PCR using the first strand cDNA synthesis from independently isolated RNA samples. A cDNA first strand was synthesized using Taq-Man Reverse Transcription Reagents (Applied Biosystems, Forster City, CA, USA; Ref: N808-0234) following the manufacturer's instructions. Two micrograms of total RNA was converted into cDNA. Each cDNA was diluted 40 times and 5 μl of cDNA was used for three-step PCR. The number of PCR cycles was optimized for the 14 genes validated. An 18s ribosomal RNA (forward: atgataactc gacggatcgc, reverse: cttggatgtggtagccgttt; cycles) was used as control for RT-PCR experiments. The genes selected for validation included up-regulated, down-regulated, and genes with no change in expression in response to salinity stress. All PCR were repeated two or three times for biological replicates at each sampling time point. The genes whose expression was validated were: SRF-type transcription factor (forward: ccaagcatgaggcaacaactg, reverse: cctatacgtgttgctacctgccag; 32 cycles), asparaginase (forward: gagaaccacaacaacaacggc, reverse: ttccacccatcac caatctcc; 34 cycles), delta-l pyrroline-5-carboxylate syn thetase (forward: gagaaccacaacaacaacggc, reverse: ttccacc catcaccaatctcc; 32 cycles), phosphoethanolamine methyl transferase (forward: ccaggaggattacgacgacatc, reverse: ttaca aagcgagtctcgctcg; 32 cycles), MtN3 (forward: gccttcaccttt ggcatccta, reverse: gctgagaaccaggaagaaggaca; 32 cycles), RD22 (forward: gttccaggtgctcaaggtgaa, reverse: actccgta caagctggcgata; 32 cycles), allene oxide synthase (AOS) (forward: tcatacatagccggtgcaggttt, reverse: cgacatgaacatcg agagcca; 30 cycles), HSP17.8 (forward: agaag gaggacgc caagtacgt, reverse: ggcactccaccactttatcaca; 34 cycles), selenium binding protein (forward: ttcttcgtggatttcgg ca, reverse: ccgtctgacaagtatttccagaca; 32 cycles), CCAAT-binding transcription factor (forward: tgatgcaacctagaa cagcgg, reverse: tcccgagtctgacaatcaatctc; 32 cycles), BFRUCT3 (forward: ggttgatcactccatcgtgga, reverse: caaa cattgcttgccggtc; 34 cycles), *O*-methyltransferase (forward: tccctcgtcccactatcatacc, reverse: aacctttccccccatttcg; 32 cycles), allene oxide cyclase (AOC) (forward: gcaacacacgga gattcattca, reverse: acag catgtacttcggcgacta; 32 cycles), NHX1 (forward: tacggtttt ctgcctctgtcaca, reverse: acaa catctggtcatactgccg; 32 cycles), and Na^+ transporter (forward: cctaccggcataaggagcagat, reverse: tttgctggaagatggag caa; 32 cycles).

Results

We analyzed the expression of ~22,400 transcripts in barley using the Barley1 GeneChip from Affymetrix. We specifically targeted the early seedling stage, which is highly susceptible to salinity stress with significant impact on yield. Because of the gradual nature and moderate level of salinity applied to the plants, no gross visual symptoms of stress were observed at the time of tissue harvest for expression analysis. However, we observed senescence in the older leaves after 1 week under stressed conditions. These plants survived the salinity stress and exhibited emergence of new leaves on the main shoot and tillering. These observations indicated that the level of stress achieved in our experiment was pertinent to the affected agricultural environments. We sampled shoot and root tissue from control and salt-stressed plants for Na^+ levels. The ion analysis data are presented in Table 1. A significant difference in Na^+ ion concentration was observed between controls and stressed plants in roots and shoot samples even after a relatively short period of salt exposure. Na^+ concentration in the root tissue was lower than in shoot tissue under salinity stress.

Genes responsive to salinity stress in barley

To identify the genes responding to salinity stress, we used DChip software with a fold change criterion of 1.5-fold as described in Materials and methods. The data from biological replicates were pooled, with consideration of measurement accuracy according to the recommendations of Li and Wong (2003). The measurement accuracy approach down-weighs the expression values with large standard errors (3 standard deviations), rather than uses a simple average of replicates. We decided to use this approach to compensate for the relatively high degree of variation inherent in experiments conducted under greenhouse conditions. The tissue was sampled for expression analysis from control and stressed plants at three time points (3, 8, and 27 h) after reaching the final salinity level around 15.2 dSm^{-1}. A total of 261 probe sets corresponding to 339 unigenes were induced in response to salinity treatment at any of the three time points. The number of

Table 1 Root and shoot Na^+ concentration from control and salinity-stressed plants from three replicate experiments

Replicate no.	Root control	Root stressed	Shoot control	Shoot stressed
1	132	550	65	945
2	200	587	179	879
3	169	782	176	869
Mean±SD	167±34	639 ±124	140±64	897±41

Five randomly selected plants from each treatment were used for ion analysis. Na^+ concentration is expressed as mmol kg^{-1} of dried tissue

probe sets down-regulated at any of the time points was 234 (311 unigenes). The overlap between the probe sets and their represented unigenes, up- or down-regulated at the three time points, is shown in Fig. 2a,b, respectively. We found a significant overlap (25 probe sets) among the genes induced at the three time points. Twenty-five probe sets represented 22 unique genes based on annotations (Table 2). Of these, two probe sets did not have a sequence match to the database. A somewhat equivalent overlap was also observed among the genes down-regulated at the 3-, 8-, and 27-h time points. The number of probe sets representing annotation-based unique genes was 13 (Table 3). Four of the probe sets did not have a sequence match to the database searched. Two obvious numerical features that can be seen in Fig. 2 are the relatively higher number of probe sets induced at 27 h and down-regulated at 8 h. It is pertinent to point out the lack of a one-to-one relationship between a probe set and the putative gene identified based on the annotation method described in Materials and methods. Often, multiple probe sets have the same annotation (BLAST hit to same rice or *Arabidopsis* loci) and hence are proposed to represent the same gene. Therefore the number of probe sets in Fig. 2 are likely an overestimate of the number of genes responding significantly to salinity stress in barley. Additionally, a single probe set can represent multiple unigenes (see Materials and methods). This results in a higher number of unigenes represented by a given number of probe sets (Fig. 2a,b). The expression values for differentially expressed probe sets at 3-, 8-, and 27-h time points are provided as electronic supplementary data sheets (S1, S2, and S3). The annotation of the up-regulated and down-regulated probe sets at the three time points is also available as electronic supplementary material (S4 to S9).

