

Transcriptional Profiling Implicates Novel Interactions between Abiotic Stress and Hormonal Responses in *Thellungiella*, a Close Relative of *Arabidopsis*^{1[W]}

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Thellungiella, an *Arabidopsis* (*Arabidopsis thaliana*)-related halophyte, is an emerging model species for studies designed to elucidate molecular mechanisms of abiotic stress tolerance. Using a cDNA microarray containing 3,628 unique sequences derived from previously described libraries of stress-induced cDNAs of the Yukon ecotype of *Thellungiella salsuginea*, we obtained transcript profiles of its response to cold, salinity, simulated drought, and rewatering after simulated drought. A total of 154 transcripts were differentially regulated under the conditions studied. Only six of these genes responded to all three stresses of drought, cold, and salinity, indicating a divergence among the end responses triggered by each of these stresses. Unlike in *Arabidopsis*, there were relatively few transcript changes in response to high salinity in this halophyte. Furthermore, the gene products represented among drought-responsive transcripts in *Thellungiella* associate a down-regulation of defense-related transcripts with exposure to water deficits. This antagonistic interaction between drought and biotic stress response may demonstrate *Thellungiella*'s ability to respond precisely to environmental stresses, thereby conserving energy and resources and maximizing its survival potential. Intriguingly, changes of transcript abundance in response to cold implicate the involvement of jasmonic acid. While transcripts associated with photosynthetic processes were repressed by cold, physiological responses in plants developed at low temperature suggest a novel mechanism for photosynthetic acclimation. Taken together, our results provide useful starting points for more in-depth analyses of *Thellungiella*'s extreme stress tolerance.

Thellungiella salsuginea, also known as *Thellungiella halophila*, is an emerging model species for the molecular elucidation of abiotic stress tolerance (Bressan et al., 2001; Wong et al., 2005). *Thellungiella* is native to harsh environments, and to date two ecotypes are being developed for research.

The Shandong ecotype of *Thellungiella* grows in the high-salinity coastal areas in eastern China and has been proposed to be an appropriate relative of *Arabidopsis* (*Arabidopsis thaliana*) for studies of salinity tolerance mechanisms (Bressan et al., 2001; Inan et al., 2004). On the other hand, the Yukon ecotype was isolated

in the Takhini Salt Flats near Whitehorse in the Yukon Territories, Canada, a subarctic and semiarid region (Warwick et al., 2004) characterized by multiple simultaneous abiotic stresses, including cold, drought, and high salinity. Under controlled conditions, both ecotypes can tolerate salinity as high as 500 mM NaCl (Inan et al., 2004; E.A. Weretilnyk, personal communication). In addition, the Yukon ecotype of *Thellungiella* survives freezing to -18°C (M. Griffith, M. Timonin, and M. Saldanha, unpublished data) and can withstand water losses in excess of 40% of its fresh weight (E.A. Weretilnyk, personal communication), which are conditions far more extreme than those tolerated by *Arabidopsis*. Moreover, like *Arabidopsis*, *Thellungiella* displays many features characteristic of a genetic model system, including a short life cycle, self-fertility, transformability, and small genome size. Particularly important for this research is its high sequence similarity to *Arabidopsis* (Wang et al., 2004; Wong et al., 2005).

Transcript-profiling experiments using the *Arabidopsis* GeneChip array or full-length cDNA microarrays have shown that extensive changes occur in the transcriptome of *Arabidopsis* in response to drought, cold, or salinity stresses (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002b). In fact, Kreps and coworkers reported that approximately 30% of the

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transcriptome on the Arabidopsis GeneChip 8 K oligoarray changed in association with stress treatments (Kreps et al., 2002).

Since expressed sequence tag analyses of *Thellungiella* clones revealed 90% to 95% identities between *Thellungiella* and Arabidopsis cDNA sequences (Wang et al., 2004; Wong et al., 2005), Arabidopsis microarrays have recently been used to perform systematic analyses of the gene expression profile in *Thellungiella* undergoing abiotic stress treatments (Taji et al., 2004; Volkov et al., 2004). Due to the specificity of the Arabidopsis GeneChip 22 K oligoarray, only one-fifth of the probes showed a significant signal after hybridization with Shandong ecotype mRNA. Of these, only 19 genes (0.5% of probe sets showing significant signals) were significantly up- or down-regulated after 5 d of 100 mM salt treatment. When an Arabidopsis full-length 8 K cDNA microarray was used in a comparative genomics study in salt tolerance between Arabidopsis and the Shandong ecotype of *Thellungiella*, only six genes were found to be differentially expressed by salt treatments in *Thellungiella* (Taji et al., 2004). This is attributed to a constitutive high level of expression of stress-responsive genes in Shandong ecotype in comparison to Arabidopsis and was proposed to be contributing to the extreme salt tolerance of *Thellungiella* (Taji et al., 2004). The constitutive high level of expression of stress-responsive genes in Shandong ecotype was also reported by a more recent study using a 25 K Arabidopsis 70-mer oligoarray that compared the transcript profiles of Arabidopsis and *Thellungiella* subjected to NaCl treatment (Gong et al., 2005). Despite these findings, the transcriptional responses of *Thellungiella*, in particular, the Yukon ecotype, to drought, cold, and rewatering after drought have yet to be explored.

Previously, we have reported the generation of 3,628 nonredundant cDNAs derived from stress-induced libraries of the Yukon ecotype of *Thellungiella* (Wong et al., 2005). These sequences were used to construct a cDNA microarray that has representative coverage of *Thellungiella* genome and is enriched for genes whose expression is associated with stress. Here we have used this microarray to perform an initial analysis of the molecular response of the Yukon ecotype of *Thellungiella* subjected to cold, exposure to salt, simulated drought, or simulated drought treatment followed by rewatering. The resulting expression profiles, though incomplete as they are limited to the transcripts represented by the array, provide a framework of molecular processes underlying abiotic stress tolerance in *Thellungiella*, which can be complemented at the level of proteins and metabolites.

Our experimental design primarily focuses on a slow and prolonged stress treatment applied to plants under controlled conditions (see "Materials and Methods") so as to approximate the type of exposure to individual stresses encountered by *Thellungiella* in the field. This approach contrasts with previous microarray studies in that they have focused on rapid, short-

term abiotic stress treatments to identify genes involved in signaling pathways and transcriptional regulation of abiotic stress-induced genes in plants (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002b; Taji et al., 2004). A consideration in this approach is that a rapidly imposed stress can result in a greater number of injury-related responses (for review, see Bray, 2002) and may not reveal adjustments required for long-term survival under stressful environmental conditions.

This study compares three stresses (cold, low water availability, and saline conditions) as well as recovery from water deficits in *Thellungiella*, using an expression-profiling strategy. We identify some intriguing features associated with *Thellungiella*'s stress responses. The lower than expected degree of overlap among genes responsive to drought, cold, or salinity suggested that there are relatively few common end responses triggered by these stresses. In addition to activating the expression of some well-known stress-responsive genes, *Thellungiella* was found to down-regulate a large number of biotic stress-related genes under drought and salinity treatments. This infers the employment of a precise defense strategy under conditions of osmotic stress. A large number of photosynthetic-related transcripts were repressed in responses to cold stress. However, plants developed at low temperature demonstrated a high tolerance to photoinhibition of photosynthesis, which is difficult to reconcile based on known photoprotective processes. This suggests an important and perhaps novel photosynthetic response of *Thellungiella* to low temperature.

