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Exogenous trehalose alters *Arabidopsis* transcripts involved in cell wall modification, abiotic stress, nitrogen metabolism, and plant defense

Hanhong Bae[a,*], Eliot Herman[b], Bryan Bailey[a], Hyeun-Jong Bae[c] and Richard Sicher[a]

[a]USDA-ARS, Plant Sciences Institute, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA
[b]USDA-ARS, Plant Genetics Research Unit, Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132, USA
[c]Research Institute for Environmental Engineering, 1322 College of Agriculture & Life Sciences, Chonnam University, 300 Yongbong-dong, Gwangju, Korea 500-757

Correspondence
*Corresponding author,
e-mail: rbae@asrr.arsusda.gov

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Trehalose exists in most living organisms and functions as a storage carbohydrate and as an osmoprotectant in yeast, fungi, and bacteria. Trace amount of endogenous trehalose was detected in flowering plants, and the trehalose biosynthetic pathway was essential for embryo maturation in *Arabidopsis*. Conversely, exogenous trehalose was toxic to higher plants and severely curtailed root and shoot growth. In the current study, 30 m*M* trehalose was added to 2-week-old liquid cultures containing *Arabidopsis thaliana* (Columbia ecotype) seedlings. Densely stained granular particles were detected in the extracellular space of cotyledons and roots of trehalose-treated seedlings using transmission electron microscopy. Expression levels of 91 transcripts were altered by 1–6 h of trehalose treatment using DNA microarray analysis, and 65 of these encoded either known proteins or putative proteins with known functions. The exogenous trehalose treatment altered transcript levels of transcription factors, cell wall modification, nitrogen metabolism, and stress-related, defense-related, and fatty acid biosynthesis genes. Many of the transcripts altered by exogenous trehalose treatment were associated with the ethylene and methyl jasmonate-signaling pathways. The above findings suggested that trehalose, or metabolites derived from trehalose, are important regulators of plant gene expression in higher plants.

Introduction

Trehalose (1,1 α-D glucopyranosyl α-D-glucopyranoside) is an important storage carbohydrate and stress protectant in yeast, bacteria, and certain fungi (Elbein et al. 2003, Gancedo and Flores 2004, Goddijn and Smeekens 1998, Wingler 2002). In higher plants, the biosynthetic pathway is composed of two enzymes (reviewed in Elbein et al. 2003, Goddijn and van Dun 1999). First, trehalose-6-phosphate (T6P) is synthesized from glucose-6-phosphate and uridine-5-diphosphoglucose

Abbreviations – ABA, abscisic acid; BGL1, endo-1,3-β-D-glucanase; CGS1, cystathionine γ-synthase 1; ChiB, basic endochitinase; FER, ferritin; FUT, xyloglucan fucosyltransferase; GAPDHc, cytosolic glyceraldehyde-3-phosphate dehydrogenase; GS-2, plastidic glutamine synthetase; HY2, phytochromobilin synthase; LOX2, lipoxygenase-2; NIT, nitrilase; NR, nitrate reductase; PIP, plasma membrane intrinsic protein; PHD, Plant Homeodomain; PLD, phospholipase D; PME, pectin methylesterase; PR, pathogenesis related; PRXR2, peroxidase 50; SAMT, S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase; SDS, sodium dodecylsulphate; T6P, trehalose-6-phosphate; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

by the action of trehalose phosphate synthase (TPS). The free sugar is then generated by trehalose-6-phosphate phosphatase. Trehalase is a hydrolytic enzyme that cleaves trehalose into two glucose moieties. The inhibition of trehalase by the fungicide, validamycin A, resulted in the accumulation of trehalose and reductions in sucrose and starch (Müller et al. 2001). These findings suggested that the trehalose biosynthetic pathway may regulate carbohydrate allocation in higher plants (Müller et al. 2001). Prior studies showed that trehalose was present in desiccation-tolerant, lower plants including the club moss, *Selaginella lepidophylla* (Fischer and Kandler 1975), and the leafy liverwort, *Plagiochila asplenoides* (Suleiman et al. 1979). Although only trace amounts of trehalose were detected in higher plant tissues (Garg et al. 2002, Goddijn et al. 1997), genes involved in trehalose metabolism were nearly ubiquitous among higher plant species (Eastmond and Graham 2003, Goddijn and Smeekens 1998).

