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Large-scale expression profiling and physiological characterization of jasmonic acid-mediated adaptation of barley to salinity stress

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ABSTRACT

Barley (*Hordeum vulgare* L.) is a salt-tolerant member of the Triticeae. Recent transcriptome studies on salinity stress response in barley revealed regulation of jasmonic acid (JA) biosynthesis and JA-responsive genes by salt stress. From that observation and several other physiological reports, it was hypothesized that JA is involved in the adaptation of barley to salt stress. Here we tested that hypothesis by applying JA to barley plants and observing the physiological responses and transcriptome changes. Photosynthetic and sodium ion accumulation responses were compared after (1) salinity stress, (2) JA treatment and (3) JA pre-treatment followed by salinity stress. The JA-pre-treated salt-stressed plants accumulated strikingly low levels of Na^+ in the shoot tissue compared with untreated salt-stressed plants after several days of exposure to stress. In addition, pre-treatment with JA partially alleviated photosynthetic inhibition caused by salinity stress. Expression profiling after a short-term exposure to salinity stress indicated a considerable overlap between genes regulated by salinity stress and JA application. Three JA-regulated genes, arginine decarboxylase, ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) activase and apoplastic invertase are possibly involved in salinity tolerance mediated by JA. This work provides a reference data set for further study of the role of JA in salinity tolerance in barley and other plants species.

Key-words: photosynthesis; salt stress.

INTRODUCTION

Under field conditions, plants are challenged by multiple stresses, both abiotic and biotic. Abiotic stresses such as drought, high and low temperatures, and salinity, are major factors limiting crop productivity. In the field, plants usually experience more than one stress at a given time. To survive these unfavorable conditions, plants employ adaptive tolerance mechanisms. These mechanisms are often triggered by signalling pathways, which have components shared by multiple stress conditions (Xiong, Schumaker & Zhu 2002). Evidence for common components (usually sensory and regulatory genes) has been provided by studies using transgenic plants where the expression of a single gene is amplified or attenuated. Such plants demonstrate greater tolerance or sensitivity to multiple stresses such as drought, salinity and cold than their wild-type counterparts (Kovtun *et al*. 2000; Chen & Murata 2002; Rizhsky, Liang & Mittler 2002). Additionally, microarray studies have revealed the extent of transcriptional overlap among abiotic stresses 1Kreps *et al*. 2002; Ozturk *et al*. 2002; Seki *et al*. 2001).

The interaction among signalling pathways is not limited only to abiotic stresses, but extends to responses against biotic challenges such as pathogens, herbivory and wounding (Knight & Knight 2001). Phytohormones such as abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid (SA) appear to be critical components of complex signalling networks and are being incorporated into current models of stress response (Kunkel & Brooks 2002; Zhu 2002). Increased levels of ABA in response to salinity are known to induce genes, which alleviate salinity and osmotic stress (Serrano & Gaxiola 1994). Additionally, Lehmann *et al*. (1995) reported increased levels of ABA and JA in barley plants under osmotic stress. Osmotic stress is a component of salinity stress that disturbs the water balance of plants. The role of ethylene-mediated biosynthesis of JA in the wounding response has been reported in *Arabidopsis* (Laudert & Weiler 1998). Exposure to increased ethylene induced the expression of the biosynthetic gene allene oxide synthase (AOS). More recently, tissue-specific co-activation of JA and ethylene-related genes during programmed cell death (PCD) was suggested by Sreenivasulu *et al*. (2006). Interactions between abiotic stress and wounding responses have been demonstrated by a large-scale microarray study recording expression changes in response to wounding (Cheong *et al*. 2002). These researchers have reported a number of salinity stress and JA-regulated genes induced by wounding.

Barley is rated among the salt-tolerant crop species in the tribe Triticeae (Shannon 1985; Munns 2005). Response of barley to JA and osmotic/salinity stress treatment has been

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the focus of several studies (Maslenkova, Miteva & Popova 1992; Lehmann *et al*. 1995; Tsonev *et al*. 1998; Ortel *et al*. 1999). Tsonev *et al*. (1998) reported that pre-treatment of barley plants with JA before salinization ameliorated the inhibitory effects of salinity on growth when compared to a direct salt stress. Dombrowski (2003) reported a wounding–JA salinity interaction in tomato, where salt stress induced wound-related genes through the activation of the octadecanoid pathway. Salt stress also increases the JA levels in roots of rice plants and in leaves of *Iris hexagona* (Moons *et al*. 1997; Wang, Mopper & Hasenstein 2001). Most of these studies however, focus on the osmotic stress component of the salt stress usually involving the shock treatment with sudden increase in salt in the growth medium of whole plants or floating of barley leaf discs in salt solution. The ion-specific component of the salinity stress in relation to JA over a period of time remains unexplored.

We have previously reported that genes involved in JA biosynthesis show increased abundance during salt stress (Walia *et al*. 2006). In that study, we hypothesized that JA plays a role in salt stress adaptation in barley. Here we test that hypothesis by applying JA to barley plants and by observing its effect on salinity tolerance. In addition, we also examined the extent of interaction as indicated by gene expression overlap between JA and salinity stress response of barley using transcriptome profiling.

MATERIALS AND METHODS

Plant material and experimental conditions

Barley seeds [*Hordeum vulgare* L. cv. Golden Promise (spring barley)] were initially provided by Peggy Lemaux (University of California, Berkeley). Seed stocks were multiplied in the field at the University of California, Riverside. Seeds were surface sterilized using bleach and Tween 20 (Sigma), washed several times with deionized water and germinated on moistened filter paper in glass crystallization dishes for 2 d in darkness (22–25 °C). The plants were grown in a greenhouse at US Salinity Laboratory, US Department of Agriculture-Agricultural Research Service (USDA-ARS), at Riverside, CA, USA (33°58′24″N, 117°19′12″W) in September and October, 2004. Germinated seeds were transferred onto two circular plastic grids with cheesecloth sandwiched between the grids. The grids were placed on plastic tanks (25 L volume) containing aerated half-strength Hoagland's solution, with double iron (50 gL^{-1}). The air temperatures ranged from 31 to 40 °C during daytime and from 17 to 26 °C during night-time. The light averaged 1150 μmol m^{-2} s^{-1}. The pH of the solution was maintained within the range of 5.0–6.5 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 caused by evapotranspiration. Reflective insulation material was used to keep the nutrient solution temperature between 21 and 25C.