RESULTS

Physiological Characterization of Simulated Drought and Salinity Treatments

Water and solute potential measurements were carried out for well-watered control plants and plants that were subjected to simulated drought or saline conditions (see "Materials and Methods"). We consistently found, under the experimental conditions used, that *Thellungiella* was visibly wilted after 3 d of withholding water, when the leaf relative water content is about 65%. As shown in Table I, the leaf solute potentials became more negative as the plants were salinized or subjected to simulated drought, an indication that the solutes have become more concentrated under these conditions. In contrast to the salinity treatment, solute accumulation for drought-treated plants is accompanied by a loss of turgor, but both turgor and solute concentrations return to values comparable to those for control plants upon rewatering. Thus, our osmotic stress treatment strategies elicit stress-related responses as monitored by changes in plant water relations under the controlled experimental conditions we utilized.

Table I. Physiological characterization of *Thellungiella* subjected to osmotic stress

Leaf water potential (ψ_w), solute potential (ψ_s), and relative water content (RWC) measurements were carried out for well-watered *Thellungiella* and those that were subjected to saline or simulated drought conditions in growth cabinets. N/A indicates RWC measurements of leaves from salt-stressed plants are not available.

Treatment	Ψ_w	Ψ_s	RWC
	MPa	MPa	
None (4-week-old well-watered plant)	-0.94 ± 0.11	-1.43 ± 0.11	83.9 ± 1.0
300 mM NaCl	-2.62 ± 0.16	-2.86 ± 0.13	N/A
Drought	-1.99 ± 0.14	-1.99 ± 0.14	64.1 ± 2.8
Drought + rewater	-1.06 ± 0.06	-1.39 ± 0.12	84.5 ± 2.0

Identification of Stress-Responsive Genes

To identify stress-associated genes using the *Thellungiella* cDNA microarrays, we have employed analysis methods that avoid the determination of transcriptional changes using cutoffs based upon fold-changes. Instead, an empirical Bayesian strategy was used to assess the significance of each treatment for each gene as outlined in "Materials and Methods" (Labbe, 2005; Labbe and Thompson, 2005).

Using this statistical test, we found the abundance of 101, 76, 22, and 46 transcripts to be significantly changed under drought, cold, high-salinity, and rewatering conditions, respectively. These transcripts were assigned a Munich Information Center for Protein Sequences (MIPS) protein code based on the best BLAST match. They range in fold-change relative to the corresponding control from 0.09 to 31 (Table II). The former corresponds to a gene encoding a member of the protease/lipid transfer protein (LTP) family (At2g10940), while the latter is a sequence annotated as *rab18* (At5g66400). The number of transcripts regulated by salinity was less than one-fifth and one-third of that of drought- and cold-regulated genes, respectively. This low level of transcriptional change has also been reported in two previous microarray studies of salinity effects using the Shandong ecotype of *Thellungiella* (Taji et al., 2004; Volkov et al., 2004).

We performed semiquantitative reverse transcription (RT)-PCR analysis on six randomly selected genes to check the validity of the microarray analysis (Fig. 1; Supplemental Table I). The transcript that has the best BLAST match to Arabidopsis UBQ10 (At4g05320) has similar intensity values across all datasets and hence was used as an internal control. In all cases, the expression profiles obtained by semiquantitative RT-PCR were in agreement with those provided by the microarray analysis (Table II).

The Venn diagrams in Figure 2 show the commonalities of transcript changes among the stresses studied. These could be distributed into shared and stress-specific responses. Based on their gene expression patterns, the transcripts represented in Figure 2A were further classified into 13 clusters. The annotation and fold-change for all the corresponding transcripts for each cluster are listed in Table II. Rewatering-responsive transcripts were compared with drought-responsive

transcripts in a Venn diagram in Figure 2B, and their expression profiles were classified into six different groups as listed in Supplemental Table II.

Stimulus-Specific Responses

Clusters 1 and 6 contain a total of 61 transcripts that among the three stresses only respond to drought treatment. They represent 59% of all the 101 drought-regulated mRNAs. The greatest fold of induction was detected for a transcript encoding a homolog to a putative Arabidopsis alkaline α -galactosidase that contains the PFAM profile of raffinose synthase (At3g57520), an enzyme involved in the biosynthesis of the raffinose family of oligosaccharides. Various genes previously reported to be abscisic acid (ABA) induced (Seki et al., 2002a) were also found to be up-regulated by drought. These ABA-responsive genes encode proteins that include galactinol synthase (At1g56600), ERD14 (At1g76180), LEA protein (At1g52690), protein phosphatase 2c (At1g72770), RD22 (At5g25610), and nodulin MtN3 family (At5g13170; Seki et al., 2002a), implicating the role of ABA in regulating drought responses in *Thellungiella*. This is consistent with the well-established role of ABA in drought stress (for review, see Zhu, 2002). Increases in transcript abundance were also found for genes involved in reactive oxygen species scavenging. It is well documented that abiotic stresses lead to overproduction of reactive oxygen species that cause damage to other molecules and cell structures (for review, see Apel and Hirt, 2004). For example, increased transcripts of *metallothionein* (At3g09390), *peroxidase 42* (At4g21960), and *glutathione S-transferase* (At2g02390) encode proteins that could potentially function as scavengers for reactive oxygen species that arise as a consequence of drought.

Cluster 6 contains transcripts that are repressed under drought and, interestingly, these include defense-related transcripts that encode β -1,3-glucanase (At3g57240) and osmotin-like protein (At4g11650). Similar transcripts have been reported to be induced by abiotic stimuli in pepper (*Capsicum annuum*) or Arabidopsis (Hong and Hwang, 2002; supplemental data in Seki et al., 2002a; supplemental data in Seki et al., 2002b).

Table II. Clustering of transcripts expressed differentially under drought, cold, or salinity treatment

A total of 148 transcripts are clustered using STATISTICA (Stats Soft) into 13 clusters as listed.

MIPs ^a	Gene ^b	Ratio of Expression ^c		
		Drought	Cold	Salinity
Cluster 1: Drought-induced transcripts				
At4g04020	Plastid-lipid-associated protein PAP, putative/fibrillin, putative	2.37		
At1g13930	Expressed protein	2.45		
At5g61820	Expressed protein	2.48		
At1g76180	Dehydrin (ERD14)	2.48		
At4g23630	Reticulon family protein (RTNLB1)	2.54		
At5g23750	Expressed protein	2.57		
At3g15190	Chloroplast 30S ribosomal protein S20, putative	2.71		
At1g76590	Zinc-binding family protein	2.74		
At4g19230	Cytochrome P450 family protein	2.75		
At2g18960	ATPase 1, plasma membrane type, putative/proton pump 1	2.96		
At2g02390	Glutathione S-transferase ζ 1 (GSTZ1) (GST18)	3.05		
At4g21960	Peroxidase 42 (PER42) (P42) (PRXR1)	3.15		
At1g52380	Ran-binding protein 1 domain-containing protein	3.16		
At1g27540	F-box family protein	3.21		
At3g57020	Strictosidine synthase family protein	3.28		
At2g18050	Histone H1 to H3 (HIS1-3)	3.34		
At1g52690	LEA protein, putative	3.37		
At5g42050	Expressed protein	3.4		
At5g60360	Cys proteinase AALP	3.47		
At1g72770	Protein phosphatase 2C P2C-HA/PP2C P2C-HA (P2C-HA)	3.51		
At5g53870	Hypothetical protein	3.75		
At5g42800	Dihydroflavonol 4-reductase	3.77		
At3g03640	Glycosyl hydrolase family 1 protein	3.9		
At5g25610	Dehydration-induced protein RD22	4.23		
At1g80130	Expressed protein	4.23		
At1g56600	Galactinol synthase, putative	4.55		
At3g09390	Metallothionein protein, putative (MT2A)	4.61		
At4g33550	Protease inhibitor/seed storage/LTP family protein	5.74		
At5g13170	Nodulin MtN3 family protein	6.12		
At3g57520	Alkaline α -galactosidase, putative	7.75		
Cluster 2: Drought- and cold-induced transcripts				
At2g05440	Gly-rich protein	2.52	4.52	
At4g14690	ELIP, putative	2.55	9.62	
At4g02280	Suc synthase, putative	2.56	2.87	
At1g11910	Aspartyl protease family protein	2.62	3.12	
At3g12490	Cys protease inhibitor, putative/cystatin, putative	2.64	2.85	
At1g54100	Aldehyde dehydrogenase, putative/antiquitin, putative	2.7	2.55	
At1g67360	Rubber elongation factor (REF) family protein	3.33	2.72	
At2g42530	Cold-responsive protein/cold-regulated protein (cor15b)	3.38	12.05	
At4g20260	DREPP plasma membrane polypeptide family protein	3.76	2.67	
At2g39800	Delta 1-pyrroline-5-carboxylate synthetase A/P5CS A (P5CS1)	3.89	3.79	
At4g33150	Lys-ketoglutarate reductase	5.04	3.45	
At5g15970	Cold-regulated protein COR6.6 (stress-induced protein KIN2)	5.53	8.47	
At1g20440	Dehydrin (COR47)	5.93	4.76	
At5g15650	Reversibly glycosylated polypeptide-3	6.64	4.81	
At1g20450	Dehydrin (ERD10)	8.01	2.41	
At1g77120	ADH	12.92	7.25	
At3g22840	ELIP	13.59	16.13	