Because of its low concentration, the function of trehalose in higher plants remains unclear. Nevertheless, trehalose metabolism has received considerable research attention in recent years. Higher plants have been engineered to manufacture trehalose with the goal of mitigating abiotic stress. Trehalose protected biomolecules in vitro, and isolated proteins and membranes were stabilized by trehalose either through hydrogen bonding to polar residues or by water displacement (Colaco et al. 1992, Wingler 2002). Transformed rice plants that overexpressed a fusion gene containing *OTSA* and *OTSB*, the *Escherichia coli* genes, responsible for trehalose biosynthesis accumulated a 200-fold excess of trehalose and showed increased tolerance to drought, salt, and cold (Garg et al. 2002). Also, Lee et al. (2003) introduced a yeast *TPS1* gene into tobacco chloroplasts and observed a substantial increase in both trehalose synthesis and drought tolerance.

Evidence is mounting that T6P has important regulatory functions in higher plants (Eastmond and Graham, 2003, Schluepmann et al. 2004). In yeast cells, T6P inhibited hexokinase activity and altered rates of glycolysis (Blázquez et al. 1998). Although a T6P-dependent inhibition of hexokinase was not observed in higher plants (Eastmond et al. 2002), enhanced photosynthesis rates per unit leaf area occurred in transgenic tobacco-expressing *OTSA* (Paul et al. 2001). Conversely, photosynthesis rates were reduced in tobacco plants overexpressing *OTSB,* a bacterial phosphatase specific for T6P. Also, T6P was an essential factor in embryo maturation based on studies performed with a transposon insertion mutant of *Arabidopsis* (Eastmond et al. 2002, Schluepmann et al. 2004). Transcripts of *TPS* were induced by prolonged darkness, suggesting that T6P was involved in plant responses to starvation (Thimm et al. 2004).

Exogenous trehalose altered the development of higher plants, redirected carbohydrate metabolism in affected tissues, and induced or suppressed specific genes. Leaf and root growth in *Arabidopsis* was severely reduced by growth on media containing trehalose, and the effects of trehalose were greater in the presence of the trehalase inhibitor, validamycin A (Müller et al. 1998, Wingler et al. 2000). Exogenous trehalose induced the expression of sucrose: fructan-6-fructosyl-transfrerase in barley leaves (Müller et al. 2000), the large subunit of adenosine diphosphate (ADP)-glucose pyrophosphorylase in *Arabidopsis* (Wingler et al. 2000), and increased sucrose synthase and alkaline invertase activities but concomitantly reduced acid invertase activity in soybean roots (Müller et al. 1998).α-Trehalase activity in *Arabidopsis* was strongly induced by infection with the trehalose-producing pathogen *Plasmodiophora brassicae* (Brodmann et al. 2001), and applying trehalose directly to wheat leaves induced the activities of various defense-response proteins and conferred resistance to powdery mildew (Reignault et al. 2001). Also, increased T6P levels were correlated with the induction of six stress response genes, mostly transcription factors and protein kinases, in a transcription profiling study of *Arabidopsis* (Schluepmann et al. 2004). The above findings suggested that T6P functioned as an important signal molecule controlling carbohydrate metabolism during higher plant development.

There is widespread agreement that carbohydrates, including trehalose and T6P, have the capacity to regulate gene expression (Jang and Sheen 1997). However, only a handful of genes that respond to exogenous trehalose treatment have been identified to date. The objective of the current study was to employ a broader screening method based on DNA microarray technology to identify genes in *Arabidopsis* that were affected by exogenous trehalose. Using these methods, we were able to identify transcripts for 91 new genes with altered expression levels between 1 and 6 h of treatment with exogenous 30 m*M* trehalose. In agreement with prior results (Schluepmann et al. 2004), trehalose treatment altered the expression of several stress-related genes. Transcripts of three nitrilases and four genes involved in amino acid metabolism were repressed by trehalose treatment. Furthermore, in agreement with ultrastructural evidence, the expression of several cell wall and membrane-associated genes were altered by trehalose treatment.