Biological processes impacted by salinity

In order to identify the broad biological themes represented by the set of differentially expressed genes responding to salinity stress in barley, we utilized the GO knowledge developed for *Arabidopsis*. To identify the functional shifts in the up- and down-regulated genes, we derived a nonredundant list of *Arabidopsis* loci based on best BLASTx hit of the responsive probe sets to the TIGR translated *Arabidopsis* genome database. There were 175 *Arabidopsis* loci representing up-regulated probe sets and 114 representing down-regulated probe sets. The functional categorization of each locus was generated using the feature available from MIPS MatDB, which currently curates 26,642 annotated *Arabidopsis* genes. The two major categories that were identified as significantly changing among the up-regulated probe sets were (1) cell rescue, defense, and virulence (2.11×10^{-11}), and (2) interaction with cellular environment (2.73×10^{-5}). Several subcategories significantly enriched in the salinity-induced gene list included stress response (2.93×10^{-6}); heat shock response (0.00086); disease, virulence, and defense (2.99×10^{-5}); detoxification (0.0072); cellular sensing and response (2.91×10^{-5}); and osmosensing (0.00024). Some of the subcategories from the down-regulated gene lists, showing significant shift based on *P* values relative to the categorized set of *Arabidopsis* genes, include disease, virulence, and defense (0.00052); defense-related proteins (0.00088); nucleus (1.90×10^{-5}); and organization of chromosome structure (3.38×10^{-6}). Complete lists of GO along with *P* values are provided for up-regulated and down-regulated gene lists as electronic supplementary data sheets S10 and S11. This functional analysis approach afforded enough resolution to filter out some groups that have been directly or indirectly associated with response of plants to stress. As a next step, we transitioned from a broad functional examination of the data to an approach involving expression-based clustering of coregulated genes.

Hierarchical clustering of salinity-induced genes

To identify groups of genes with similar expression patterns, we applied a hierarchical clustering algorithm to the list of genes induced at any of the time points in response to salinity stress. This analysis enabled us to identify several gene expression patterns characterizing different time points. Two of the clustering patterns are shown in Fig. 3a,b. Genes belonging to the cluster in Fig. 3a characterize a common response to salinity stress at

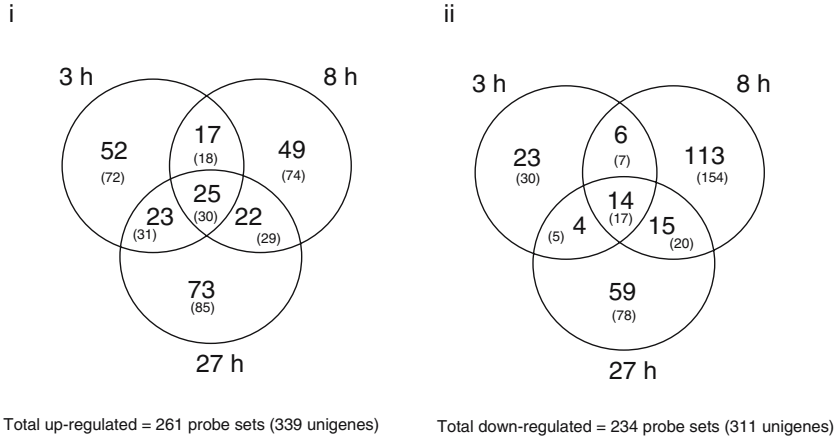

Fig. 2 Venn diagram of responsive probe sets at 3, 8, and 27 h. (**a**) Overlap among the salt-induced probe sets and corresponding "unigenes." (**b**) Down-regulated probe sets in response to salt stress at the three time points. Unigenes are consensus sequences of clusters and singletons derived from CAP3-assembled expressed sequence tags (EST) sequences used for the design of Barley1 GeneChip

Table 2 List of differentially expressed genes identified by microarray analysis of salinity-stressed barley plants which are up-regulated

Putative function	Probe set	*E* value	3 h	8 h	27 h
Family II extracellular lipase	Contig10206_at	6×10^{-48}	1.04	1.09	0.93
Ovarian fibroin-like substance-1	Contig10522_at	4×10^{-47}	1.56	1.5	1.21
Photosystem II 10 kDa polypeptide	Contig11477_at	5×10^{-56}	0.88	1.03	1.16
Expressed protein	Contig11993_at	5×10^{-36}	1.4	1.13	0.82
Calmodulin-2/3/5 (CAM5) (TCH1)	Contig1339_at	5×10^{-77}	0.85	0.76	0.88
Expressed protein	Contig13866_at	1×10^{-159}	1.08	0.97	0.98
Expressed protein	Contig15186_at	4×10^{-23}	1.45	1.13	1.71
Phosphoethanolamine *N*-methyltransferase 2	Contig2189_at	9×10^{-26}	1.11	1.14	0.84
Protease inhibitor/lipid transfer protein	Contig23122_at	1×10^{-35}	0.96	1.39	1.13
Acid phosphatase, putative	Contig2430_at	3×10^{-45}	1.0	0.94	1.33
Glycine-rich protein	Contig3198_s_at	7×10^{-32}	0.92	1.14	0.84
Polyamine oxidase, putative	Contig3212_s_at	0	1.32	0.86	1.3
delta 1-Pyrroline-5-carboxylate synthetase A	Contig3814_at	0	1.46	1.98	1.5
C-4 sterol methyl oxidase	Contig6208_at	1×10^{-116}	2.3	1.38	1.84
Myb family transcription factor	Contig6946_at	8×10^{-75}	1.13	0.79	0.8
Heat shock transcription factor family protein	Contig6968_at	1×10^{-45}	0.81	0.75	0.94
Protease inhibitor/lipid transfer protein	Contig7967_at	5×10^{-45}	1.24	1.18	0.99
Nodulin MtN3 family protein	Contig8708_at	3×10^{-72}	1.92	2.2	2.1
Arginine/serine-rich protein (SR45)	Contig9382_at	2×10^{-10}	2.31	1.23	3.06
No hit	HS05D20u_s_at	–	0.7	1.15	0.98
No hit	HVSMEc0014K14f	–	0.98	1.57	1.35
Sugar transporter	rbaal31j11_s_at	3×10^{-66}	1.4	1.92	1.19