(Table continues on following page.)

Table II. (Continued from previous page.)

MIPS ^a	Gene ^b	Ratio of Expression ^c		
		Drought	Cold	Salinity
Cluster 3: Drought- and cold-induced, and salinity-repressed transcripts				
At5g45890	Senescence-specific Cys protease SAG12	5.73	12.2	0.33
Cluster 4: Cold-induced transcripts				
At4g16170	Expressed protein		2.04	
At3g55610	Delta 1-pyrroline-5-carboxylate synthetase B		2.18	
At3g56210	Expressed protein		2.18	
At3g21670	Nitrate transporter (NTP3)		2.25	
At1g13440	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic		2.28	
At3g15310	Expressed protein		2.33	
At4g13250	Short-chain dehydrogenase/reductase (SDR) family protein		2.49	
At1g17420	LOX, putative		2.5	
At5g13930	Chalcone synthase (naringenin-chalcone synthase)		2.52	
At1g17100	SOUL heme-binding family protein		2.65	
At1g74050	60S ribosomal protein L6 (RPL6C)		2.69	
At2g39460	60S ribosomal protein L23A (RPL23aA)		2.71	
At1g62570	Flavin-containing monooxygenase family protein		2.74	
At2g29630	Thiamine biosynthesis family protein/thiC family protein		2.98	
At2g23600	Hydrolase, α/β -fold family protein		3.11	
At5g23060	Expressed protein		3.13	
At1g06430	FtsH protease, putative		3.18	
At2g24280	Ser carboxypeptidase S28 family protein		3.21	
At3g22120	Protease inhibitor/seed storage/LTP family protein		3.42	
At2g02100	Plant defensin-fusion protein, putative (PDF2.2)		3.68	
At3g26210	Cytochrome P450 71B23, putative (CYP71B23)		3.82	
At5g64570	Glycosyl hydrolase family 3		3.82	
At4g02920	Expressed protein		4.9	
At5g44420	Plant defensin protein, putative (PDF1.2a)		6.33	
At4g12470	Protease inhibitor/seed storage/LTP family protein		11.49	
Cluster 5: Drought-, cold-, and salinity-induced transcripts				
At5g52300	Low-temperature-induced 65-kD protein	3.88	3.29	11.76
At5g59310	LTP4	6.53	3.33	2.54
At5g66400	Dehydrin RAB18-related protein (sp P30185)	31.27	3.41	10.42
Cluster 6: Drought-repressed transcripts				
At4g02380	LEA3 family protein	0.24		
At5g38410	Rubisco small subunit 3b	0.28		
At5g14120	Nodulin-related protein	0.29		
At1g72610	Germin-like protein (GER1)	0.29		
At3g57270	Glycosyl hydrolase family 17 protein	0.32		
At4g35010	Glycosyl hydrolase family 35 protein	0.32		
At2g06850	Xyloglucan:xyloglucosyl transferase	0.33		
At2g02860	Suc transporter/Suc-proton symporter (SUC3)	0.34		
At5g65730	Xyloglucan endotransglycosylase, putative	0.34		
At3g04790	Ribose 5-phosphate isomerase related	0.34		
At4g22310	Expressed protein	0.35		
At5g01590	Expressed protein	0.35		
At3g26650	Glyceraldehyde 3-phosphate dehydrogenase A, chloroplast	0.35		
At4g15510	PSII reaction center PsbP family protein	0.36		
At1g09340	Expressed protein	0.36		
At1g21120	O-methyltransferase, putative	0.37		
At3g29590	Transferase family protein	0.37		
At1g76080	Thioredoxin family protein	0.37		
At2g26250	β -Ketoacyl-CoA synthase family (FIDDLEHEAD) (FDH)	0.37		
At3g57240	β -1,3-Glucanase (BG3)	0.38		

(Table continues on following page.)

Table II. (Continued from previous page.)

MIPS ^a	Gene ^b	Ratio of Expression ^c		
		Drought	Cold	Salinity
At3g02170	Expressed protein	0.38		
At3g16370	GDSL-motif lipase/hydrolase family protein	0.39		
At1g58684	40S ribosomal protein S2, putative	0.4		
At3g21055	PSII 5-kD protein, putative	0.41		
At1g28380	Expressed protein	0.41		
At1g11860	Aminomethyltransferase, putative	0.43		
At2g44490	Glycosyl hydrolase family 1 protein	0.43		
At2g39330	Jacalin lectin family protein	0.44		
At4g24620	Glc-6-phosphate isomerase, putative	0.45		
At1g49750	Leu-rich repeat family protein	0.52		
At4g11650	Osmotin-like protein (OSM34)	0.53		
Cluster 7: Drought- and cold-repressed transcripts				
At2g10940	Protease inhibitor/seed storage/LTP family protein	0.09	0.21	
At3g16530	Legume lectin family protein	0.15	0.21	
At1g20340	Plastocyanin	0.34	0.25	
At1g71710	Inositol polyphosphate 5-phosphatase, putative	0.38	0.32	
At5g64040	PSI reaction center subunit PSI-N precursor	0.41	0.37	
At5g25980	Glycosyl hydrolase family 1	0.46	0.38	
Cluster 8: Drought-, cold-, and salinity-repressed transcripts				
At5g54270	Light-harvesting chlorophyll <i>a/b</i> -binding protein	0.36	0.26	0.33
At1g15820	Chlorophyll <i>a/b</i> -binding protein, chloroplast (LHCB6)	0.43	0.31	0.28
Cluster 9: Cold-repressed transcripts				
At1g58270	Meprin and TRAF homology domain-containing protein		0.15	
At3g09260	Glycosyl hydrolase family 1 protein		0.21	
At3g54890	Chlorophyll <i>a/b</i> -binding protein/LHCI type I		0.23	
At1g61520	Chlorophyll <i>a/b</i> -binding protein/LHCI type III		0.24	
At3g61470	Chlorophyll <i>a/b</i> -binding protein/LHCA2		0.26	
At5g54160	<i>O</i> -methyltransferase 1		0.28	
At3g47470	Chlorophyll <i>a/b</i> -binding protein 4, chloroplast/LHCI type III		0.29	
At1g08380	Expressed protein		0.33	
At5g40890	CLC-a chloride channel protein		0.33	
At4g11310	Cys proteinase, putative		0.34	
At1g52030	Myosinase-binding protein, putative (F-ATMBP)		0.35	
At5g64040	PSI reaction center subunit PSI-N precursor		0.37	
At5g20230	Plastocyanin-like domain-containing protein		0.37	
At2g06520	Membrane protein, putative		0.37	
At4g10340	Chlorophyll <i>a/b</i> -binding protein CP26, chloroplast/LHClc		0.39	
At4g22880	Leucoanthocyanidin dioxygenase/anthocyanidin synthase		0.39	
Cluster 10: Salinity-induced and cold-repressed transcripts				
At1g52400	β -Glucosidase, putative (BG1)		0.38	2.43
At2g22170	Lipid-associated family protein		0.14	2.04
Cluster 11: Salinity-repressed transcripts				
At1g31580	Expressed protein			0.38
At3g27690	Chlorophyll <i>a/b</i> -binding protein (LHCB2:4)			0.39
At3g50820	Oxygen-evolving enhancer protein, chloroplast, putative			0.4
Cluster 12: Drought- and salinity-repressed transcripts				
At1g06680	PSII oxygen-evolving complex 23 (OEC23)	0.41		0.42
At1g61660	Basic helix-loop-helix family protein	0.4		0.39
At1g75040	Pathogenesis-related protein 5 (PR-5)	0.31		0.35
At2g39730	Rubisco activase	0.42		0.41
At2g43590	Chitinase, putative	0.18		0.29
At3g01500	Carbonic anhydrase 1, chloroplast	0.33		0.32