Materials and methods

Plant materials

Arabidopsis thaliana (L.) Henyh seeds of the Columbia ecotype were sterilized with one-third strength

commercial bleach for 10 min and were then rinsed five times with sterile, deionized water. Approximately 50 seeds were transferred to sterile 250-ml Erlenmeyer flasks containing 50 ml of 1× strength Murashige-Skoog (MS) basal salts medium supplemented with 1× strength vitamin B5, 1% (w/v) sucrose, and 0.1 m*M* MES-NaOH (pH 5.7) buffer. Seedlings were grown in controlled environment chambers under continuous fluorescent light providing 150 ± 10 μmol m^{-2} s^{-1} photosynthetically active radiation measured at flask height. Air temperature was a constant 22°C, and the flasks were agitated continuously at 100 rev min^{-1} on a rotary shaker. After 2 weeks' growth, sufficient amounts of a filter-sterilized trehalose solution (Sigma, St. Louis, MO) were added to one-half of the flasks to yield a final concentration of 30 m*M* (approximately 1% w/v). An equivalent amount of sterile, deionized water was added to the control flasks. Seedlings were harvested at 0, 0.5, 1, 2, and 6 h after treatment with trehalose commenced. Harvested seedlings were filtered through a stainless steel sieve, rinsed thoroughly with deionized water, and immediately frozen in liquid N_2 to stop metabolism. Samples were stored at −80°C if necessary.

Sterilized *Arabidopsis* seeds also were sown on 100-mm Petri dishes containing MS basal salts plus IX strength vitamin B5 but minus sucrose as described above and supplemented with 0.8% (w/v) agar for transmission electron microscopic (TEM) analysis. One-half the seeds were sown on solid medium containing 30 m*M* trehalose. After 2 days in the dark at 4°C, seedlings were grown in controlled environment chambers for 2 weeks as described above.

Transmission electron microscopy

Whole *Arabidopsis* seedlings were removed from the agar medium and were fixed for 2 h at room temperature by immersion in 50 m*M* Na^+-cacodylate buffer, pH 7.0, containing 2.5% glutaraldehyde (v/v). The fixed seedlings were stored in a refrigerator at 4°C overnight in 50 m*M* Na^+-cacodylate buffer, pH 7.0, and were then rinsed six times for 1 h each with the same solution. The samples were post-fixed with 2% buffered OsO_4 for 2 h, dehydrated in 100% ethanol, and infiltrated with Spurrs low-viscosity-embedding resin. *Arabidopsis* cotyledons or roots were sectioned (90 nm) on a Riechert/AO Ultracut microtome with a Diatome diamond knife and were mounted on 200 mesh Ni grids. The mounted sections were stained with 4% uranyl acetate and 3% lead citrate and were viewed at 75 kV with an H-7000 Electron Microscope (Hitachi High Technologies America, Pleasanton, CA).

DNA microarray analyses

Equipment and supplies provided with the LifeArray™ System from Incyte Genomics (Palo Alto, CA) were used to perform the DNA microarray experiments. Each LifeArray contains 7942 cDNA clones representing over 6000 different genes/clusters on a glass slide. The cDNA clones were originated from cDNA libraries of all major organs and tissues of 4-week old (rosettes and roots) and 6-week old plants (including the inflorescence). Each glass slide is composed of four subarrays that are positioned in two rows and columns.