The association between probe sets and the gene they represent is based on best BLAST hits to NCBI nr, TIGR Arabidopsis ,or TIGR rice databases. The fold change at the three time points sampled is expressed as $\log_2$. Fold changes smaller than 1.5-fold (~0.6-fold change in $\log_2$) are marked as (–) for nonsignificant changes. Probe sets that were redundant are included only once in this list

all three time points. Some of the probe sets which were commonly induced at all time points (Fig. 2a) were also found in this cluster. Examples of such genes include nodulin MtN3 family protein, heat shock transcription factor, calmodulin-2/3/5 (CAM5), and phosphoethanolamine methyltransferase, among others. Genes belonging to the cluster in Fig. 3b were expressed at higher levels at the 8-h time point in control as well as stressed samples relative to other time points. Interestingly, most of the genes in this cluster were related to stress. The stress-related genes included dehydrin 5, salt stress-induced ESI3, abscisic acid- and stress-induced protein, and a cold-regulated protein. It is noteworthy that 3- and 27-h samples were exactly 24 h apart, such that any circadian influence

Table 3 List of differentially expressed genes identified by microarray analysis of salinity-stressed barley plants which are down-regulated

Putative function	Probe set	*E* value	3 h	8 h	27 h
Organic cation transporter	Contig17575_at	1×10^{-98}	−1.09	−0.84	−1.05
No hit	Contig19515_at	–	−0.68	−0.89	−0.88
Peroxidase, putative	Contig2113_at	1×10^{-98}	−1.57	−1.99	−1.23
No hit	Contig21937_at	–	−0.97	−0.98	−1.15
Transcription factor MADS31	Contig24884_at	1×10^{-73}	–	−1.18	−1.29
No hit	Contig3185_s_at	–	−0.9	−1.42	−1.72
Protease inhibitor/lipid transfer protein	Contig3778_x_at	2×10^{-31}	−2.03	−1.31	−1.18
Expressed protein	Contig4544_s_at	1×10^{-7}	−0.74	−1.05	−0.8
L-Asparaginase	Contig8739_at	1×10^{-141}	−0.98	−0.78	−1.05
Hypothetical protein	Contig9923_at	9×10^{-10}	−0.85	−1.31	−0.85
No hit	HK04G05r_at	–	−0.81	−0.86	−0.8
Expressed protein	HU14G14r_s_at	6×10^{-22}	−1.25	−0.77	−1.58
Peroxidase	HVSMEm0005P05r2	4×10^{-27}	−1.3	−0.83	−2.39

The fold change at the three time points sampled is expressed as $\log_2$. Fold changes smaller than 1.5-fold (~0.6-fold change in $\log_2$) are marked as (–) for nonsignificant changes. Probe sets that were redundant are included only once in this list