(Table continues on following page.)

Table II. (Continued from previous page.)

MIPS ^a	Gene ^b	Ratio of Expression ^c		
		Drought	Cold	Salinity
At5g01980	Zinc-finger (C3HC4-type RING finger) protein family	0.36		0.42
At5g21274	Calmodulin-6 (CAM6)	0.33		0.36
At5g41550	Disease resistance protein (TIR-NBS-LRR class), putative	0.29		0.35
Cluster 13: Salinity- and cold-repressed transcripts				
At5g01530	Light-harvesting chlorophyll <i>a/b</i> -binding protein		0.33	0.41
At1g31330	PSI reaction center subunit III family protein		0.37	0.4

^aMIPS protein code. ^bAnnotation for all the corresponding transcripts based on best BLAST match for each cluster is given. ^cRatio of expression is the normalized signal intensity of treated sample over that of untreated control and corresponds to an average value from six replicates for each probe set.

We found 25 transcripts to be specifically expressed following a 3-week exposure to cold treatment (Cluster 4; Table II). Among these transcripts are 17 cold-responsive transcripts that encode a putative lipoxygenase (LOX; At1g17420), two members of the plant defensin protein (PDF1.2a and PDF 2.2), and chalcone synthase (At5g44420; At2g02100; At5g13930; Cluster 4; Table I). According to AraCyc, the Arabidopsis Biochemical Pathway tool (<http://www.arabidopsis.org/tools/aracyc/>), this putative LOX is predicted to be involved in the biosynthesis of the phytohormone jasmonic acid (JA; Bell and Mullet, 1993). The up-regulation of a LOX with a concomitant increase of PDF1.2a, a known JA-mediated defense marker (Penninckx et al., 1998), strongly implicates an increase of JA levels in response to cold. In addition, expression of chalcone synthase, a key enzyme in phenylpropanoid biosynthesis that is known to be transcriptionally regulated by JA (Richard et al., 2000) was also found in the same cluster. To date, only one report describes the changes in the Arabidopsis transcriptome associated with short- as well as long-term cold stresses (Fowler and Thomashow, 2002). Intriguingly, neither the transcript for the JA biosynthetic enzyme nor PDF1.2 is reported to be up-regulated by long-term cold treatment in Arabidopsis (supplemental data in Fowler and Thomashow, 2002). However, it is important to note that the long-term cold stress in that study was only 7 d in duration (Fowler and Thomashow, 2002).

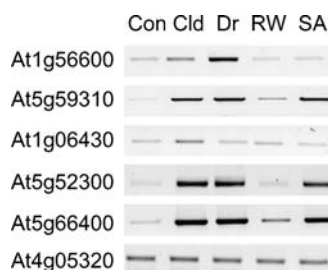


Figure 1. Verification of microarray analysis by RT-PCR. RT-PCR analysis was carried out under linear amplification conditions for six orthologs of Arabidopsis as shown. An ortholog of Arabidopsis UBC10 (At4g05320) was used as an internal control. Con, Control; Cld, cold; Dr, drought; SA, salinity.

Our analysis identified 26 cold-repressed transcripts (Cluster 9; Table II); one-half encode products that have putative functions associated with photosynthesis. These include genes that encode chlorophyll *a/b*-binding proteins, plastocyanin, and PSI subunit proteins. Similar genes have been reported to be repressed in Arabidopsis "cold" microarray studies (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002b). Based on these findings, we further examined plants that were allowed to develop at low temperature for their tolerance to photoinhibition (Fig. 3A) and their capacity for nonphotochemical quenching (NPQ), a major photoprotective mechanism (Fig. 3B; Müller et al., 2001). It is well established that development at low temperature results in an increased tolerance to photoinhibition (Huner et al., 1993; Gray et al., 1996, 2003), in part due to a reprogramming of photosynthetic carbon metabolism (Stitt and Hurry, 2002; Gray and Heath, 2005). Plants of *Thellungiella* grown under standard conditions demonstrated a 54% reduction in the maximum quantum yield of PSII photochemistry as measured by the F_v/F_m ratio in response to the 4-h photoinhibitory treatment (Fig. 3A). In contrast, plants developed at low temperature exhibited only a 10% reduction in F_v/F_m in response to the same photoinhibitory treatment (Fig. 3A). The capacity for NPQ formation was observed to be slightly higher in the plants developed at low temperature, although the difference was minimal (Fig. 3B).

Surprisingly, only three genes were specifically responsive to the salinity treatment. These transcripts are represented by Cluster 11 (Table II) and are all down-regulated relative to the control. Two of the genes encode products associated with photosynthesis (chlorophyll *a/b*-binding protein and oxygen-evolving enhancer protein), whereas the third is an expressed protein of unknown function.

Shared Responses

Clusters 2, 3, 5, 7, 8, 10, 12, and 13 consist of transcripts that are differentially regulated by more than one stress treatment relative to the control; the

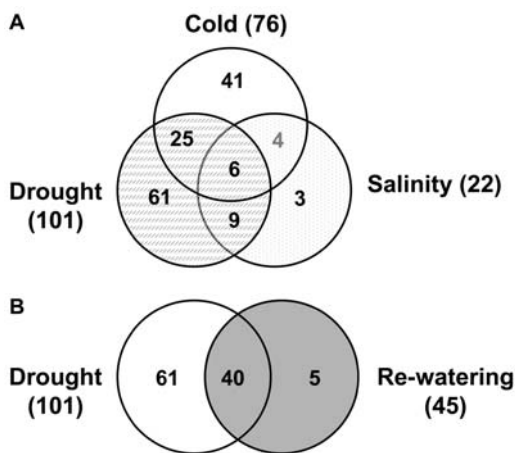


Figure 2. Changes in transcript abundance in *Thellungiella* subjected to abiotic stress. A and B, Venn diagrams showing the number of transcripts differentially regulated during drought, cold, or high-salinity treatment (A), or drought or rewatering after drought (B). The total number of transcripts for each treatment is indicated in parentheses.

