Analysis of Seaweed Extract-induced Transcriptome Leads to Identification of a Negative Regulator of Salt Tolerance in Arabidopsis

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Abstract. Successful development of plants resistant to salinity stress is problematic as a result of the complex polygenic natures of salt tolerance. Previously, alkaline extracts of the brown seaweed *Ascophyllum nodosum* have shown promise in enhancing plant tolerance toward abiotic stresses. To understand the underlying molecular mechanisms, the whole genome transcriptome of Arabidopsis undergoing salt stress was analyzed by microarray analysis after treatment with the chemical components of *A. nodosum* extracts (ANE). Treatment with ANE induced many positive regulators of salt tolerance in addition to downregulating numerous other genes. Using T-DNA insertion mutants within these downregulated genes, we examined the potential for a novel source of enhanced NaCl tolerance through removal of negative regulators of NaCl stress responses within Arabidopsis. Several potential target mutations were identified with enhanced salt-tolerant phenotypes. A T-DNA insertion within the promoter of a putative *Pectin Methyl Esterase Inhibitor* (PMEI) gene (*At1g62760*) was found to be resistant to salinity stress and was further characterized. This T-DNA insertion mutant was designated as *pmei1-1*. The phenotype of *pmei1-1* seedlings included increased primary root growth in vitro and improved biomass accumulation under NaCl stress. Additionally, modified transcript levels of dehydration-responsive genes, including *RD29A*, were observed in *pmei1-1* plants. Taken together, these results suggest a role for PME1 as a negative regulator of NaCl resistance and that chemical stress-induced transcriptome analysis may lead to identification of additional novel regulators of abiotic stress tolerance in plants, the use of which would have significant implications for agriculture globally.

Plants are sessile organisms that are faced with a range of abiotic stresses throughout their lifespan, which are increasing on a global scale. High salinity is one of the major abiotic stress factors that significantly reduce crop yield and productivity. High NaCl conditions adversely affect plant growth through increased osmolarity, ion toxicity (i.e., Na⁺, Cl⁻, and SO₄²⁻), nutritional imbalance, and oxidative stress (Turkan and Demiral, 2009). The main toxic effects of Na⁺ include inhibition of enzyme activities and disruption of intracellular K⁺/Na⁺ homeostasis (Zhu, 2002). To cope with high salinity, plants have developed a number of mechanisms, including altered gene expression pathways through hormone regulation

for ion exclusion or decreasing water potential within the plant to cope with the added salts (Kronzucker and Britto, 2011). Molecular genetic approaches, aimed at dissecting the complexity of NaCl stress responses in plants, have provided new understanding to the biological processes involved in perception and signal transduction of environmental cues perceived by the plants (Sreenivasulu et al., 2007). Microarray studies established that plant response to NaCl involved subtle alterations in the expression of genes involved in oxidative, osmotic pathway, vacuolar antiporters (Seki et al., 2002; Taji et al., 2004), transcription regulators (Kreps et al., 2002), and cell wall-related genes (Walia et al., 2005).

Production of plants with superior agronomically important traits, including tolerance to high NaCl conditions, has been a focal theme of plant biotechnology. There have been numerous attempts to improve salinity tolerance in glycophilic plants that typically involved overexpression of genes, upregulated during stress conditions; including genes involved in production of compatible solutes, sodium exclusion, and reactive oxygen scavenging (reviewed in Arzani, 2008; Ashraf and Akram, 2009; Stamm et al., 2011; Wally et al., 2011). Recently research focused on growth promotion