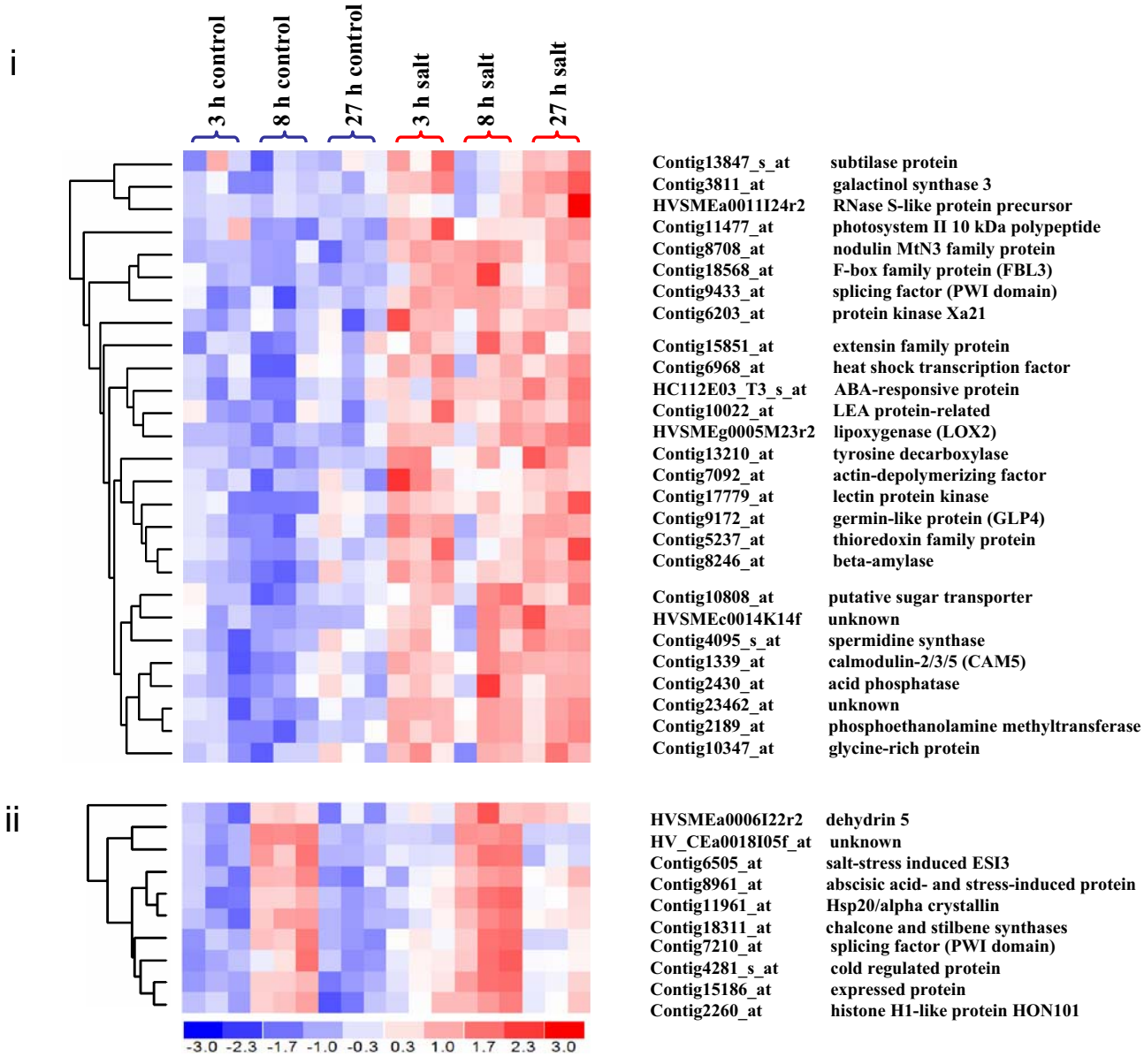

Fig. 3 Hierarchical clustering performed on salinity-induced probe sets. Two branches from the dendrogram are selected for discussion because of interesting patterns. Sets of three columns represent the standardized expression values of three biological replicates for each treatment. The expression values are standardized based on the mean value of the probe set across all 18 samples. For each probe set, *red*, *blue*, and *white* indicates increased, decreased, and equal expression level, respectively. These colors are representative of a relative scale (−3 to +3) derived from the mean of expression values divided by the standard deviation for a given probe set; they do not indicate the absolute values of expression levels used for identifying differential expression. A *P* value threshold of 0.001 was used for calling significant gene clusters. (**a**) Gene cluster of probe sets induced at 3, 8, and 27 h time points under salinity stress. (**b**) A set of probe sets strongly up-regulated at 8 h time point in control as well as stressed samples. Most of the genes represented by these probe sets are related to abiotic stress

on gene expression would be less of a factor when comparing these two time points than when considering also the 8-h time point.

We further studied the gene lists generated by this microarray work to identify any specific biochemical pathways or sets of genes known to have functional associations and responding to salinity stress in barley. A particularly noteworthy feature of the early salinity stress response of barley at seedling stage was the change in expression level of genes related to jasmonic acid (JA) biosynthesis and jasmonic acid-responsive genes (JRGs) (Table 4). We also found a large number of genes reported to be associated with various abiotic stresses such as low temperature, heat stress, and drought stress. Multiple genes associated with response of plants to biotic stress were also responding to salinity stress.

Table 4 List of differentially expressed genes identified by microarray analysis of salinity-stressed barley plants which are involved in jasmonic acid (JA) biosynthesis or known to respond to increased level of endogenous JA

Putative function	Probe set	E value	3 h	8 h	27 h
Allene oxide synthase	Contig3096_s_at	0	0.82	–	–
Lipoxygenase 1	Contig12574_at	3×10^{-61}	1.29	0.85	0.67
probable lipase	Contig6611_at	1×10−129	0.97	0.62	–
Lipoxygenase 2.1	Contig2306_s_at	0	0.71	–	–
12-Oxophytodienoate reductase (OPR2)	Contig2330_x_at	2×10−83	–	−0.97	–
Selenium binding protein	Contig2837_at	0	0.88	0.78	–
Glutathione *S*-transferase	Contig3295_at	1×10−121	0.89	–	–
23 kDa Jasmonate-induced protein 1	Contig1675_s_at	1×10−122	0.86	–	–
Glutamine-dependent asparagine synthetase 1	HV11O04r_s_at	0	0.86	–	–
alpha-Hordothionin	S0001100181F11F1	5×10−45	0.75	–	–
Protease inhibitor	HA10J11u_x_at	1×10−04	1.01	–	–
Lectin protein kinase	Contig17779_at	3×10−24	0.80	–	–
O-methyltransferase	Contig4910_at	1×10−125	0.86	–	1.34
Plant defensin-fusion protein (PDF2.5)	Contig15988_at	0.006	0.80	–	–
Protein phosphatase 2C	Contig13161_at	1×10−116	0.96	0.69	–
Thionin	Contig1579_s_at	5×10−75	−0.88	−0.63	–
Alcohol dehydrogenase (ADH)	Contig431_at	1×10−108	−0.91	−0.86	–

The fold change at the three time points sampled is expressed as $\log_2$. Fold changes smaller than 1.5-fold (~0.6-fold change in $\log_2$) are marked as (–) for nonsignificant changes

Salinity stress and jasmonic acid pathway

Jasmonic acid is an important stress hormone in plants. Plants synthesize JA when exposed to pathogens or other stress conditions. JA is the terminal product of the octadecanoid pathway. Genes involved in the biosynthesis of JA were prominently found to be responsive to salinity stress treatment in barley under our experimental conditions. The genes encoding enzymes involved in the biosynthesis which were up-regulated in stressed plants included lipase (phospholipase), lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) (Table 4). However, the enzyme 12-oxo-phytodieonic acid reductase, involved in converting 12-oxo-10,15(*Z*)-octadecatrienoic acid (OPDA) to OPC-8:0, is significantly down-regulated at the 27-h time point. The JA biosynthetic pathway is shown in Fig. 4a with responsive genes in bold face. We also found a number of known JRGs with altered expression under salinity stress. These included jasmonic acid-induced proteins (JIPs), hordothionins, *O*-methyltransferase (OMT), glutathione S-transferase, and selenium binding protein. We performed hierarchical clustering on the JA biosynthesis and JA-responsive genes that were identified from the gene lists. The resulting heat map is shown in Fig. 4b. Based on our observation of differentially expressed gene lists, we found that salt stress in barley induces JA pathway related genes and, not surprisingly, genes known to be induced by increased levels of endogenous JA. It appears that the induction of the JA pathway is a prominent characteristic of 3 h sampling time point.