The JA pre-treatment was initiated on day 8 after germination by transferring the tank into an adjacent greenhouse maintained at similar conditions and by adding JA (J2500; Sigma-Aldrich, St. Louis, MO, USA) dissolved in water to a final concentration of 12 μM in the tank (Fig. 1). The transfer of the tank was carried out to avoid JA volatiles from affecting the expression of the control and salinity-stressed plants. On day 10 after germination, a salinity stress of ~ 18 dS m^{-1} was imposed gradually in four equal steps on the plants pre-treated with JA and on a tank in the first greenhouse. $CaCl_2$ was added with salt to maintain a molar ratio of Na^+ : Ca^{2+} at 10:1 on molar basis. $CaCl_2$ was added to prevent salt-induced Ca^{2+} deficiencies in the plants. On day 12, another tank with non-salinized plants was transferred to the second greenhouse and treated with JA. On day 14 after germination, crown and growing point tissue from ~ 12 plants from each treatment was harvested and snap frozen for RNA extraction. Therefore, tissue material from ~ 12 plants per treatment constituted a single replicate. The experiment was conducted in two independent biological replicates.

Phenotypic measurements

Net photosynthetic rate per unit area, stomatal conductance to CO_2 (g_c) and transpiration rate of the youngest fully expanded leaf were measured between 1000 and 1200 h on day 14 after planting using a Li-Cor 6400 Photosynthesis System (Li-Cor Biosciences, Lincoln, NE, USA). The following conditions for leaf gas measurements were used: photosynthetic photon flux density, 1200 μmol m^{-2}; chamber CO_2 concentration, 380 μmol CO_2 mol^{-1}; leaf temperature, 27 °C; and chamber vapour concentration, 20 mmol H_2O mol^{-1}. Twenty-four plants per treatment were harvested for fresh weights and root length measurements on day 14. Shoot tissue from eight plants was obtained for each treatment for ion analysis. Plants were washed with deionized water, dried in a forced air oven (70 °C) then ground into fine powder. Shoot Na^+ concentrations were determined on nitric-perchloric acid digests by inductively coupled plasma (ICP) optical emission spectrometry (Perkin-Elmer Co., Norwalk, CT, USA).

Target preparation and processing for GeneChip analysis

RNA samples were processed as recommended by Affymetrix Inc. (Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix Inc., Santa Clara, CA) at the Core Instrument Facility at University of California, Riverside. Total RNA was initially isolated from frozen shoot tissue using TRIzol reagent. The RNA was purified by passing through an RNAeasy spin column (Qiagen, Chatsworth, CA, USA) and on-column DNaseI treatment. Eluted total RNAs were quantified with a portion of the recovered total RNA and adjusted to a final concentration of 1 μg/μL. Labelling and hybridization were performed at the Core

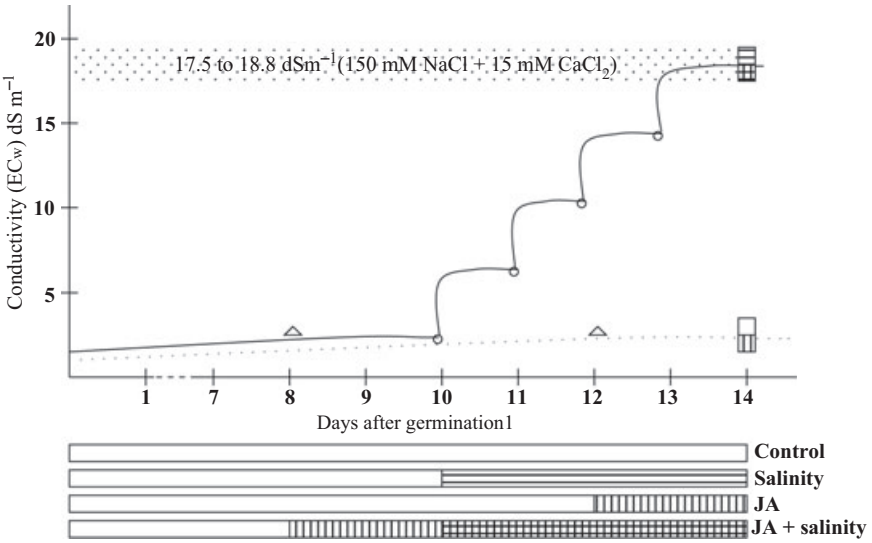

Figure 1. Experimental design of salinity stress and jasmonic acid (JA) treatments. Salinity stress was imposed 10 d after germination (see horizontal bars). A final salinity level of ~ 18 dS m^{-1} (150 mM salt) was reached by a four-step addition of salt and $CaCl_2$. A Na : Ca molar ratio of 10:1 was maintained during salt additions. One set of plants was pre-treated with JA on day 8, followed by a salinity stress on day 10 as previously described. JA treatment was applied on day 12 to plants in another tank. The plants were moved to the second greenhouse for the rest of the experiment before application of JA in both treatments. Conductivity in the control tank and JA-treated tanks is represented by a dotted line, and the salinized tanks by a solid line. Circles represent addition of salts; triangles represent JA application (12 μM), and squares represent the harvest time points. The crown and growing point of the main shoot were harvested for expression analysis 14 d after germination. EC_w, electrical conductivity.

Instrumentation Facility at the University of California, Riverside. 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) 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 that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded 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 labelling kit (T7) (Enzo Diagnostics Inc., Farmingdale, NY, USA). Fifteen micrograms of the resulting biotin-tagged cRNA was fragmented to strands of 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 5 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 [streptavidin-phycoerythrin (SAPE)] on an Affymetrix Fluidics Station 400, followed by scanning on a Hewlett-Packard GeneArray scanner (HP, Palo Alto, CA, USA).