through increased cytokinin levels under the control of stress-induced promoters with varying levels of success (Havlova et al., 2008; Rivero et al., 2007, 2010). Although these logical biotechnological methods to improve abiotic stress tolerance continue to hold promise, novel functional genomics approaches to isolate genes for abiotic stress tolerance are required for further improvement in this field. In earlier studies, it was observed that addition of commercial extract from the brown macroalgae, ANE, induced salt stress and drought tolerance in plants (Nabati et al., 1994; Spann and Little, 2011). To understand the molecular basis of *A. nodosum*-elicited salt stress tolerance, microarray analysis was performed. It was observed that the seaweed components elicited a genome-wide transcriptional response by upregulating several known, positive regulators of NaCl tolerance (Prithiviraj et al., unpublished data). Additionally, there were many genes that were downregulated during NaCl stress on addition of ANE. Further functional phenotypic screening of Arabidopsis knockout mutants for some of the downregulated genes during salt stress were examined under the premise that these might represent new novel regulators involved for NaCl tolerance in plants. We present the screening of 18

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T-DNA knockout mutations of the downregulated genes with detailed characterization of one gene, *Atlg62760*, which encodes for a putative PME1 that exhibits heightened resistance toward salt stress.

MATERIALS AND METHODS

Salt resistance screening. The *Arabidopsis* Col-0 T-DNA insertional mutants from seaweed extract (To et al., 2007) transcriptome downregulated genes were obtained from the Arabidopsis Biological Resource Center stock center (Columbus, OH; Table 1). Rapid assessment of salt stress tolerance was conducted as previously described (Achard et al., 2006; Verslues et al., 2006); 4-day-old plants were grown on half-strength Murashige and Skoog (MS) medium (Sigma, St. Louis, MO) supplemented with 0.1% (w/v) sucrose solidified with 0.8% (w/v) agar and were transferred onto half-strength MS plates containing either 0 or 125 mM NaCl. The plates were then maintained in a growth chamber at 22°C with a 16-h light/8-h dark cycle with a light intensity of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Root elongation rates were measured at 3, 5, and 7 d after transfer, marking the progress of root growth at each time and measured with Image J software (Research Services Branch, National Institutes of Health, Bethesda, MD). Root elongation of the T-DNA lines was compared with the Col-0 controls and analyzed using analysis of variance Tukey's honestly significant difference test $P < 0.05$ with SPSS 15.0 software (Leads Technology).

Quantitative reverse transcription–polymerase chain reaction analysis. Total RNA was isolated using the monophasic RNA extraction method (Chomczynski and Sacchi, 1987). Two micrograms of RNA was treated with 2 units of RQ1 DNase (Promega) according to the manufacturer's instructions. cDNA was synthesized using an Applied Biosystems high-capacity cDNA synthesis kit (Applied Biosystems) using the manufacturer's protocols. Relative transcript levels were assayed by real-time polymerase chain reaction (PCR) analysis using gene-specific primers on a StepOne™ Real-Time PCR System (Applied Biosystems) using SYBR gene reagent power SYBR (Applied Biosystems). Primer sequences were taken from the Roche Universal ProbeLibrary (Roche Diagnostics Corp.). Wherever possible, the primers would flank an intron-spanning region. All amplicon length ranges were between 68 and 120 bp. The reaction mixture contained 5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), 20 ng of cDNA, and 250 nM of each of the forward and reverse primers. The following default program was used: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each and a dissociation stage of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. RNA relative quantification analyses were performed using 7300 System SDS software (Applied Biosystems). The list of primers used is shown in Table 2. The data represent the mean $\pm$ SE of $n = 3$ independent experiments using

Table 1. Initial screening of Arabidopsis T-DNA lines for increased salt resistance.