Genes related to other abiotic stresses

On examination of the expression profiles of genes responding to salinity stress, we found a number of known abiotic stress related genes. The largest group of induced genes related to abiotic stress fall into the category of heat shock proteins (HSPs). HSPs are believed to facilitate growth and survival of plants under severe heat stress (Lee and Vierling 2000). The induction of HSPs was a prominent characteristic of the 8- and 27-h time points. Some of the genes induced at these two time points included HSP18.1 class I, small heat shock protein HSP17.8 class II, HSP81-1, and HSP101 among others. A HSP70/*dnaK* gene was up-regulated while a *dnaJ* gene was down-regulated at 8 h under stress. Genes encoding heat shock related proteins induced at all three time points included a heat shock transcription factor and a HSP20/alpha crystallin protein (Table 2).

We found some genes known to respond to other abiotic stress conditions including dehydration, osmotic stress, and exposure to low temperature. A probe set annotated as dehydrin 5 (HVSMEa0006I22r2) was induced at 3- and 27-h time points. Another dehydration-responsive gene, RD22 (basd2306_s_at), was induced at 3- and 27-h time points. The expression of the probe set HM09B02r_s_at, annotated as RD22, was down-regulated at the 8-h time point. A gene with late embryogenesis abundant (LEA) group 1 protein domain was also induced at the 27-h time point. Among the low temperature-induced genes, Contig6505_at, annotated as low temperature-induced protein blt101/salt stress-induced ESI3, was induced at the 3-h

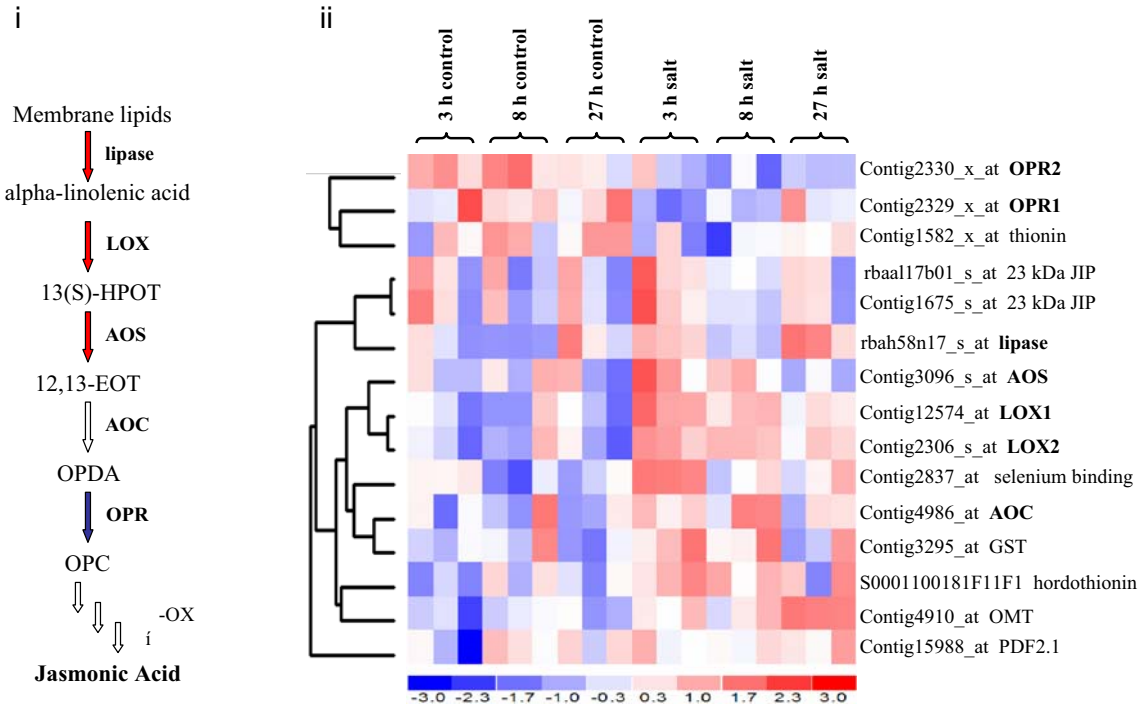

Fig. 4 Jasmonic acid (JA) biosynthesis related genes and known jasmonic acid responsive genes are induced by salinity treatment in barley. (**a**) Biosynthesis pathway leading to JA. The enzymes for which the genes are up- and down-regulated in response to salinity are symbolized by *red* and *blue arrows*, respectively. (**b**) Hierarchical clustering of JA-related genes and some genes known to respond to JA treatment. Gene names listed in *bold* are the ones involved in biosynthesis. *OPR* 12-Oxo-phytodienoic acid reductase, *AOS* allene oxide synthase, *AOC* allene oxide cyclase, *LOX* lipoxygenase, *GST* glutathione *S*-transferase, *JIP* jasmonic acid-induced protein, *OMTO*-methyltransferase, *PDF* plant defensin fusion protein. The method used for clustering is described in the legend to Fig. 3

time point. A cold-regulated protein 2 (Contig 1337_s_at) was induced at the 27-h time point. At 27 h, a CCAAT-binding transcription factor (CBF-B/NF-YA) protein was up-regulated. Phosphoethanolamine methyltransferase (Contig2189_at), a precursor of osmolyte glycine betaine and involved in choline biosynthesis, was commonly induced at all three time points. Contig4281_s_at represents a phosphatidylethanolamine-binding family protein that was up-regulated at 3- and 27-h time points. Another gene induced at all three time points and having a role in osmotic adjustment is delta 1-pyrroline-5-carboxylate synthetase. Among the genes down-regulated in response to salinity, a CBF1-like protein of barley, an osmotic stress responsive protein, and a calcineurin B-like (CBL) interacting protein kinase 2 (CIPK2) were identified at the 3-h time point.