Data analysis

The scanned images from GeneChips, two independent biological replicates of the experiment, were examined for any visible defects. 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 carried out using DChip software (Li & 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 Perfect Match (PM) model. After expression values were computed, genes with extremely low values were assigned a value equivalent to the average value of the lowest 10th percentile of all the genes that were called absent. This step prevents the overestimation of fold changes for very weakly expressed genes. The expression values were $\log_2$ transformed after calculating the expression index. For comparative analysis of samples from controls, salt stress, JA treated and JA-pre-treated salt stress samples, we used a two-pronged approach. First comparison was performed using DChip software itself where we considered at least a twofold change as an indication of significant change of gene expression for up-regulation or down-regulation. A second comparison for identifying differentially expressed genes was carried out by Significance Analysis of Microarrays (SAM) software

(Tusher, Tibshirani & Chu 2001) using two-class unpaired analysis with 1000 permutations, > 2-fold change and a sliding scale for false discovery and delta values. For this, the expression values of all the ~ 22 700 probe sets were imported into SAM from DChip. SAM assesses the false discovery rate, and provides a statistical significance *q* value (corrected for multiple comparisons). At least 92% of the induced genes called significant by SAM were also identified by DChip. We used this robust set of genes identified by both criteria (intersection of SAM and DChip) for discussion in the present work. The same data pre-processing and analysis strategy was implemented for the *Arabidopsis* methyl jasmonate (MeJA) treatment data set generated by Hideki Goda, Shigeo Yoshida and Yukihisa Shimada for AtGenExpress Consortium and obtained from The Arabidopsis Information Resource (TAIR).

For performing hierarchical clustering, expression levels of selected probe sets were standardized to have a mean of 0 and SD of 1 in all samples and were then used to calculate 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 analysis

The probe sets, which showed significant differential expression under salt-stress treatment, were annotated using HarvEST:Barley (version 1.34) assembly 21 (http://harvest.ucr.edu/). The output from HarvEST includes the best Basic Local Alignment Search Tool (BLAST) hit from the non-redundant (nr) database of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), and best BLAST hits from rice (version 3) and *Arabidopsis* databases of The Institute for Genomic Research (TIGR) (http://www.tigr.org/). Besides a description of the best hit, output also includes the genome location (chromosome and base pair position) of the best BLAST hit gene models in rice and *Arabidopsis*. The annotations for probe sets from Affymetrix 25 K *Arabidopsis* GeneChip were obtained from the microarray elements search tool available at TAIR web site.

Expression validation by semi-quantitative RT-PCR

Some of the expression profiles obtained from chip hybridizations were further validated by semi-quantitative RT-PCR using the first strand cDNA synthesis from RNA samples used for GeneChip hybridizations (Fig. 2). 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-fold, 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, atgataactcgacggatcgc; reverse, cttggatgtggtagccgttt; cycles) was used as control for RT-PCR experiments. The genes selected for validation included those with increased, decreased or unchanged abundance in response to the treatments. The primer sequences for the genes validated are listed in Supplemental Table S1.

RESULTS

Phenotypic responses

To determine if pre-treatment with JA could mitigate salinity stress in our system, we measured the level of relative stress induced by salinity, JA and JA-pre-treated salinity treatments. For this, we focused on two physiological components, namely Na^+ ion accumulation and photosynthesis, which are believed to be involved in the salt adaptation response of glycophytes. The shoot Na^+ concentrations were measured 14, 21 and 28 d after germination (Table 1). During this period, the JA-pre-treated salinity-stressed plants maintained a lower Na^+ concentration compared with salinity-stressed plants when measured on day 21($P = 0.007$) and day 28 ($P = 0.0002$). No difference in Na^+ accumulation was observed on day 14 (1 d after reaching the final salt level).

Because increasing salt levels can affect carbon fixation, we measured the net photosynthetic rate of the youngest fully expanded leaf on day 14. The photosynthetic rate declined in response to all three treatments when compared to control plants (Table 1). JA-treated plants maintained a photosynthesis rate only slightly lower than that of control plants. Further analysis indicated that the decline in the net rate of photosynthesis was significantly less in JA-pre-treated salinity-stressed plants (~ 18% reduction) relative to salinity-stressed plants with no prior JA treatment, where a decrease of 40% was observed. Salinity stress reduced the stomatal conductance by 67%. Transpiration correlated well with stomatal conductance values. Taken together, these data indicate that JA treatment prior to salinization can partially alleviate the effects of salinity stress in barley, at least with respect to photosynthesis.

Gene expression analysis

Having established that a JA pre-treatment before salinity stress improves some physiological responses thought to be involved in salt tolerance of the barley plants, we decided to conduct a transcriptome analysis. The main objectives were to (1) measure the extent of common regulation between salinity stress and JA treatment, and (2) to identify genes that are potentially involved in the improved salt tolerance of pre-treated barley. Transcriptome analysis was performed after exposure of the plants to salinity stress, JA and JA pre-treatment preceding salinity stress (Fig. 1). We employed the Barley1 GeneChip (Close *et al*. 2004) to

Table 1. Phenotypic responses of barley to salinity stress, jasmonic acid (JA) treatment and JA pre-treatment plus salinity stress

Shoot Na^+ concentration measured from barley plants 14, 21 and 28 d after germination[a]			
Treatment	Day 14 (mmol kg^{-1} DW)	Day 21 (mmol kg^{-1} DW)	Day 28 (mmol kg^{-1} DW)
Control	44.4 ± 4.3	76.5 ± 3.6	63.5 ± 7.5
Salinity	662.7 ± 55.7	1462.2 ± 151.6	1572 ± 87.8
JA	55.6 ± 11.5	62.5 ± 1.6	53.6 ± 5.2
JA + salinity	584.2 ± 39.7	1160.7 ± 62.6	1196 ± 97.5

Photosynthetic parameters measured on youngest fully expanded leaf of 14-day-old barley seedling[b]			
Treatment	Photosynthesis (μmol m^{-2} s^{-1})	Conductance (mol H_2O m^{-2} s^{-1})	Tr (mmol H_2O m^{-2} s^{-1})
Control	21.3 ± 2.9	0.43 ± 0.10	6.58 ± 2.06
Salinity	12.7 ± 1.52	0.14 ± 0.03	2.30 ± 0.41
JA	19.4 ± 1.18	0.57 ± 0.08	8.11 ± 0.74
JA + salinity	17.3 ± 1.42	0.24 ± 0.04	3.65 ± 0.54

[a]Mean values of four replicates ±SE. Each replicate for ion analysis constituted two plants. The JA and JA + salinity treatments were significantly different ($P < 0.001$) for days 21 and 28.
[b]Mean values of seven replicates ±SE from 14-day-old plants.
DW, dry weight.

identify differentially expressed genes. The pre-processing and higher-level statistical analysis of array data from two independent biologically replicated experiments is detailed in the Materials and Methods section. The expression levels of 11 key transcripts identified by this analysis were validated by semi-quantitative RT PCR (Fig. 2). The microarray and RT PCR results were consistent with each other when using the same RNA samples.