Gene name	T-DNA line	Insertion location	Accession	Increased root length
Wall-Associated kinase 1	Salk_107175	Exon	AT1G21250	–
Wall-Associated kinase 1	Salk_137260	Promoter	AT1G21250	–
Glycine-rich RNA binding protein	Salk_047963c	Exon	AT4G39260	–
Glycine-rich RNA binding protein	Salk_149544	Exon	AT4G39260	–
Glycine-rich RNA binding protein	Salk_114101	Promoter	AT4G39260	+
Cellulose synthase like e1	Salk_072106	Exon	AT1G55850	–
Cellulose synthase like e1	CS25016	Exon	AT1G55850	–
Cellulose synthase like e1	Salk_123441	Exon	AT4G24000	–
Cellulose synthase like e1	Salk_008597c	Exon	AT4G24000	–
Pectin methylesterase inhibitor	Salk_072421	Promoter	AT1G62760	++
Pectin methylesterase inhibitor	Salk_007858c	Promoter	AT1G62760	++
Pectin methylesterase inhibitor	Salk_084836c	Promoter	AT1G62760	+
RNA binding protein	Salk_078245	Exon	AT5G61030	–
RNA binding protein	Salk_078241	Exon	AT5G61030	–
RNA binding protein	Salk_007592	Exon	AT5G61030	–
RNA binding protein	Salk_098675	Exon	AT5G61030	+
RNA binding protein	Salk_078146	Intron	AT5G61030	–
RNA binding protein	Salk_007455	Promoter	AT5G61030	–

Screening of T-DNA insertion mutants from genes identified from microarray downregulated during salt stress after application of organic fraction of *A. nodosum* seaweed extracts (B. Prithiviraj, unpublished data). Lines were assessed for increased root length, growing on half-strength Murashige and Skoog MS media supplemented with 125 mM NaCl. Lines were scored (–) no increase, (+) 10% increase in root length, or (++) more than 20% increase compared with controls. Thirty seedlings were scored from two repeated experiments.

Table 2. Primers used for quantitative reverse transcription–polymerase chain reaction.

Primer	Sequence (5'–3')	Accession number
Act2-F	ttgtttgtttcatttcccttttg	AT3G18780
Act2-R	gcagacgtaagtaaaaaaccagaga	AT3G18780
Pme1-F	gectccaattcgtcaaaaa	AT1G62760
Pme1-R	cgaactacttccaacacgtg	AT1G62760
RD29a-F	tgcaccaggcgtaacaggta	AT5G52310
RD28a-R	taatcggaagacacgacagga	AT5G52310
RAB18-F	cttgccgatttgggatacagc	AT1G43890
RAB18-R	cgtgtctcttctgtcacatc	AT1G43890

the delta-delta Ct method using Act2 as the endogenous control. Each data point was determined in triplicate in each of the three biological replicates and presented as mean $\pm$ SE.

Genetic analysis of T-DNA insertion alleles. Homozygous plant lines for SALK_072421 were confirmed by PCR amplification of total genomic DNA isolated by using CTAB method of Genomic DNA isolation (Weigel and Glazebrook, 2002) left and right gene-specific primers, LP (5'–CGAATCTTGAA GCGAAGTCAC 3'), RP (5'–TCCATTGCT AAAATTTACACG 3'), and LBb1 (5'–GCG TGGACCGCTTGCTGCAACT–3') were obtained from the SIGnAL web site. Homozygous lines were verified by diagnostic PCR using LP and RP primers that did not result in any amplification product whereas heterozygous lines resulted in a PCR product. Furthermore, PCR-using primers LBb1 with both LP and RP primers were used to confirm the T-DNA insertion with the homozygous mutants producing a signal amplicon of ≈ 700 bp, heterozygous lines producing two amplicons of ≈ 700 and 900 bp, while the homozygous wild types produced a single amplicon of 900 bp.

Coexpression analysis. Coexpression analysis was performed using the Arabidopsis Coexpression Tool (ACT; <<http://www.arabidopsis.leeds.ac.uk/ACT>>).

Statistical analysis of the significance of overrepresentation of Gene Ontology terms in a gene list was performed using the corresponding tool at the ACT web site (Jen et al., 2006; Manfield et al., 2006).

Salt stress tolerance in soil. Seeds of wild-type and *pme1-1* mutant were sown directly in potting soil. Fifteen uniform plants from the wild-type and *pme1-1* populations were selected and grown in the greenhouse using a standard irrigation regime. To assess salt stress tolerance in soil, 3-week-old wild-type and mutant plants were salt-stressed (100 mM) for 4 weeks at weekly intervals.