Discussion

Experimental design and data analysis

In this work we profiled the transcriptome of a commonly studied barley genotype under salinity stress at the susceptible seedling stage using Barley1 GeneChip. This microarray is currently the platform with the largest number of barley genes that can be probed simultaneously. One of the key features of this experiment was the design of the salinity stress experiment. As saline soils tend to be highly variable, we decided to conduct the experiment in salinized nutrient solutions so that plants would experience a constant salinity level at the root zone. The stage of imposition of stress was the young seedling stage, which largely determines plant stand density in the salinity-affected fields and ultimately grain yield. A salinity level in the vicinity of 15 dSm^{-1} was imposed on the plants. This level of stress is similar to what is likely to be found in affected agricultural systems in the Central Valley of California. Munns (2002) has pointed out that a salinity level equivalent to 175 mM NaCl or higher, imposed as a shock, is likely to cause plasmolysis of epidermal root cells resulting in an artifactual flow of salt into the plant. Thus to avoid profiling the mRNA population responding to such an effect, we gradually imposed a salinity stress over a 4-day period. This also reduced the osmotic effect of salt stress, which is predominant during the early stages of stress imposition. Hence, with our experimental design we intended to target the ionic effects of salinity stress rather than the osmotic shock effects. To achieve this, we timed our sampling points based on the physiological status of plants in response to addition of salinity. We specifically targeted the reducing growth rate phase (3 h), which is attributed to water loss, growth rate recovery phase (8 h), and ion-specific responses (27 h). These time-based responses have been proposed by Munns (2002) for most plants including barley. In exploratory experiments pre-

ceding the experiment described here, we found the salinity increment ranging from 11 to 15 dSm^{-1} to be the range where visible symptoms of stress first appear.

Since the experiment was conducted under greenhouse conditions, we expected and observed a certain degree of variation in our data between biological replicates. Uncontrollable variables occurred in the greenhouse, such as changes in light intensity from cloud cover, the position of shadows cast by support architecture at different times of day, and surges in air flow from cooling fans responding to fluctuations in temperature. Therefore, we analyzed our data for identifying differentially expressed genes using the DChip feature where replicate arrays are pooled considering measurement accuracy to obtain average value for each probe set (Li and Wong 2003). This approach of data analysis results in the identification of differentially expressed genes with higher confidence levels.

Genes induced by salinity stress

In contrast to earlier studies, we did not find a very large number of genes responding to salinity stress in barley. This is likely a result of the gradual nature and moderate level of stress imposed in our experiment. The maximum numbers of genes were up-regulated in response to stress at the 27-h time point. This is roughly the period when the growth rate of plants under stress has recovered from short-term osmotic effects, resuming at a new lower level (Munns 2002). A large number of probe sets are down-regulated in stressed plants at the 8-h time point. However, this apparently higher number can be attributed in part to overrepresentation of probe sets for histones and heat shock proteins on the Barley1 GeneChip that were responding at 8 h. A similar report of high level of redundancy for a histone H4 (39 probe sets) was reported by Ibrahim et al. (2005), who attributed it to the overseparation of allelic sequences during stringent EST cluster assemblies described by Close et al. (2004). From a total of 261 probe sets induced at any time point, 22 probe sets did not have a BLAST hit to databases used for annotations. Some of these probe sets may represent genes whose sequences have significantly diverged from orthologs of related crop species and can be a potential starting points for exploration into the barley-specific response to salinity at the gene expression level.

Salinity-induced jasmonic acid response in barley

Jasmonic acid and its derivates are collectively called jasmonates (JAs) and are formed from OPDA, which belongs to the group of compounds called octadecanoids. Jasmonates play an important role in developmentally or environmentally induced changes in gene expression. In our experiment, genes involved in JA biosynthesis and JRGs respond conspicuously to salinity stress. A role of JA in adaptation of barley to saline conditions was proposed by Tsonev et al. (1998). They reported that pretreatment of barley seedlings with JA before the imposition of a salt stress results in the amelioration of salinity-induced inhibition of growth, photosynthesis, and RuBPC activity. In another physiological study, Maslenkova and Toncheva (1996) showed increased salinity tolerance of barley seedlings when treated with methyl-jasmonate. This observation was based on the measurement of photosynthetic parameters, and the authors proposed tolerance to result from increased stability of photosynthetic membrane structures upon treatment with JA. In a more recent study in *Arabidopsis* involving gene expression analysis of plants under K^+ starvation, JA biosynthesis and JRGs were induced as a prominent response to low K^+ (Armengaud et al. 2004). This finding is particularly interesting because high Na^+ concentrations are known to compete with and displace cellular K^+ from binding sites, a situation somewhat parallel to K^+ starvation induced in the *Arabidopsis* report. Based on our results alone, we cannot prove a direct role for JA in the salinity stress response in barley. However, it is possible that JA may be involved in ion homeostasis in the plants, considering its induction in response to both salinity stress and a nutritional deficiency.

Jasmonic acid may function as a "master switch" for stress-induced signaling pathway leading to changes in gene expression (Wasternack et al. 1998). However, JA also acts in coordination with other plant hormones such as ABA, ethylene, and salicylic acid. It has been suggested that JA and ABA affect gene expression in a synergistic manner through more or less independent signaling pathways (Ortel et al. 1999). In this light, our finding that three ABA-related genes, ABA-responsive gene (3 and 8 h), ABA-induced protein, ABA7 (8 h), and ABA- and stress-induced protein (27 h) up-regulated in response to salinity may be important. This indicates that the ABA signaling pathway is also activated along with JA pathway in response to salinity stress. This conclusion is consistent with another microarray study comparing the transcriptome of salt cress with *Arabidopsis* (Taji et al. 2004). These investigators suggested that JA and ethylene signaling maybe up-regulated in salt cress even under normal growth conditions.

Salinity regulated genes

In addition to genes related to specific biochemical pathways, we found other genes that were responsive to salinity stress. When viewed broadly, some of these genes fall into categories such as transporters and membrane proteins, cell wall-related proteins, transcription factors, and abiotic and biotic stress-related genes.