Genes commonly regulated by salinity and JA

Salinity stress, JA treatment and a JA-pre-treatment followed by salinity stress induced markedly different numbers of probe sets. Figure 3a illustrates the overlap among the probe sets induced by the three treatments. A total of 44 probe sets increased abundance by all three treatments. These commonly detected genes are potentially important for salt tolerance as these may exert a priming effect by acclimating plants before they actually experience stress. Information on selected genes that were commonly induced is summarized in Table 2. These include photosynthesis and stress response-related genes such as the small subunit of ribulose-1,5-bisphosphate carboxylase, carbonic anhydrase (CA), glutathionone S-transferase and a water stress-induced tonoplast intrinsic protein. The transcript levels of most genes in Table 2 is higher in JA-pre-treated plants compared with plants exposed directly to salinity stress. We found considerable overlap between the genes that were induced by salinity stress and JA treatment; of the 124 salinity stress up-regulated probe sets, 48 (38%) were also induced by JA treatment. Figure 3b shows the overlap among the genes that have decreased abundance of transcript by each of the treatments (Supplemental Table S2).

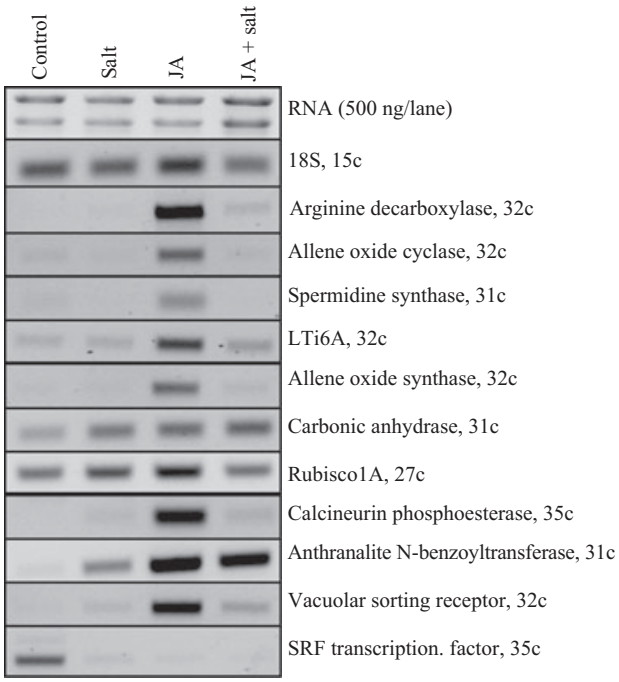

Figure 2. Validation of several genes identified by microarray study using semi-quantitative PCR. The genes validated included jasmonic acid (JA) biosynthesis-related allene oxide synthase (AOS) and allene oxidase cyclase (AOC), genes associated with salt-stress response such as arginine decarboxylase, and LTI6A, and genes related to photosynthesis such as ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) and carbonic anhydrase (CA). Included also were genes induced and repressed by all three treatments. The number of PCR cycles (c) used for individual genes are listed next to the gene name.

Candidate genes for salinity tolerance

To identify candidate genes that may be mediating the JA-induced component of salt tolerance in barley, we mined the data for specific expression pattern across the treatments. For example, we looked for genes that decreased abundance in response to salinity stress but did not change their expression when pre-treated with JA. This set is important because it potentially includes genes that may ameliorate the inhibitions caused by salt stress upon induction by JA pre-treatment. We found eight probe sets that fulfilled this criterion. One of the probe sets represented glutathione S-transferase 1 (Contig2975_s_at). This finding is important in light of a report by Roxas *et al*. (1997), where tobacco seedlings over expressing glutathione S-transferase and glutathione peroxidase had improved growth performance under salt stress compared to wild-type seedlings. Another probe set (Contig11241_at) representing an apoplastic invertase has also been linked to salt tolerance (Fukushima *et al*. 2001). Increased transcript level of apoplastic invertase is known to increase sugar levels (glucose and fructose) in the cytoplasm of the source cells primarily by interfering with transfer of sugar from them.

We also searched for genes that were induced in JA-pre-treated then salt-stressed samples but were not responsive to salinity stress treatment alone. Any gene in this set with association to salt tolerance may explain, at least in part, the physiological basis of observed salt tolerance in JA-pre-treated plants. This gene set included 60 probe sets expected to represent JA responses. A list of probe sets induced by JA treatment and JA-pre-treated salinity-stressed samples but not by salinity stress alone is presented (Table 3). From this set, we found the increased transcript abundance of an arginine decarboxylase 2 (ADC2) in the JA-pre-treated sample particularly intriguing. Arginine decarboxylase catalyzes the first step of polyamine biosynthesis. The expression of ADC2 in *Arabidopsis* is associated with salt tolerance through accumulation of putrescine (Urano *et al*. 2004). Besides ADC2, genes such as ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) activase, fasciclin-like arabinogalactan (FLA10) and a jacalin lectin protein have also been linked to salinity stress/photosynthesis and are regulated by JA.