RESULTS

Identification of salt-tolerant mutants from the NaCl phenotype screen. From microarray data collected studying the NaCl stress-alleviating alterations in gene expression, from the organic fraction of the alkaline extract of *A. nodosum*, we selected the six genes that were maximally downregulated and for which functions of salt resistance had not previously been explained (B. Prithiviraj, unpublished data). Multiple T-DNA insertion lines were selected for each downregulated gene, comprising

insertions within the promoters, introns, and exons (Table 1); they were subjected to a functional NaCl phenotypic screen. To select appropriate T-DNA lines with altered NaCl phenotypic response, we performed the root gravitropic bending assay comparing the T-DNA lines to controls at 0 and 125 mM NaCl. Through the root screening, we identified five T-DNA lines, which exhibited enhanced root growth at 125 mM NaCl as compared with the Col-0 Arabidopsis controls. Of the NaCl-resistant T-DNA lines, three (Salk_072421, Salk_007858c, and Salk_084836c) were within the putative promoter of *Atlg62760* that encodes for a putative PME1 gene. Because all three T-DNA insertions of *Atlg62760* were found to be resistant, we selected the T-DNA lines for further investigation.

Reverse transcription–polymerase chain reaction analysis of *Atlg62760* expression reveals that T-DNA mutants are reduced function mutants. To confirm the exact insertion site of T-DNA and to rule out the possibility of chromosomal rearrangements, both T-DNA/genomic DNA junctions of the insertion allele were sequenced. The T-DNA insertion was found 53 bp upstream of the

ATG translational start site within the promoter region of *Atlg62760*; similarly, the T-DNA insertion of SALK_007858C was found in the promoter region of *Atlg62760*. SALK_072421 and SALK_007858C alleles were designated *pmei1-1* and *pmei1-2*, respectively. The T-DNA line Salk_084836c was identified 218 bp upstream of the ATG translational start site and was designated *pmei1-3* (Fig. 1). Quantitative real-time PCR analysis was performed to analyze the expression of *Atlg62760* in these mutant lines. Transcript levels of *Atlg62760* are substantially reduced in *pmei1-1*, 2, 3 (Fig. 2).

Altered sensitivity of *pmei1-1* to NaCl stress in comparison with wild-type Col-0. There were no visible morphological differences between wild-type Col-0 and *pmei1-1* Arabidopsis when grown under optimal conditions. Salt responses were characterized as per Verslues et al. (2006) by transferring 4-day-old seedlings to half-strength MS plates containing NaCl concentrations (i.e., 0, 50, 75, 100, 125, and 150 mM), measuring the primary root growth at 3 and 7 d after transfer, and determining the daily growth rate. We observed that the primary root growth rate

increased in *pmei1-1* plants, as compared with controls, when the NaCl concentration was between 75 and 125 mM. Root growth rates were $\approx 20\%$ higher in *pmei1-1* at 75 mM, 100 mM, and 125 mM NaCl, respectively (Fig. 3A). However, at NaCl concentrations greater than 150 mM, there was no increase in *pmei1-1* root growth rate (data not shown). Similarly, when the total rosettes of salt-grown plants were measured, *pmei1-1* plants accumulated 40% and 50% higher fresh weight at 75 and 100 mM NaCl, respectively, when compared with Col-0 (Fig. 3C).

To determine whether the altered salt stress response in *pmei1-1* plants also occurred in plants grown in soil, 2-week-old plants were grown in peat pellets and subjected to salt stress by root irrigation with 100 mM NaCl for ≈ 3 weeks (at weekly intervals). After 3 weeks of root irrigation with NaCl solution, the Col-0 plants exhibited symptoms of chlorosis and necrosis, whereas the *pmei1-1* plants maintained a healthy phenotype (Fig. 4).