Shoot and root ion data indicate a significant increase in the Na^+ content when plants were grown in saline solution for a relatively short period (Table 1). Salt-tolerant species such as barley are known to accumulate Na^+ roughly at the rate of 5 mM day^{-1} (Rawson et al. 1988; Munns 2002). As the cytoplasm usually constitutes only 10% of the cellular volume, Na^+ is expected to accumulate at roughly 50 mM

day^{-1} in the cytoplasm if the ions are not transferred into the vacuoles (Munns 2002). Therefore, based on the shoot Na^+ concentrations in our experiment, we expected to detect up-regulation of sodium transporters in the shoot tissue. Surprisingly, none of the several well-characterized sodium transporters or antiporters was identified as significantly induced (at least 1.5-fold) in our experiment. However, one sodium transporter (Contig11770_at) was up-regulated 1.4-fold at the 3- and 27-h time points upon salinization (Fig. 5). We further investigated the expression of several antiporters present on Barley1. Examination of the raw as well as processed expression values of five antiporters indicated that all of the probe sets had a present call, but none of them were differentially expressed in response to salinity treatment at any time point. The expression level of *Nhx1* represented on the array as probeset Contig16875_at was confirmed by RT-PCR validation (Fig. 5). Our data indicating that antiporters are not differentially expressed in response to salinity stress are consistent with those of Fukuda et al. (2004), who reported that *HvNhx1* does not respond to salt stress in the shoot tissue of barley.

A number of probe sets with sequence matches to transporter molecules had a significant change in gene expression in response to salinity stress. An inward rectifying potassium channel (KAT1) was induced at the 3-h time point in response to salinity. KAT1 is structurally similar to animal *Shaker* K^+ channels and does not depend on external K^+ concentration for inward rectification (Latorre et al. 2003). This may have relevance to salinity tolerance in barley plants as it has been suggested that maintenance of a low Na^+/K^+ ratio is more important than low Na^+ in certain plant species (Gorham et al. 1997; Dubcovsky et al. 1996). Another gene induced at 3 h was the alpha-hordothionin precursor. A barley hordothionin treatment was reported to induce rapid ion fluxes (Ca^{2+} and K^+) and alter lipid bilayers in *Neurospora crassa* (Thevissen et al. 1996). In our experiment, we found a decreased shoot and root K^+ concentration in salinity-stressed barley (data not shown). Other transporters included a nitrate transporter, down-regulated at 8 h, and a sugar transporter molecule up-regulated at all three time points. Another molecule whose expression was commonly repressed at all time points was the organic cation transporter.

We found several known drought-, low temperature-, and salinity stress-induced genes from our expression data. Osmotic and oxidative stresses are the likely common components of these abiotic stresses (Chinnusamy et al. 2004). A family of transcription factors, known as C-repeat binding factors (CBFs) or dehydration responsive element binding factors (DREBs), are known to improve freezing, drought, and salt tolerance (Hasegawa et al. 2000). A gene encoding for a CBF (Contig8396_at) was induced at the 27-h time point. Expression level of barley HvCBF1, however, is down-regulated by salinity stress at the 3-h time point. HvCBF1 contains an AP2 DNA-binding domain, highly homologous to the cold, inducible *Arabidopsis* CBF/DREB1 proteins (Xue 2003).

Another aspect that has emerged more recently is the central role of calcium signaling in response to various abiotic stresses (Knight and Knight 2001). One level of regulation of calcium signaling is achieved through calcium binding proteins such as calmodulins (CaM) and calcineurin B-like (CBL) proteins (Kudla et al. 1999; Zielinski 2002). A probe set annotated as calmodulin (CAM5)/touch-induced protein (TCH1) was induced at all three time points. However, a CBL-interacting protein kinase 2 (CIPK2) was down-regulated at the 3-h time point. The CIPK2 protein is known to interact strongly with CBL3 in *Arabidopsis* (Kim et al. 2000).

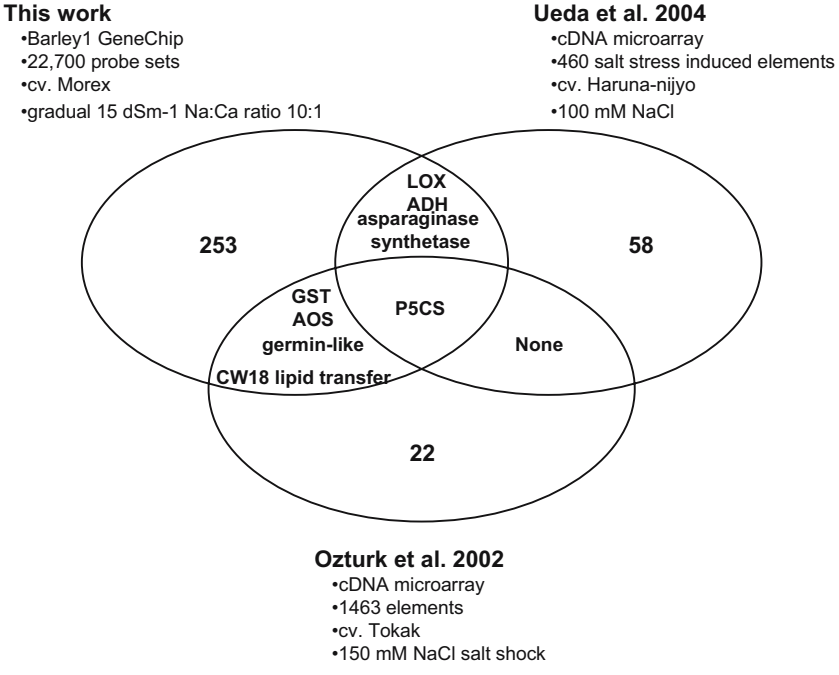

Fig. 5 Induced genes in barley under salinity stress in studies by Ozturk et al. (2002), Ueda et al. (2004), and this work. The total number of genes reported to be induced in response to salt stress in the shoot tissue for each work is provided on the non-overlapping section of the Venn diagram. The three experiments used different barley genotypes, plant age at sampling, and mode of salinity imposition. The platform used for differential expression analysis is also different in all three studies. *LOX* Lipoxygenase, *ADH* aldehyde dehydrogenase, *GST* glutathione *S*-transferase, *AOS* allene oxide synthase, *P5CS* delta-1-pyrroline-5-carboxylate synthetase

High salinity levels result in a change in external osmotic potential. A universal response to such changes in osmolarity of plants is the increase in levels of osmolytes in an effort to maintain a continued supply of water (Hasegawa et al. 2000). In our data we found the expression level of delta-pyrroline-5-carboxylase synthase (P5CS) increase in stressed samples at all time points. P5CS is the rate-limiting enzyme for accumulation of proline in plants (Kavi Kishor et al. 1995). Proline levels are known to increase in response to salinity stress in many plants. Another osmoprotectant known to confer tolerance to plants against salinity and drought is glycine betaine (Rhodes and Hanson 1993). Glycine betaine in certain plants is manufactured by the two-step oxidation of choline, a vital metabolite in plants required for the manufacture of membrane phospholipids phosphoatidycholine (Rhodes and Hanson 1993). The key enzyme of the plant choline biosynthesis is phosphoethanolamine *N*-methyltransferase (McNeil et al. 2001). The probe sets representing phosphoethanolamine *N*-methyltransferase were up-regulated at all time points, indicating an increase in choline and likely glycine betaine levels in barley in response to salinity. Based on these data, osmoprotection appears to be one of the mechanisms triggered in barley as a relatively early response.