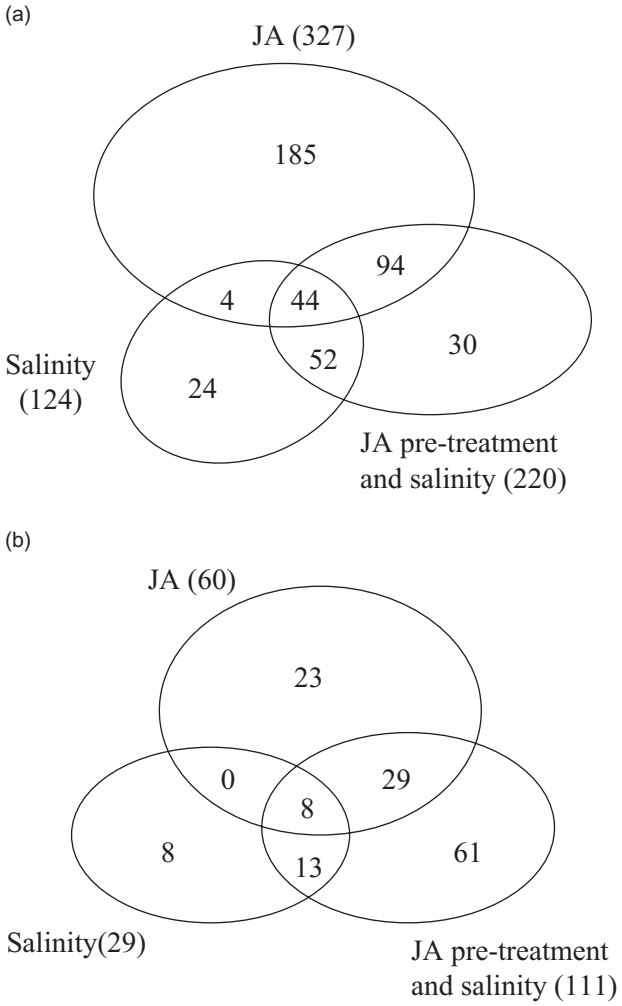

Figure 3. Number of differentially expressed probe sets. (a) Probe sets induced at a significant level (twofold or more) by salinity stress, jasmonic acid (JA) and JA pre-treatment before salinity stress, and the overlap among the treatments. (b) Number of overlapping probe sets significantly down-regulated by any of the three treatments.

JA-regulated genes

JA modulated the expression of a larger number of genes, significantly up-regulating 327 probe sets (Fig. 3, Supplemental Table S3). A total of 138 of these probe sets (42%) were commonly induced between JA and JA-pre-treated salinity stress samples.

It is noteworthy that JA positively induced the expression of genes involved in JA biosynthesis, indicating a positive feedback mechanism (Fig. 4a). Among the genes exhibiting increased transcript abundance are multiple isoforms of lipoxygenases (LOXs), which catalyze the oxygenation of α-linolenic acid. The isoforms up-regulated include LOX1, LOX2:Hv:1, LOX2:Hv:2 and LOX2:Hv:3. Other genes encoding for AOS2, allene oxide cyclase (AOC) and 12-oxophytodienoate reductase 3 (OPR3) were significantly responsive to JA. A heat map generated by hierarchical clustering of genes associated with JA biosynthesis pathway is shown in Fig. 4b. Note that the heat map display shows relative expression across all the samples and not absolute expression levels for each sample.

We identified 60 probe sets with reduced transcript abundance on JA treatment. Of these, nearly half showed the same trend in JA-pre-treated salinity-stressed plants. Three JA-repressed genes were associated with chromosomal structure or modification. These included two histones (H2A and H4) and a regulator of chromosome condensation (RCC1). Several light-inducible genes such as early light-inducible proteins (ELIPs) HV58 and HV90, a putative LHY protein, CCA1 and a signal transducer of

Table 2. Selected genes commonly induced by salinity stress, jasmonic acid (JA) treatment and JA-pre-treated salinity-stressed barley plants

Putative function	Probe set ID	fc N	fc J	fc JN
Rubisco small subunit 1A	Contig308_x_at	2.2	2.2	2.3
Carbonic anhydrase (CA)	Contig897_s_at	3.4	2.4	3.7
Hsp20/alpha crystallin family	Contig2006_s_at	2.0	3.2	3.0
Lipoxygenase (LOX) 2.1 (LOX2:Hv:1)	Contig2306_s_at	4.5	8.9	9.5
Acid phosphatase	Contig2430_at	2.4	9.9	6.2
Laccase (LAC2-1)	Contig2622_at	2.2	5.3	4.0
Peptidylprolyl isomerase (FKBP77)	Contig2720_at	2.8	4.7	4.2
Glutathione S-transferase	Contig3295_at	2.4	2.4	3.6
Apyrase (APY2)	Contig3332_at	2.5	5.7	5.3
Expressed protein	Contig7690_at	2.7	15.1	6.7
Pre-mRNA splicing SR protein (RSR-1)	Contig9382_at	10.6	2.1	9.6
Anthranilate N-benzoyltransferase	Contig9897_at	2.5	35.2	11.2
Oxidoreductase NAD-binding domain protein	Contig11420_at	4.2	23.9	4.4
Expressed protein	Contig11993_at	5.0	12.2	10.4
LOX I	Contig12574_at	3.8	10.6	8.1
Water stress-induced tonoplast intrinsic protein	Contig19393_at	3.4	5.4	4.3
dnaK protein	Contig22700_at	2.3	2.4	3.3
Calcineurin-like phosphoesterase protein	Contig26476_at	4.0	26.5	8.8
23 kDa JIP (P32024)	Contig1675_s_at	2.1	8.5	3.8
No hit	HK04A15r_at	2.5	23.5	5.6

Putative function is based on Basic Local Alignment Search Tool (BLAST) hits against The Institute for Genomic Research (TIGR) rice and *Arabidopsis* and National Center for Biotechnology Information (NCBI) non-redundant (nr) databases. Fold change (fc) for each probe set under salinity stress (N), JA treatment (J) and JA pre-treated salinity stress (JN).
Rubisco, ribulose 1·5-bisphosphate carboxylase/oxygenase; JIP, jasmonate-induced protein.

phototropic response (RPT2) had reduced transcript abundance. At least eight probe sets represented putative transcription factors. JA pre-treatment repressed the transcript levels of relatively large numbers of genes. Most notable were several light-regulated and/or photosynthesis-related genes. These include genes encoding a putative LHY protein, CCA1, chlorophyll *a* and *b* (cab)-binding protein/LHCII type I, cab-binding protein Cab-3B, two ELIPs, light-induced protein CPRF-2 and a protein related to signalling of phototropic response (RPT2).