Reverse transcription–polymerase chain reaction shows altered salt stress signalling in *pmei1-1*. To identify possible interactions between known stress adaptation pathways and PME1, known candidate genes were selected based on their established differential transcriptional regulation in response to salt stress treatment. The transcriptional abundance of these genes was studied in *pmei1-1* plants in the presence at 0, 50, and 100 mM NaCl. *RD29A* and *RAB18* were chosen because they are well-characterized salt stress-responsive genes (Jakab et al., 2005) in addition to examining the stress-induced response of *PME1* during salt stress. The transcript abundance of *RD29A* was slightly lower in *pmei1-1* plants after both 24 and 96 h of treatment with both 50 and 100 mM NaCl compared with Col-0 plants (Fig. 5). There was a slight increase in the basal levels of *RD29a* transcripts during the unstressed (mock) treatments, increasing by up to 30% by 96 h (Fig. 5A), whereas there was no significant difference in the constitutive *RAB18* levels (Fig. 5B). Furthermore, there was a substantial decrease in the induced levels of both *RD29a* and *RAB18* after treatment of either 50 or 100 mM NaCl (Fig. 5A–B). We confirmed that *PME1* transcripts levels are increased after salt stress treatment with nearly a 40-fold increase found 96 h after treatment with 100 mM NaCl (Fig. 5C).

Coexpression analysis to reveal the pathway network for *Atlg62760*. Patterns of coexpression have been used to offer insight into a number of biological processes such as networks of functionally related genes (Wei et al., 2006), convergent gene pairs (Krom and Ramakrishna, 2008), functionality of a single gene in a multigene family (Ho et al., 2007), and also identification of interacting proteins (Fraser et al., 2004). Genes coexpressed and antiexpressed with *Atlg62760* were identified using the Arabidopsis Coexpression mining ACT tool (Manfield et al., 2006). The results of this analysis are depicted in Supplementary Tables 1A and 1B. In general, the positively coexpressed genes with annotated function were

Fig. 1. Physical map of the *Atlg62760* locus and T-DNA insertion sites. Broken lines indicate the coding region of the gene with the promoter segment; arrow indicates the predicted transcription direction, Salk_072421 (*pmei1-1*), Salk_007858c (*pmei1-2*), and Salk_084836c (*pmei1-3*). *Atlg62760* encodes a 939 bp continuous gene lacking introns. The insertion sites are indicated in the promoter regions of *Atlg62760v*.

Fig. 2. Quantification of *Atlg62760* transcripts in *pmei* insertional mutants. Quantitative real-time polymerase chain reaction analysis of *Atlg62760* transcripts in wild-type (Col-0) and mutant (*pmei1-1*, *pmei1-2*, and *pmei1-3*) lines relative to control. Data represent the means and $\pm$ SE values of three independent biological replicates.

Fig. 3. The mutation in *pmei* (*Atlg62760*) imparts NaCl tolerance in Arabidopsis. (A) Root length growth rate on salt-containing half-strength Murashige and Skoog (MS) medium. Seedlings were transferred to half-strength MS medium at the indicated mM concentration of NaCl. Root elongation was determined after 3, 5, and 7 d and calculated as the elongation rate (mm·d⁻¹). Error bars indicate $\pm$ SE of three independent experiments (n = 30). (B) Fresh weight of 4-day-old seedlings growing on indicated NaCl containing half-strength MS medium for an additional 15 d. Error bars indicate $\pm$ SE of two independent experiments (n = 30).

Fig. 4. *pmei-1* mutant Arabidopsis plants are tolerant to salt stress. Representative photograph of Col-0 wild-type and *pmei-1* mutant plants exposed to salt stress (100 mM) for 4 weeks. Col-0 plants exhibited symptoms of ion toxicity (left) compared with *pmei-1* plants that were relatively tolerant and appeared green (right).

mostly related to senescence, transcription, nucleotide recovery during senescence, and amino acid regeneration; on the other hand,

negatively correlated genes were overrepresented in ATP-ase activity (Supplementary Tables 1B and 1C).