corresponding transcripts for each class are listed in Table II. As shown in Figure 2A, there was greater overlap between the drought- and salinity-induced changes as compared to the changes caused by drought and cold stresses. Out of 101 and 22 transcripts differentially regulated by drought treatment and salinity, respectively, there was an overlap of 15 sequences (68% of salinity-regulated transcripts), and all were repressed by both treatments. Based on best BLAST match, the corresponding gene products can be categorized under three major functional groups: signaling, photosynthesis, and defense related. A member of the basic helix-loop-helix transcription factor family (At1g61660) and a calmodulin-6 (At5g21274) represent the signaling group, whereas a putative chitinase (At2g43590), a pathogenesis-related protein (At1g75040), and a disease resistance protein (At5g41550) constitute the defense-related group. Meanwhile, 41% of the cold-regulated transcripts were also responsive to drought treatment. Among them were well-known

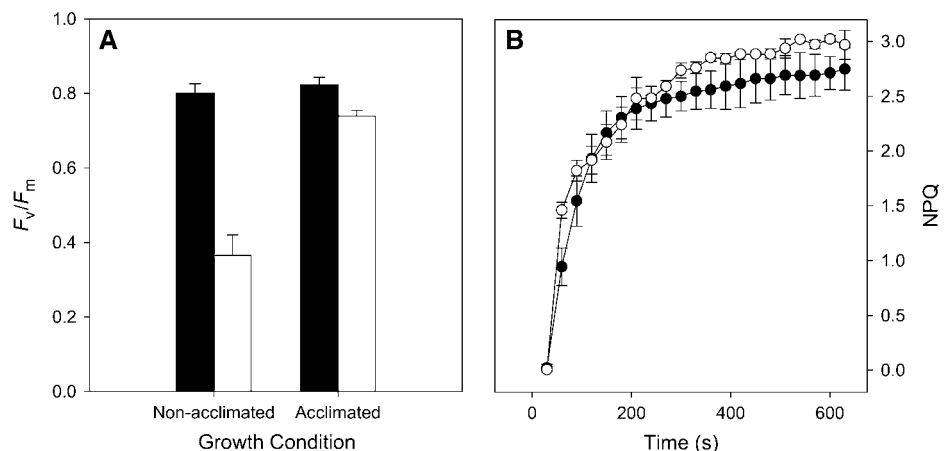
cold- and drought-induced genes, such as *ELIP* (early light inducible), *cor15b* (cold-regulated protein), *cor47*, *erd10* (early responsive to dehydration), *cor6.6*, *Adh* (alcohol dehydrogenase), and *P5CS A* (delta 1-pyrroline-5-carboxylate synthetase A; At2g39800), a gene encoding a product associated with Pro biosynthesis (Kreps et al., 2002; Seki et al., 2002b).

There is a higher percentage (68%) of genes that are also drought regulated in the salinity dataset when compared to that of the cold dataset (41%). This extensive overlap between the drought- and salinity-induced changes hints of commonalities between stress responses elicited by the drought and salinity treatments we used, suggesting cross talk between responses triggered by these stresses. This is consistent with previous observations on the greater overlap of drought- and high salinity-responsive gene expression in *Arabidopsis* (67%) in comparison to that of drought and cold (41%; Seki et al., 2002b).

There were four transcripts that are cold and salinity regulated (Clusters 10 and 13; Table II). Genes encoding a putative β -glucosidase (At1g52400) and a member of lipid-associated protein family (At2g22170) were up-regulated under salinity treatment but repressed by cold (Cluster 13; Table II). No known function or molecular basis of their antagonistic responsiveness has been established for either corresponding protein.

The expression of 149 transcripts changed in association with at least one stress treatment; only six of these responded to all stress treatments (Fig. 2A). This suggests that the stress-responsive mechanisms in *Thellungiella* are divergent for each of these stresses, as reflected by the lack of sequence overlap in our previously reported expressed sequence tag collections of stress-induced libraries (Wong et al., 2005). Transcripts elevated by all three stresses encode the low temperature-induced 65-kD protein (RD29B; At5g52300), a lipid transfer protein 4 (LTP4; At5g59310), and the dehydrin Rab18 (At5g66400). The increase of *rd29b* and *rab18* transcripts by abiotic stress is similar to that of *Arabidopsis*. For example, Seki and coworkers reported a greater than 2-fold increase for *rd29b* under all three stress treatments relative to the controls (Seki

Figure 3. Photosynthetic responses of *Thellungiella*. Plants were grown under standard growth conditions (Non-acclimated; ●) or developed at low temperature (Acclimated; ○) as described in "Materials and Methods." A, Photoinhibition at low temperature. Plants were exposed to 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 4°C for 4 h, and the F_v/F_m ratio was determined pretreatment (black bars) and posttreatment (white bars). B, Induction of NPQ. Induction occurred at room temperature with a photon flux of 1,400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. All values represent means \pm SD, $n = 2$ to 3.



et al., 2002b). Though an increase of *rab18* transcripts was not found in the same study (Seki et al., 2002b), a modest increase in the protein level has been reported in *Arabidopsis* after 7 d of cold treatment (Mantyla et al., 1995). Meanwhile, *LTP4* transcripts were highly abundant in a salt-induced library of the Shandong ecotype of *Thellungiella* (Wang et al., 2004), and our microarray analysis revealed that *LTP4* mRNAs, in addition to being induced by salt, are also induced by cold or drought treatment.

Drought and Rewatering Dataset

There were a total of 106 transcripts identified to be drought and/or rewatering responsive (Fig. 2B). Among these, 45 transcripts were differentially regulated by rewatering treatment when compared to the drought-treated control. The remaining 61 transcripts that were up- or down-regulated by drought were unchanged in rewatered plants relative to the drought sample.

We identified 19 rewatering-repressed and drought-inducible transcripts, and 21 rewatering-inducible and drought-repressed transcripts (Supplemental Table II). The former includes transcripts with homology to that encoding *Arabidopsis* dehydration-induced protein ERD10 (At1g20450), COR47 (At1g20440), and a LEA protein (At1g52690), while the latter consists of genes that may be repressed by drought and released from the repression by rewatering. This includes photosynthesis-related proteins and is consistent with photosynthesis being important in the process of recovery following rewatering.

DISCUSSION

Our microarray study primarily focused on *Thellungiella* exposed to various stresses over the course of days or weeks to identify genes that may be mechanistically involved in acclimation or stress resistance in the steady state. Among the 149 transcripts that changed in response to drought, cold, or salinity, only six were regulated by all three stresses (Fig. 2A). This is in spite of the fact that osmotic stress and the associated oxidative stress appear to be common consequences of exposure to drought, salinity, and cold (Apel and Hirt, 2004). The fact that the time period used to develop suitable drought and cold or salinity samples is not equivalent may have contributed to this finding. Nevertheless, the low degree of overlap among the transcripts associated with each stress suggests that the end responses triggered by these stresses are rather specific and suited to the particular stress conditions encountered.