Whole *Arabidopsis* seedlings were grown in liquid culture for 14 days as described above. Samples harvested after 1 and 6 h of trehalose treatment were ground to a fine powder under liquid N_2 using a mortar and pestle. Note that samples from 1- and 6-h treatments were combined in this study to simulataneously identify both rapid and relatively slower transcript responses to exogenous trehalose. Equal amounts of powdered tissue from the 1- and 6-h samples were combined, and total RNA was extracted from trehalose-treated and -untreated samples using Trizol reagent (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was purified using an Oligotex mRNA mini kit (Qiagen, Alameda, CA), and the resultant mRNA was reverse-transcribed with 5′-Cy3- or 5′-Cy5-labeled random 9-mers. Reactions were incubated at 37°C for 2 h with 200 ng of poly(A)$^+$ RNA using the LifeArray™ probe-labeling kit. The reactions were terminated by incubation at 85°C for 5 min. The paired reactions (e.g. matched normal tissues vs. trehalose-treated tissues) were mixed and column-purified to remove unreacted dye and primers. The purified probes were hybridized to the DNA microarrays and incubated at 60°C for 6 h in a custom-built chamber. During hybridization, specific yeast cDNAs were used as a control. These PCR products are printed on every array to monitor the success of hybridization. The microarrays were then washed three times in wash buffers provided by the vendor to remove unreacted probe. The washed microarrays were scanned using both Cy3 and Cy5 channels with a ScanArray 4000 at 1-μm resolution (Perkin Elmer, Boston, MA). The microarray digital images were then normalized using INCYTE GEMTOOLS software (version 2.5.0) provided by the vendor. GEMTOOLS software uses a gridding and region-detection algorithm. Local background surrounding each image element was calculated and was subtracted from the total element signal. The mean Cy3/Cy5 ratio was calculated and was used to balance and normalize the signals. The resultant ratio of Cy3 and Cy5 signal intensities indicated the relative expression levels between the two samples.

Three independent biological replications were conducted with three independent samples and three microarrays. Statistical differences ($P \leq 0.05$) were determined at ± 1.8 times the baseline response in all three replications. LifeArrays and GEMTOOLS software are proprietary products from Incyte Genomics and are no longer sold. More detailed information about the construction and the software-based statistical analysis of these microarrays is not public information. The microarray experiments conformed to the MIAME guidelines (http://www.mged. org/Workgroups/MIAME/miame_checklist.html). The conformance to the MIAME guide for describing microarray experiments was listed in UDSA website (http://www. arsusda.gov/acsl/news/rsicher.html). Transcripts that were induced or repressed by the trehalose treatment were annotated and categorized using Gene Ontologies from The Institute of Genomic Research (http://www.tigr.org) and The Arabidopsis Information Resource [TAIR (http://www.arabidopsis.org)]. Gene list, ID, and other data have been deposited on the web as supplementary data (TAIR, http://www.arabidopsis.org, submission number ME00370).

Northern blot analyses

Two-week old *Arabidopsis* samples from liquid cultures were exposed to 30 m*M* exogenous trehalose for 0, 0.5, 1, 2, and 6 h, and RNA was extracted as described above. Total RNA (7 μg) was denatured at 50°C for 30 min in an equal volume of NorthernMax-Gly™ loading dye (Ambion Inc., Austin, TX) and was separated by electrophoresis for 1.5 h at 70 V on 1.3% agarose gels. The running buffer consisted of 0.3 *M* Bis-Tris [bis (2-hydroxyethyl) imino-tris (hydroxymethyl)-methane], 0.1 *M* PIPES (Piperazine-N,N′-bis-2-ethanesulphonic acid), and 0.01 *M* ehylene diamine tetraacetic acid (EDTA) at pH 8.0. The separated RNA was transferred to membranes (Zeta-Probe®·GT Bio-Rad Laboratories, Hercules, CA), and cross-linking was performed with a UV-Stratalinker 8600 (Stratagene, La Jolla, CA). Specific probes were amplified using gene-specific primers (Table 1), and the amplified PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Probes (35 ng) were labeled with [α-^{32}P] dCTP using a Random Primed DNA Labeling Kit™ (Roche Diagnostics, Inc., Indianapolis, IN), and unreacted label was removed by gel filtration (Edge Gel Filtration Cartridges™, Edge BioSystems, Gaithersburg, MD). Blots were pre-hybridized in ExpressHyb™ solution (BD Biosciences Clontech, Palo Alto, CA) at 68°C for 1 h, and the probes were denatured at 95–100°C for 5 min. Blots were incubated with labeled probes in 15 ml of fresh ExpressHyb™ solution for 2 h at 68°C with continuous shaking. Blots were washed according to the ExpressHyb™ protocol and imaged at 200 μm resolution on a Typhoon 8600 Variable Mode Imager (Molecular Dynamics/Amersham-Pharmacia Biotech, Sunnyvale, CA). Following imaging, blots were washed in 10 m*M*