DISCUSSION

Isolating and characterizing genes of agronomic importance are both crucial because the world's agricultural productivity is increasingly affected by abiotic factors, particularly salinity and drought. New methods that rely on function-based screening approaches have been developed to identify novel regulators of stress tolerance (Du et al., 2008; Papdi et al., 2008) in plants. We performed functional screening of six different genes, which were downregulated in Arabidopsis during salt stress by organic extracts of commercial *A. nodosum* extracts and a potential novel regulator of NaCl tolerance was identified. In this article, we report the isolation of loss-of-function mutants (*pmei-1*) by forward functional screening. *Atlg62760* mRNA levels showed an induction in 75 mM and 100 mM NaCl treatments and declined at higher NaCl concentration (150 mM). In this case, *pmei-1* (in Col-0 background) showed a clear phenotype at both 75 and 100 mM NaCl.

Pectin methylesterase (PME; electrical conductivity 3.1.1.1) is a ubiquitous enzyme in the plant kingdom; however, its role in plant growth and development is not yet clearly understood. PME is reportedly involved in a multitude of physiological processes such as fruit maturation, microsporogenesis and tube pollen growth, cambial cell differentiation, seed germination and hypocotyl elongation, reproductive development, and plant defense (reviewed in Pelloux et al., 2007). Studies suggested that a reduction in PME activity altered cation levels in solanaceous plants (Pilling et al., 2004; Tieman and Handa, 1994). Interestingly, the ion-binding capacities of cell walls from PME-inhibited plants were specifically modified because they preferentially bound more sodium but less potassium. Pilling et al. (2004) also suggested that modifications of ion partitioning were the basis for reduced elongation rates in PME-inhibited plants. PME-catalyzed methanol production might either diffuse or be catabolized into formate, the substrate of formate dehydrogenase, which is upregulated by methanol sprays or environmental stresses such as wounding, cold, and drought in potato leaves (Pelloux et al., 2007). PME-induced methanol production might be more generally involved in plant stress signaling. Interestingly, *RD29A*, a stress-induced gene in the salt stress response of Arabidopsis, showed high basal activity in *pmei-1* plants.

The results of anticoverexpression analysis revealed an overrepresentation of genes with a functional role involving ion and transmembrane transport aided by ATPases. Plant survival under NaCl stress depends on activity of membrane transport systems, ATPases, secondary active transporters, and channels (Sze et al., 1999). Increased ATPase-mediated transmembrane proton transport is an early plant cell response to salt stress and is involved in maintaining ionic balance (Ratajczak et al., 1994; Sze et al., 1999). It is possible that ATPase-mediated H⁺ transport may have a role in regulating the transport of Na⁺, leading to

Fig. 5. Expression of stress responsive genes after application of NaCl in Col-0 and *pmei1-1* plants. Four-week-old soil-grown Arabidopsis plants were treated with 50 or 100 mM NaCl for the indicated time of exposure (24 and 96 h). The transcript levels were normalized to the Col-0 at 24 h after mock (water) treatment. Data represent the means and $\pm$ SE values of three independent biological replicates.

lower sodium accumulation in the cytosol of *pmei1-1* plants. Also, the *pmei1-1* mutation did not change the kinetics of the stress gene induction as compared with the Col-0 Arabidopsis. This might suggest independent mechanisms, possibly through ATPase action. Therefore, it appears that NaCl tolerance in *pmei1-1* plants might be, at least in part, affected by the differential regulation of coexpressed genes.

Commercial seaweed extracts are known to induce abiotic stress tolerance in plants (Rayirath et al., 2009; Spann and Little, 2011; Zhang and Ervin, 2004). However, the molecular responses elicited by the extracts and the nature of the chemical components present in those extracts are poorly characterized. Analysis of the molecular mechanisms of action can lead to unraveling novel mechanisms and genes that regulate abiotic stress tolerance in plants in general. By using organic extracts of *A. nodosum* extract that impart salt tolerance in Arabidopsis, we have identified a *pmei* as a novel, negative regulator of NaCl tolerance, suggesting that such an approach may potentially be useful in improving plant abiotic stress tolerance.

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