Table 3. Selected genes induced by jasmonic acid (JA) treatment and JA pre-treatment followed by salinity stress but not by salinity stress separately

Putative function	Probe set	fc J	*q*-value J	fc JN	*q*-value JN
Rubisco activase	Contig1019_at	3.7	11.12	7.0	22.62
Hydroperoxide lyase (HPL1)	Contig12785_x_at	2.3	9.28	2.0	41.70
Chloroplast precursor (LOX2:Hv:3)	Contig23795_at	3.4	9.28	3.2	19.05
Lipoxygenase (LOX) 2.2 (LOX2:Hv:2)	Contig2305_at	10.7	9.28	4.1	19.05
Allene oxide synthase (AOS2)	Contig3096_s_at	4.1	9.28	2.0	40.94
Arginine decarboxylase 2 (ADC2)	Contig5994_s_at	14.3	9.28	5.0	9.52
Fasciclin-like arabinogalactan (FLA10)	Contig6155_at	7.1	11.12	3.6	19.05
JIP	Contig2899_at	7.6	11.12	4.0	19.05
Phenylalanine ammonia-lyase (PAL1)	Contig1805_s_at	8.7	9.28	2.3	26.26
Agmatine coumaroyltransferase	Contig5638_at	8.7	11.12	2.0	41.70
Calcineurin-like phosphoesterase protein	Contig2289_s_at	8.2	11.12	5.0	19.05
Polyamine oxidase	Contig3212_s_at	10.7	11.12	15.8	19.05
Strictosidine synthase (YLS2)	Contig2900_at	6.1	11.12	2.7	19.05
O-methyltransferase 2	Contig4910_at	9.9	11.12	7.3	22.62
Jacalin lectin family protein	Contig7886_at	108.3	8.56	11.9	9.09
23 kDa JIP (P32024)	Contig1675_s_at	8.5	11.12	3.8	19.44

Putative function is based on Basic Local Alignment Search Tool (BLAST) hits against The Institute for Genomic Research (TIGR) rice and *Arabidopsis* and National Center for Biotechnology Information (NCBI) non-redundant (nr) databases. Fold change (fc) for each probe set after JA treatment (J) and JA pre-treated salinity stress (JN). The statistical significance of differential expression of individual probe set is expressed as *q*-value (%).
Rubisco, ribulose 1·5-bisphosphate carboxylase/oxygenase; JIP, jasmonate-induced protein.

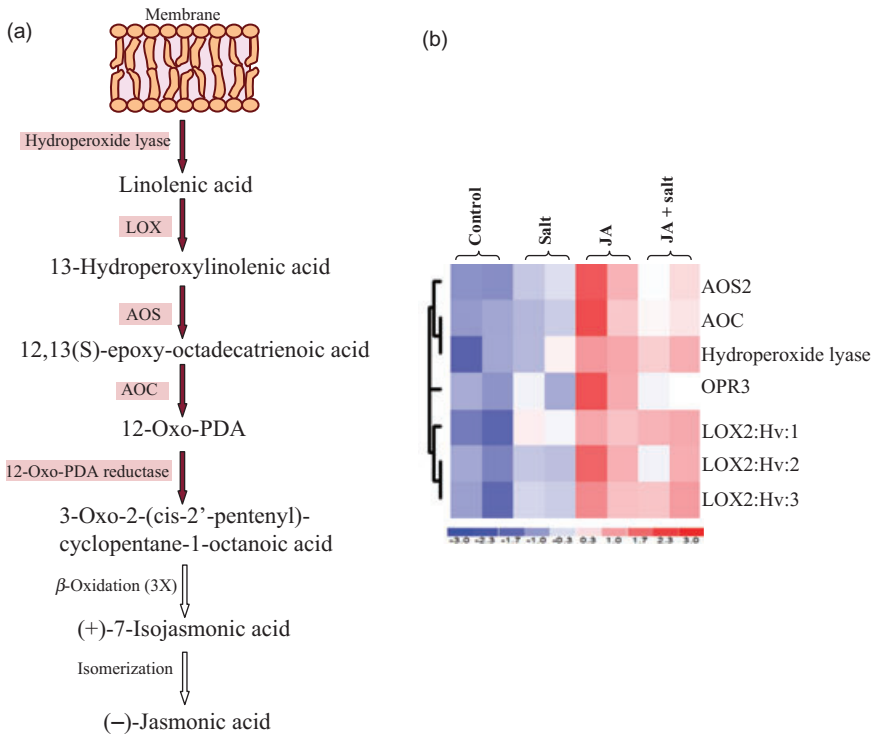

Figure 4. Jasmonic acid (JA) treatment induced the expression of several of the genes involved in biosynthesis of JA. (a) The simplified pathway leading to JA biosynthesis. Enzymes encoded by genes that were up-regulated in response to JA application are highlighted. (b) Heat map based on hierarchical clustering ($P > 0.001$) of JA biosynthesis-related genes. The relative expression across the samples for a given probe set was scaled (−3 to 3) based on mean and does not represent expression levels in comparison to controls. Dark red represents highest expression and dark blue represents lowest relative expression, while white represent no change. LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, oxophytodienoate reductase 3; OXO-PDA, oxo-phytodienoate acid.

DISCUSSION

In this work, we used whole-plant phenotypic characterization to establish a role that JA assumes in salinity tolerance of barley, through sodium ion homeostasis and sustained photosynthesis and transpiration. To explore the molecular basis of this JA-mediated salt tolerance, we performed transcriptome profiling. The results from the transcriptome analysis define a framework on which further detailed characterization of genes can be performed and provide a reference data set for such work.