Salinity-Induced Transcript Changes in Halophyte

The salinity dataset contains the fewest transcripts that were stress regulated when compared to drought

and cold datasets. This corresponds to less than 1% of the sequences represented by our array. A similar low degree of transcriptional change was revealed by other "stress" microarray studies in *Thellungiella* (Taji et al., 2004; Volkov et al., 2004). Furthermore, many well-known drought- and salt-responsive genes were differentially regulated by drought treatment but not by high salinity in *Thellungiella*. For example, the accumulation of raffinose, an osmolyte in response to drought, can be inferred from the increased level of the transcripts galactinol synthase (At1g56600) and raffinose synthase (At3g57520), but neither transcript was found in the dataset of salinity-induced transcripts. Raffinose family oligosaccharides have been proposed to act as osmoprotectants in some plant species to allow osmotic adjustment of plant cells exposed to water deficit (for review, see Rathinasabapathi, 2000). Other proposed roles for these compounds include free radical scavenging, protection from photoinhibition, and metabolic detoxification (Orthen et al., 1994; Pharr et al., 1995), all of which may help a plant to survive various environmental stresses. However, the actual role of these osmolytes in cellular stress tolerance remains equivocal and has recently been challenged (Tunnacliffe and Lapinski, 2003; Zuther et al., 2004).

Nevertheless, the low degree of mRNA changes in salt-acclimated *Thellungiella* is intriguing. The degree of fold-change for salinity stress-responsive transcripts relative to the control under our experimental conditions may be far less pronounced and hence lie beyond the detection sensitivity of microarray analysis. While it is possible that more dramatic transcriptional changes occur in the roots instead of leaves or at an earlier time point following salt treatment, this seems unlikely given that microarray experiments using roots and 2-h salt-treated tissues have been performed by others with similar results (Taji et al., 2004; Volkov et al., 2004). An alternative explanation is offered by the report that salt tolerance in *Thellungiella* is associated with specific features of high selectivity for K^+ accumulation and not Na^+ after NaCl application, a response that is unlike its glycophytic relative *Arabidopsis* (Volkov et al., 2004). Thus, this predicts that the innate ability of *Thellungiella* to maintain ion homeostasis may allow its adaptation to a high-salinity environment with few transcriptional changes, in contrast to *Arabidopsis*. The remarkable halophytic nature may also be closely linked to the finding that many of these stress-inducible genes in *Arabidopsis* are constitutively overexpressed in *Thellungiella* and hence offered a greater innate tolerance to salt stress (Taji et al., 2004).

Abiotic Stress and Photosynthesis

The photosynthetic system in higher plants is highly susceptible to abiotic stresses (Huner et al., 1998). Under low temperatures, for instance, absorbed light energy cannot be used productively due to the inhibition of photosynthetic CO_2 assimilation and other metabolic processes (Huner et al., 1998). Such energy

imbalance leads to an overexcitation of the photosynthetic apparatus that in turn increases the potential for photoinhibition and subsequently photooxidative damage (Huner et al., 1998). To avoid the energy imbalance resulting from abiotic stress, plants are able to down-regulate genes associated with photosynthetic light harvesting to reduce light energy absorbed. It is therefore not surprising that we found various photosynthesis-related genes repressed in our datasets, an observation that is consistent with previous similar microarray studies (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002b). In fact, these genes constitute the largest functional group of transcripts (15%) identified to be down-regulated in our analysis. It is established that in *Arabidopsis*, transcripts encoding products associated with photosynthesis, such as chlorophyll *a/b*-binding proteins and Rubisco, decrease after transfer to low temperature (Strand et al., 1997). However, photosynthesis is able to recover in plants that develop at low temperature, in part due to a reprogramming of photosynthetic carbon metabolism (Strand et al., 1997; Stitt and Hurry, 2002; Gray and Heath, 2005). Stress responses often initiate transient physiological, biochemical, and molecular perturbations (Huner et al., 1993; Stitt and Hurry, 2002). These transient responses lead to stable, long-term adjustments that reflect complex developmental responses to the new conditions (Huner et al., 1993, 1998; Gray et al., 1996; Stitt and Hurry, 2002). Our observation that *Thellungiella* developed at low temperature exhibits an extremely high tolerance to photoinhibition would indicate that photosynthetic acclimation to low temperature had occurred (Fig. 3A). Whether the mechanism for this unusually high tolerance to photoinhibition is similar to that utilized by *Arabidopsis* (Gray et al., 2003) will require further experimentation.

Among the photosynthesis-related genes that were repressed are those that encode Rubisco subunits, oxygen-evolving complex, PSI and PSII subunits, and chlorophyll *a/b*-binding proteins. However, only two members of this group of genes were consistently down-regulated by all three stress treatments, and both of them belong to the chlorophyll *a/b*-binding protein family. The fact that we did not see all photosynthesis-related genes repressed in the same manner across all three stresses may reflect the different impact that these stresses have on photosynthesis in the short as well as the long term. For example, in *Arabidopsis*, down-regulation of photosynthetic gene expression after cold treatment is associated with the accumulation of hexose phosphate and soluble sugars (Strand et al., 1997, 2003). This is similar to the carbohydrate-mediated repression that is observed when sugars accumulate for other reasons (Krapp et al., 1993; Jang and Sheen, 1994; Sheen, 1994; Krapp and Stitt, 1995). In our analysis, we have observed an increased level of transcripts associated with Suc synthase under cold or drought treatment that may correlate with the carbohydrate-mediated repression of photosynthesis reported upon shifting plants to the cold (Strand et al., 1997, 2003).

Unlike light-harvesting proteins that are down-regulated by drought, cold, and high-salinity treatments, *Thellungiella* accumulates transcripts of ELIP to different levels in response to cold and drought treatment (At4g14690 and At3g22840; Cluster 2; Table II). ELIPs accumulate in thylakoid membranes during light stress, where they bind chlorophylls and carotenoids and provide protection against photooxidation (Montané and Kloppstech, 2000; Hutin et al., 2003). NPQ is a complex process considered to be a major photoprotective mechanism in all plant species by which excess light energy is released as heat (Müller et al., 2001). In barley (*Hordeum vulgare*), the accumulation of ELIPs is correlated with greater NPQ (Potter and Kloppstech, 1993; Krol et al., 1999). It is tempting to speculate that the ability of *Thellungiella* to accumulate ELIPs in response to cold or drought treatment serves a role in the dissipation of excess light energy early in the establishment of stress tolerance. However, when we examined the ability of plants developed at low temperature to generate NPQ in comparison to those grown under standard growth condition, minimal differences were observed (Fig. 3B). Thus, it is unlikely that this would account for the increased tolerance to photoinhibition that we also observed in these plants (Fig. 3A). This may suggest that alternative mechanisms exist in *Thellungiella* for dealing with energy imbalances at the level of photosynthesis in response to low temperature.

ABA and Drought/Salinity-Induced Responses

The phytohormone ABA is involved in regulating diverse plant processes, including gene expression during abiotic and biotic stresses. More specifically, ABA is known to mediate the expression of many stress-responsive genes as a result of drought or saline conditions, but may have a limited role in cold regulation (for review, see Zhu, 2002; Shinozaki et al., 2003). The apparent up-regulation of several genes known to be ABA responsive among products in our dataset associated with drought offers indirect evidence for the involvement of ABA in regulating the response of *Thellungiella* to drought.

There was a concomitant decrease of various defense-related transcripts noted in the drought and salinity datasets. Interestingly, ABA has been found to enhance disease susceptibility (Ward et al., 1989; McDonald and Cahill, 1999; Mohr and Cahill, 2003) and lead to a down-regulation in the expression of disease-resistance genes (Rezzonico et al., 1998; Audenaert et al., 2002; Anderson et al., 2004). Together, these correlative changes suggest that the down-regulation of defense-related transcripts observed in our study is likely associated with an increase in endogenous ABA levels in *Thellungiella* in response to osmotic stress, although this has yet to be directly verified.