Large-scale expression analysis of barley under salinity stress

Gene expression data for early response to salinity stress in barley has been previously reported (Ozturk et al. 2002; Ueda et al. 2004). We were interested in comparing the results from our experiment with genes identified by these two studies to examine any consensus responses to salinity stress from these investigations. We compared the lists of genes that were reported as differentially expressed with our combined list of genes induced at any time point. Surprisingly, very few common genes were identified based on annotations from the comparison. Some of the genes shared by at least two studies are shown in Fig. 6. The gene-encoding P5CS is the only gene found to be commonly up-regulated in all three studies. We found some genes that behaved differently in our experiment as compared to Ozturk et al. (2002). Genes encoding a DnaJ protein and a pathogenesis-related protein (PR-1a), reported as up-regulated by Ozturk et al. (2002), were down-regulated in our study The small overlap in the number of genes identified by all three studies is very curious. One possible explanation might be the relatively small number of genes previously probed—1,463 and 460 by Ozturk et al. (2002) and Ueda et al. (2004), respectively—compared to roughly 22,400 probed by the Barley1 GeneChip. Additionally, in all three studies, different genotypes of barley were used and the tissue was sampled at different time points after germination, and experimental differences can be critical. As evident from the current work, even a single genotype can present a strikingly different transcriptome when sampled at different time points within a short period (i.e., 3, 8, and 27 h). The elements spotted on the array used by Ueda et al. (2004) were characterized as differentially expressed under long-term salinity stress. Assaying a transcript population at early stages of salt stress may be one of the reasons for the identification of a small number of up-regulated genes. Ueda et al. (2004) used a salinity level more comparable to our experiment. However, Ozturk et al. (2002) employed a higher level of salinity (150 mM) and imposed the stress as a salt shock. Considering the differences in experimental design and methods of transcriptome analysis, it is perhaps not surprising that there are so few consistencies.

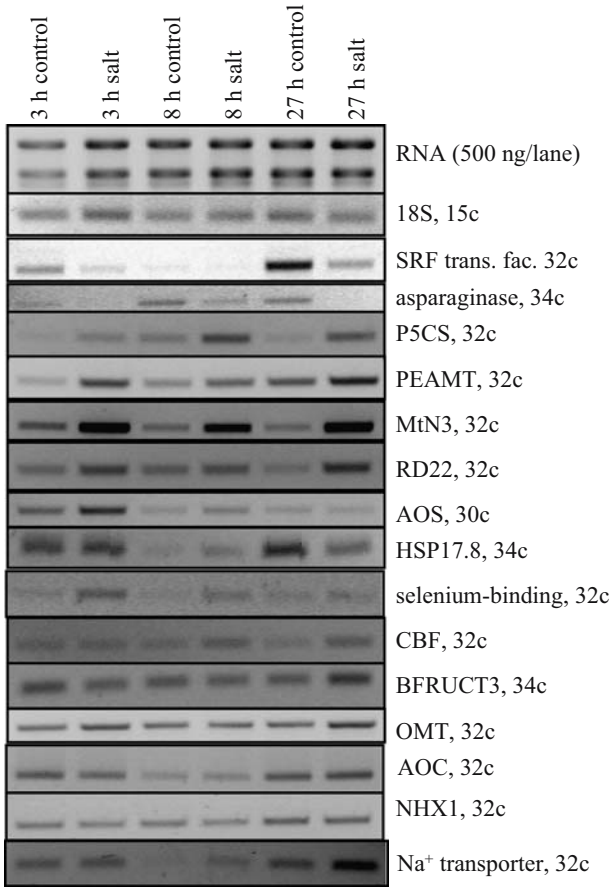

Fig. 6 RT-PCR analysis independently confirms array results. Semiquantitative RT-PCR was performed on key transcripts using RNA from biological replicates for all three time points. The gel image illustrates transcript abundance for 14 genes. The number of PCR cycles (c) used for each gene is listed next to the gene name. 18s ribosomal RNA was used as control. *P5CS* delta-1-Pyrroline-5-carboxylate synthetase, *PEAMT* phosphoethanolamine methyltransferase, *MtN3 Medicago truncatula*, nodulin, *AOS* allene oxide synthase, *HSP* heat shock protein, *CBF* CCAAT-binding transcription factor, *BFRUCT3* beta-fructofuranosidase 3, *OMT O*-methyltransferase, *AOC* allene oxide cyclase, *NHX* Na^+/H^+ antiporter

Conclusion

In this work we aimed to study the transcriptome of a barley genotype under salinity stress imposed in an agronomically relevant manner using Barley1 GeneChip.

One of the key features of the response to salinity was the induction of jasmonic acid pathway genes and genes responsive to JA and ABA. In addition, we also found a number of abiotic stress-related genes induced by salt stress, supporting the current idea of cross talk among certain components of abiotic stresses like heat, low temperature, and dehydration stress. In future studies we will address the expression level response of multiple genotypes of barley under salinity stress. We will also attempt to identify potential transcriptional differences resulting in variation in salt tolerance among different genotypes of barley.

Data availability

All expression data are available through the Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ under platform GPL1340, Series GSE3097. The list of significantly responsive probe sets along with annotations and fold changes is available as electronic supplementary Tables 3–8. Enhanced annotation for all Barley1 probe sets is available through HarvEST Barley (version 1.34) at http://www.harvest.ucr.edu.

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