JA regulates ion homeostasis in barley

The finding that JA-pre-treated stressed plants maintained a lower Na^+ concentration in shoot tissue than salt-stressed plants has linked JA to Na^+ ion homeostasis in barley. This is significant because the ability to exclude Na^+ from the shoot tissue is considered one of the major components of salt tolerance in plants. The Na^+ ion exclusion was highly significant 7 and 14 d after reaching the final salinity concentration in the culture solution. In our experiments, JA-treated plants exhibited higher stomatal conductance and transpiration rate than control plants. The lower concentration of Na^+ ion in pre-treated plants is surprising considering a higher transpiration rate, which usually results in increased ionic in-flow. The difference in Na^+ concentration cannot be explained by differences in biomass production (dilution effect) because JA-pre-treated salt-stressed plants had slightly lower shoot and root biomass than salinity treated plants, although the differences are statistically not significant. Therefore, the lower concentration of Na^+ in pre-treated plants suggests an early initiation of a highly effective salt exclusion mechanism in response to JA application.

JA pre-treatment and photosynthetic inhibitions

Salinity stress reduced the photosynthetic rate of stressed plants to about half of that measured in control plants on day 14. However, pre-treatment of barley seedlings with JA prior to imposing a salinity stress resulted in a significantly higher net photosynthetic rate relative to salinity-stressed, non-pre-treated plants. Salinity stress similarly resulted in a dramatic reduction in stomatal conductance, whereas treatment with JA resulted in greater stomatal conductance compared with control plants. Salt-stressed plants that were pre-treated with JA exhibited higher stomatal conductance than salinity-stressed plants. Photosynthetic inhibition, possibly resulting from reduced stomatal conductance, is one of the major consequences of salinity stress in plants (Wingler *et al.* 2000; Table 1). JA pre-treatment ameliorated the negative effect of salinity stress significantly. JA-induced improvement of stomatal conductance contrary to some reports where jasmonates caused stomatal closure (Satler & Thimann 1981). However, several subsequent studies indicate that the response of stomatal conductance to jasmonates depends on the concentration used (Horton 1991) and the period of exposure (Metodiev, Tsonev & Popova 1996). Here we used a concentration range (12 μM) well below what is known to cause senescence-related responses including stomatal closure.

JA-regulated genes linked to salinity tolerance

We found the response of an ADC2 to JA pre-treatment very interesting. The transcript level of ADC2 increased in plants pre-treated with JA before salinity stress, but not in plants that were directly exposed to salinity stress. Arginine decarboxylase catalyzes the first step in the conversion of arginine to putrescine in the polyamine biosynthesis pathway. Two independent mutants of ADC2 in the model plant *Arabidopsis* resulted in decreased polyamine content and consequently decreased salt tolerance of a mutant relative to respective wild types (Kasinathan & Wingler 2004; Urano *et al*. 2004). The decreased salt tolerance of the ADC2 mutant, *spe2-1*, was reflected in loss of chlorophyll from leaves (Kasinathan & Wingler 2004). The expression level of ADC2 was also elevated in the JA treatment in barley plants. The fact that ADC2 is JA induced and involved in salt tolerance but is not induced independently by salt stress to significant levels in our experiment makes it a good candidate gene for further characterization.

Another gene that came to the fore from searches for potential JA-regulated salt tolerance candidates was an apoplastic invertase. An apoplastic invertase gene from yeast ectopically expressed in tobacco resulted in increased levels of sucrose and hexoses in leaves (Fukushima *et al*. 2001). The transgenic plants were able to better withstand high levels of salt stress compared with wild type. The increased levels of sucrose in the leaves prevented inhibition of photosynthesis in the transgenic plants under salt stress. This observation is striking, considering that JA-pre-treated barley plants in our experiment also maintained higher net photosynthetic levels compared with plants exposed to salt stress alone.

The transcriptome analysis revealed a strong regulation of photosynthesis-related genes by one or more of the imposed treatments in barley. Of the several photosynthesis-related genes identified, we found the regulation of Rubisco activase during various treatments quite noteworthy. Rubisco activase transcript increased by 7-fold in JA-pre-treated salinized plants and 3.8-fold in JA treatment, but not differentially expressed at a significant level in salinity-stressed plants. Rubisco activase is an ATPase protein, which makes the active site of Rubisco catalytically competent by carbamylation with CO_2 (Salvucci & Ogren 1996).

Our expression analysis results indicate an increase in the mRNA abundance of several photosynthesis-related genes in response to JA treatment. Previous reports on response of plants to jasmonates have indicated a decrease in levels of photosynthesis-related proteins such as Rubisco and light-harvesting cab-binding proteins upon treatment with jasmonates (Reinbothe, Mollenhauer & Reinbothe 1994). Although the abundance of Rubisco small subunit and Rubisco activase transcripts increased in our experiment, the expression level of several other light-responsive genes including those encoding for cab-binding protein decreased in abundance upon JA treatment. Besides the evidence for large-scale regulation of gene expression by jasmonates from previous research, there are reports indicating jasmonate regulation at post-transcriptional and translational levels for some genes (Reinbothe, Reinbothe & Parthier 1993; Reinbothe *et al*. 1994). Post-translational regulation is one possibility that may explain the differences between our expression results and previous reports on protein abundance for photosynthesis-related genes.

In the context of photosynthesis-related gene expression, it is noteworthy that all three treatments increased the mRNA levels of CA. The physiological/biochemical role of CA is associated with the carbon-concentrating mechanism, which increases the local CO_2 concentration around the primary CO_2-fixing enzyme, Rubisco (Badger & Price 1992; Sültemeyer 1998). Conceivably, increased activity of CA by JA treatment can enable pre-treated plants to better maintain a relatively higher photosynthetic rate when subjected to salt stress. Various studies have also shown a relationship between salinity adaptation and an increase in CA activity (Latorella & Vadas 1973; Brown *et al*. 1987; Fisher *et al*. 1996). Therefore, the increased abundance of CA by JA pre-treatment 2 d before salinity stress can prime the subsequently stressed plants and make photosynthetic machinery more adaptive to salinity stress compared to directly salt-stressed plants. Less inhibited net photosynthetic rate in JA-pre-treated plants relative to salinity-stressed plants, coupled with increased transcript levels of Rubisco activase, and CA indicates a potential role of these genes in improved tolerance of barley as a consequence of JA pre-treatment.