Intriguingly, a number of reports have shown an induction of defense-related genes by similar stresses in *Arabidopsis* (supplemental data in Seki et al., 2002a;

supplemental data in Seki et al., 2002b; Zimmermann et al., 2004). It is conceivable that the ability of this *Thellungiella* ecotype to tolerate semiarid conditions in the Yukon may be associated with its strategy to more precisely coordinate the antagonistic relationship between osmotic and biotic stress responses in comparison to plants that have a lower innate tolerance of stress, like *Arabidopsis*. A more recent study using a 25 K 70-mer *Arabidopsis* oligoarray that compared the transcript profiles of *Arabidopsis* and the Shandong ecotype of *Thellungiella* subjected to NaCl treatment have reached similar conclusions (Gong et al., 2005). It is likely that ABA is among the key players that allow the employment of a specific rather than general defense strategy. Although ABA has been known to mediate osmotic stress responses in *Arabidopsis*, there are reports that ABA-biosynthesis as well as ABA-responsive genes are more highly expressed in *Thellungiella* than in *Arabidopsis* (Taji et al., 2004; Gong et al., 2005).

The relatively lower percentage of ABA-responsive genes in our cold dataset in comparison to drought, in particular, is consistent with the emerging consensus that ABA may not play a critical role in the cold response (Seki et al., 2002a; Xiong and Zhu, 2002). For ABA-responsive genes induced by both cold and drought treatments, such as *Adh*, previous studies in *Arabidopsis* have shown induction by drought to be, in part, dependent on the action of ABA but the cold effect appears to be independent of ABA (Bruxelles et al., 1996).

Cell Wall-Related Transcripts

A number of transcripts encoding cell wall-related proteins are found in the drought-responsive transcript dataset. Transcripts encoding a xyloglucan:xyloglycosyl transferase (At2g06850) and a putative endotransglycosylase (At5g65730) were down-regulated, whereas some of those encoding a potentially cell wall-associated LTP family of proteins were up-regulated (At4g33550, At5g59310) by drought treatment. Similar proteins have been implicated in cell wall loosening (Nishitani and Tominaga, 1992; Nieuwland et al., 2005). The plant cell wall plays a critical role in cell enlargement, which is an essential process in plant growth and development (Cosgrove, 2001). In addition, it has been documented that increased cell wall plasticity could potentially be a strategy employed in ameliorating the shear forces that arise with the massive loss of volume in mesophyll during drought in grasses (Balsamo et al., 2005). Perhaps this change in the cell wall can also provide a way to prevent extensive water loss during drought.

A possible explanation for the apparent antagonistic regulation of the two groups of cell wall-related transcripts observed in the drought dataset is that developmental and environmental cues may trigger the action of different groups of cell wall-loosening enzymes. In fact, there are two classes of enzymes involved in the cell wall-loosening process (for review, see Cosgrove, 2001). First, there are the primary enzymes that are directly involved in cell wall loosening as well

as extension, which takes place during growth and development (Cosgrove, 2001). There are also secondary enzymes that act to weaken the wall without inducing wall extension (Cosgrove, 2001). There is minimum growth during drought and it is therefore conceivable that transcripts coding for the primary wall-loosening and extension proteins are repressed, while those that are responsible mainly for loosening the cell wall would be up-regulated, reflecting the need of *Thellungiella* for plastic cell walls in coping with drought stress. In line with this hypothesis is the down-regulation of two transcripts predicted to form the structural constituent of cell walls (At2g10940 and At1g21120) in the drought dataset.

JA Is Implicated in Mediating Cold Responses

Our analysis revealed transcriptional changes of some JA-related gene products (*LOX*, *PDF1.2a*, *chalcone synthase*) after cold treatment. The current knowledge of JA-related genes derives mostly from work on defense mechanisms during pathogenesis and wounding, and to some extent osmotic stress (for review, see Wasternack et al., 1998). A number of studies report that JA application in the form of methyl jasmonate could be used to reduce chilling injury in fruits (Gonzalez-Aguilar et al., 2004). It is likely that tissue damage associated with low temperature in chilling-sensitive plants induces responses similar to those occurring in pathogen-wounded plants. It is known that the primary site of injury (disruption) during a freeze-thaw cycle is the cellular membranes, particularly the plasma membrane (Steponkus, 1984). Furthermore, mechanical wounding, a process known to involve JA-mediated responses, has been reported to induce the cold regulatory pathway mediated by the CBF transcription factor in *Arabidopsis* (Cheong et al., 2002). These considerations and the correlative evidence from our analysis point to a potentially novel and exciting regulatory role of JA during cold responses in *Thellungiella*. Whether JA plays a critical role in the increase in freezing tolerance that occurs with cold acclimation of *Thellungiella* has to be examined further with metabolomics analysis and reverse-genetic approaches.

Transcript Changes Associated with Rewatering

For a plant to survive a drought stress, not only does it have to overcome drought-associated damages but also injuries that occur upon rewatering as a result of rapid water uptake (Stewart, 1989). Our experiments attempted to obtain insight into processes associated with the recovery process from a water deficit to gain a better understanding of the capacity of *Thellungiella* to recover from this stress. It is interesting to observe two patterns of expression for some well-known drought-responsive genes following rewatering after drought. Some transcripts, such as those for *cor15b* and *ERD14*, remained highly expressed 2 d after rewatering,

whereas others, including *KIN2* and *COR47*, were down-regulated upon rewatering. Whether these differences identify genes that play a direct role in recovery from a water deficit needs further investigation.

CONCLUSION

Our microarray analysis provides transcript profiles of drought, cold, high-salinity, or rewatering responses in *Thellungiella*. This analysis is a timely contribution to the emerging recognition of *Thellungiella* as a model species for the molecular elucidation of abiotic stress tolerance. Our results revealed interesting features and potentially valuable traits associated with the stress responses of *Thellungiella*. However, as with all microarray analyses, the interpretation of the transcript changes requires caution. Microarrays provide a sensitive but transient snapshot of gene expression. Furthermore, the changes in mRNA levels may not correlate with changes in protein or enzyme activity levels. Nevertheless, the results obtained from our microarray analysis provide useful starting points for more thorough investigation into the molecular mechanism behind the extreme stress tolerance of *Thellungiella*.

MATERIALS AND METHODS

Plant Materials and Stress Treatments

Plants of the Yukon ecotype of *Thellungiella salsuginea* (Pall.) O.E. Schulz (Al-Shehbaz et al., 1999; Cody, 2000) were grown in controlled environments with a day/night temperature regime of 22°C/10°C. An irradiance of 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ over a 21-h daylength was provided. When the plants were 4 weeks old, they were subjected to stress treatments as described below. Plants were harvested 8 h after the lights came on in the growth chamber for the microarray experiment.

For the cold treatments, plants were shifted to a day/night temperature regime of 5°C/4°C with all other conditions constant and leaves were sampled after 3 weeks. For the photosynthetic experiments, the plants were allowed to develop under these conditions for an additional 8 weeks. Drought stress was simulated in pots by withholding water from 1-month-old plants until they wilted visibly (3 d). For the drought and rewatering treatment, plants were subjected to drought treatment and then rewatered and allowed to recover for 2 d. To have plants acclimated to high salinity, plants were watered with NaCl solutions at concentrations that increased by 50 mM increment every 3 d until the final concentration reached 300 mM; tissues were harvested 3 d later.

Physiological Characterization of Simulated Drought and Salinity Treatments

Water and solute potentials and relative water content measurements were carried out for well-watered control plants, plants that were subjected to simulated drought or saline conditions, or rewatering after water deficits as described by Weretilnyk et al. (2001).

Photosynthetic Measurements and Photoinhibition

F_v/F_m and experiments examining the rapid induction of NPQ were determined in planta at room temperature using a PAM-2000 portable chlorophyll fluorometer (Heinz Walz GmbH) as detailed by Baerr et al. (2005).