JA biosynthesis genes

Our results indicate an increase in the mRNA abundance of several key genes involved in the JA biosynthesis pathway upon exogenous application of JA. These include several isoforms of LOXs (LOX2:Hv:1, LOX2:Hv:2, LOX2:Hv:3, LOX1), AOS2, AOC and OPR3. Increased transcript abundance of AOC correlates with increased levels of oxophytodienoate acid (OPDA) in barley (Sreenivasulu *et al*. 2006). The existence of a positive feedback mechanism for JA has also been reported for other plant species such as *Arabidopsis* (Sasaki *et al*. 2001). A similar observation has also been made for ethylene biosynthesis gene 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) upon treatment with ethylene in *Pisum sativum* (Petruzzelli *et al*. 2000). Interestingly, ACO was also induced in barley plants treated with JA in our experiment.

Exogenous application of jasmonates to barley plants has been suggested to activate a separate signalling pathway from the one responding to endogenous increase in jasmonates from sorbitol treatment (Kramell *et al*. 2000). The authors measured the transcript levels of several jasmonate-induced and JA biosynthesis genes upon inducing endogenous rise in JA levels and exogenous application of jasmonates. Based on transcript abundance after 24 h in response to osmotic stress-induced rise in JA levels, they identified biosynthesis gene AOS and several jasmonate-induced proteins (JIPs) including JIP23, JIP60 and JIP37 as responsive to increased endogenous JA. In addition to the

previously mentioned set, two of the LOXs (LOX2:Hv:1 and LOX2:Hv:2) were induced by exogenous applications. The exogenous JA treatment in our experiment resulted in increased transcript abundance of genes from both categories and included AOS, JIP23, JIP60, LOX2:Hv:1 and LOX2:Hv:2. Although our exogenous JA treatment induced a set of genes consistent with Kramell *et al*. (2000), such a comparison has limitations because of differences in treatment method, concentrations of jasmonates used, and time and tissue differences.

Tsonev *et al*. (1998) previously predicted an adaptive role of JA pre-treatment in barley plants before salinization based on several physiological parameters. Our work confirms the general conclusion of Tsonev *et al*. (1998) and further shows that the advantage of pre-treatment is through improved Na^+ exclusion and relatively less-inhibited photosynthesis. However, there are several differences in the experimental set-up including JA concentration used, the age of the plants and salt stress application. The JA concentration (25 μM) used by Tsonev *et al*. (1998) was double compared to the current experiment. Tsonev *et al*. (1998) reported no significant effect of JA pre-treatment when salt stress was applied in step-wise manner. However, in the current experiment, we have showed a significant improvement upon JA pre-treatment when the salt stress is applied in a step-wise fashion (Fig. 1).

Salt tolerance and responses of barley and *Arabidopsis* to jasmonates

We were interested in comparing the transcriptional responses of barley with *Arabidopsis* to jasmonates. Such a comparison was expected to be useful considering that Barley1 array does not probe the entire transcriptome of barley, in comparison to the global evaluation afforded by *Arabidopsis* array. For this, we analyzed the Affymetrix 25 K GeneChip data from a short-term MeJA treatment on *Arabidopsis*. This data set was generated by the AtGenExpress Consortium and is available from TAIR (http://www.arabidopsis.org/). Jasmonate-responsive *Arabidopsis* genes relevant to salinity tolerance include a salt-tolerance zinc-finger protein (STZ/ZAT10; At1g27730), a sodium/potassium/calcium exchanger (At5g17860), an outward rectifying potassium channel (At4g18160) and a mechanosensitive ion channel protein (At5g19520). The gene for salt-tolerance zinc-finger protein (STZ/ZAT10) was also induced in response to wounding in *Arabidopsis* (Cheong *et al*. 2002). The STZ/ZAT10 (At1g27730) is not represented on Barley1. Some noteworthy genes up-regulated in both species include the ADC2, an osmotic stress-responsive proline dehydrogenase, ACO and an amine oxidase (Supplemental Table S4). The comparison confirmed JA-mediated regulation of arginine decarboxylase, which is associated with salinity tolerance in plants. Analysis of *Arabidopsis* data set also brings to the fore genes like the jasmonate-modulated STZ/ZAT10, which is directly associated with salt tolerance but is not represented on the barley array. STZ/ZAT is induced by salt and is negatively regulated by the salt overly sensitive (SOS) system in *Arabidopsis* (Gong *et al*. 2001).

CONCLUSION

Pre-treatment with JA improved the adaptation of barley plants to salinity stress in our experimental set-up. Photosynthesis measurements indicate an important role of JA in the adaptation of barley to a saline environment by partially mitigating the negative effects of salinity. Rubisco activase and CA are two genes identified by our expression studies, which may be important in maintaining relatively higher net photosynthetic rate under salinity stress. Rubisco activase can ameliorate the inhibitory effect of salinity stress on JA-pre-treated plants because of a higher induction level than observed in the other two treatments. In contrast, for CA which is induced by all three treatments to similar levels, the mitigation may result from a priming effect because of pre-treatment with JA. JA pre-treatment prior to salinity stress resulted in lower Na^+ accumulation, therefore linking JA to Na^+ homeostasis. We identified genes such as arginine decarboxylase and apoplastic invertase, which are directly associated with salinity tolerance and are only induced upon JA application. The microarray study has introduced JA in the regulatory framework of these genes in response to salt stress. Expression analysis of *Arabidopsis* data revealed that a salt tolerance gene, STZ, also responds to jasmonate treatment. Detailed characterization of promising JA-regulated salinity tolerance-associated candidate genes as well as the barley ortholog of STZ can be the focus of future research.

Data availability

All expression data is available through the Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ under platform GPL1340, Series GSE5605. The list of significantly responsive probe sets along with annotations is available as supplemental data. The enhanced annotation for all Barley1 probe sets is available through HarvEST Barley at http://www.harvest.ucr.edu/.

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SUPPLEMENTARY MATERIAL

The following supplementary materials are available for this article:

Table S1. Primer sequence information for genes validated with RT-PCR.
Table S2. Genes commonly down-regulated by salinity stress, jasmonic acid (JA) treatment and JA pre-treated salinity-stressed barley plants.
Table S3. Selected categories of genes induced by jasmonic acid (JA) application.
Table S4. Genes commonly induced in response to methyl jasmonate (MeJA) treatment in *Arabidopsis* and jasmonic acid (JA) treatment in barley.

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