Photoinhibition of photosynthesis was induced at 4°C by exposure to a photon flux density of 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the leaf surface for 4 h as described previously (Gray et al., 2003). Susceptibility to photoinhibition was quantified by monitoring changes in F_v/F_m .

RNA Extraction

Total RNA was extracted from only aboveground tissues as described previously (Wong et al., 2005) and were then purified using RNeasy columns (Qiagen). Three independent tissue collections and RNA extractions were performed for each of the treatments.

Amplification of cDNA Inserts and Microarray Preparation

A total of 3,628 unique clones derived from the abiotic stress libraries described previously (Wong et al., 2005) were used for the array printing. Based on sequence similarity, each of these probe sets was assigned an *Arabidopsis* (*Arabidopsis thaliana*) MIPS protein code as described by Wong et al. (2005). Target DNAs for spotting on the microarray were amplified by PCR using the SP6 and T7 promoter primers that flanked both sides of the cDNA inserts. Plasmid template (5 ng) was added to a 60- μL PCR mixture containing a total of 15 mM of each nucleotide, 60 μM of each primer, 120 mM MgCl_2 , 600 mM Tris-HCl, pH 8.8, 3 M KCl, 0.48% Nonidet P-40, and 3 units of Taq polymerase (MBI Fermentas). Inserts were amplified using 40 cycles (94°C for 30 s, 56°C for 45 s, 72°C for 2 min) with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 5 min. One microliter of each finished reaction was electrophoresed on a 1% agarose gel to confirm amplification quality and quantity. PCR products were then sent for purifications and printing on Corning GAPS II coated slides at the MicroArray Lab at the Biotechnology Research Institute (BRI), Montreal. Each target DNA was spotted in triplicate. A variety of control elements were also arrayed on the slide. These included multiple blank spots, buffer-only spots, and control DNA sequences derived from three transgenes (bacterial *ACC deaminase* gene, and fish *chemokine* and *IL-1 β* genes), housekeeping genes (*actin* and *tubulin*), and known abiotic stress-induced genes (*cor47*, *cor15a*, *rd20*, and *cbf1*).

Fluorescent Probe Preparation

Forty micrograms of total RNA was reverse transcribed to synthesize aminoallyl-labeled cDNA, followed by coupling of the aminoallyl groups to either Cyanine 3 or 5 (Cy3/Cy5) fluorescent molecules using protocol from The Institute of Genome Research (<http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml>). Anchored poly d(T) primers at a final concentration of 4 μM were used to prime the first-strand cDNA synthesis. Routinely, 2 ng of fish *IL-1 β* mRNA was spiked into the cDNA synthesis. The spiking probe added to labeling permitted an independent cross-check of the results.

Hybridization and Washing

The cDNA microarrays were hybridized with Cy3 and Cy5 fluorescently labeled probe pairs of untreated/drought 3 d, untreated/cold acclimated, untreated/salt acclimated, and rewatering after drought/drought 3 d. Three biological replicates with dye swap as technical replicate were examined for all experimental conditions. The statistical analysis was based on a total of six replicates per experimental condition (three biological replicates plus three technical replicates).

Microarrays were prehybridized, hybridized, and washed according to the protocol provided by the MicroArray Lab at BRI (http://www.bri.nrc.gc.ca/microarraylab/labelling_en.pdf). An extra step of dipping the slides in isopropanol was performed prior to air drying the slides.

Scanning and Data Analysis

ScanArray confocal scanning system and QuantArray data acquisition software (Perkin-Elmer) were used according to the manufacturer's instructions to capture the data. Normalization between the Cy3 and Cy5 fluorescent dye emission intensities was achieved by adjusting the level of the photomultiplier gains. The signal intensities for all 14,688 spots were quantified and local background fluorescence values were automatically calculated by the QuantArray program. After local background subtraction, the data were imported into an algorithm that used a Bayesian approach to assess the significance of each treatment for each gene (Labbe, 2005; A. Labbe and M.E. Thompson, unpublished data). This methodology combines Bayesian analysis and frequentist theory in the context of multiple comparisons. Using the posterior probability of overexpression as a test statistic, we are taking advantage of the flexibility brought by Bayesian models. This probability is eventually regarded in the same spirit as a *P* value in the Benjamini-Hochberg

procedure, to control for the multiplicity of the tests. By doing so, we are then taking advantage of both the control of false discoveries by using some well-known frequentist procedures and the flexibility brought by the Bayesian framework. The algorithm was implemented in an R environment for statistical analysis. Median values of triplicate spots for all probe sets were used in all calculations.

To correct for intensity-dependent bias and spatial bias simultaneously, a data transformation method called the Joint Lowess method (Cui et al., 2003) was used to normalize the data. The transformation was applied to each array individually. After normalization, differentially expressed genes between each control and treatment conditions were detected using a Bayesian model. The well-known gamma conjugate model, as described by Kendziorowski et al. (2003), was fitted to the data, and parameters were obtained in an empirical Bayes manner. Note that different parameters were considered for each of the four treatment conditions and analyses were carried out separately. For each treatment, posterior probabilities of gene overexpression were computed for each gene. It has been shown that this type of probability shares the same property of uniformity with a *P* value under the null hypothesis of no differential expression (Dudley and Haughton, 2002). Such probabilities can thus be used as an input in the Benjamini-Hochberg procedure, which usually takes *P* values as an input (Benjamini and Hochberg, 1995), to control the false discovery rate. A false discovery rate of 1% was chosen for this experiment to address the problem of multiple testing. Considering these posterior probabilities in the same spirit as a *P* value enables us to combine the best of each approach (frequentist and Bayesian) in a unique procedure. The mathematical validity of this approach has been demonstrated (Labbe, 2005; Labbe and Thompson, 2005).

The list of genes identified to be significantly up- or down-regulated was then clustered using STATISTICA (version 6.1; Stats Soft). A Euclidean matrix was used to calculate the distances, and a complete linkage method was then applied for hierarchical clustering of these genes.

RT-PCR Analysis

First-strand cDNA was prepared from 5 µg of total RNA with the Superscript RT II kit (Invitrogen) and oligo(dT)₁₈ according to the manufacturer's instructions. A 0.5-µL aliquot of the total RT reaction volume (20 µL) was used as template in a 25-µL semiquantitative RT-mediated PCR amplification reaction. Number of cycles used for the transcripts investigated was routinely between 20 and 25, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. Eight microliters of the PCR reaction was separated on 1% agarose gel containing 0.1 µg/µL ethidium bromide and visualized under UV light. The amount of amplified product was then estimated semiquantitatively using FluorChem Imaging System according to the manufacturer's instructions (Alpha Innotech).

Primers used were At1g56600F, CGAACCGTCTTTCAGCCACCG and At1g56600R, TGACGTACTGAAGCACACCGGCC; At5g59310F, AACCTAGAAAACAAAAGTCAACTAAATCT and At5g59310R, CTAGACTTGGTTGATGCTCTGT; At1g06430F, GGAGCTGATCTTGCAAACCTCTTG and At1g06430R, AGCCGACGAATCCATTAGCGAC; At5g52300F, ACA-GGATCAGCCGTGATGACG and At5g52300R, TATTTTCCCCTCCAATTC; At5g66400F, GGTTGGTTCCTTCCCAACATGGCGTCTTACCAGAA and At5g66400R, CGGAATCTTAAACGACCACCACCAGGAAGTTTATC; and At4g05320F, AGTCGACCTTCATTGGTG and At4g05320R, TGATAA-GTAAGCAGGCCG.

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