Table 1. Primer sequences used to synthesize probes for RNA blot analyses. F, forward primer; R, reverse primer; AGI, *Arabidopsis* Genome Initiative.

Gene	Sequences (5′-to 3′)	Expected size of probe (bp)	AGI No.
AtHY2	F: TGGGTTTTCAATTGGGTCAT R: CCATGGGAAAGTCTCAGCAT	520	At3g09150
AtATP-PRT1	F: CCTATCCTATCTCCGCCTCC R: AAGACGAATCTGGTCACGCT	174	At1g58080
AtMYOSIN	F: CAACGGAGGAGTTTGTTGGT R: GATCAACGGAGGAGGATCAA	368	At1g17580
Atβ-1,4-GLU	F: TCGCAACAGCTAAGGATGTG R: TCCGCAATTGAATGTGTGTT	490	At1g71380
AtNIT1	F: CATTGAGCTTTATTGTGCAC R: AATATCAGCTGTGACGAGAC	280	At3g44310
AtPRXR2	F: GTGTTCAATCGGATATACAC R: GACCAAGCTTGATCATCGAG	310	At4g37520
AtPIP1C	F: GAGAGGCAACCGATAGGTAC R: ACCAGAGATTCCAGCAGTAC	246	At1g01620
AtWRKY6	F: CTCAAATCCTTTGGCGATGT R: ACCTGAGAGACTCACCGGAA	103	At1g62300
AtPME	F: ACAACCGTAAGCTCAAGGAG R: CACATTCTCTCTGTAAACTC	272	At3g14310

Tris-HCl, pH 7.5, 1 m*M* EDTA, 1% sodium dodecylsulphate (SDS), microwaved for 10 min, scanned to verify that no probe remained, and reused.

Results and discussion

Plant growth and ultrastructure were altered by trehalose treatment

In agreement with previous reports (Müller et al. 1998, Wingler et al. 2000), 30 m*M* exogenous trehalose inhibited root growth and leaf expansion of *Arabidopsis* after 2 weeks' growth on MS agar plates (data not shown). According to Fritzius et al. (2001), root lengths of *Arabidopsis* were decreased up to 80% by exogenous trehalose in comparison with control plants. Both here and previously (Wingler et al. 2000), this inhibition of root elongation was partially reversed by high concentrations of sucrose or glucose. Changes due to exogenous trehalose treatment were also visible within cotyledons and roots of *Arabidopsis* at the ultrastructural level. In the current study, substantial numbers of densely stained particles were observed in the extracellular space of trehalose-treated cotyledons and roots of *Arabidopsis* using TEM (Fig. 1). These densely stained particles were prevalent on the external surface of cells situated near the exterior of roots and cotyledons. Trehalose treatment did not alter cellular integrity, and organellar and membranous structures in both roots and cotyledons were intact. Thylakoid membranes were detected in root tissue due to the fact that the roots obtained from agar plates in this study were exposed to illumination.

DNA microarray analyses

Transcripts of about 8000 genes representing approximately one-third of the *Arabidopsis* genome were examined in this study. Results were obtained by comparing expression patterns of 14-day-old control samples with a combined sample obtained by mixing plant material after 1 and 6 h of trehalose treatment. A total of 91 *Arabidopsis* transcripts were altered by trehalose treatment, which was defined here as a 1.8-fold increase or decrease in transcript level vs. the control. Trehalose treatment induced or repressed 48 and 43 transcripts, respectively (Table 2). Of the 48 transcripts induced by trehalose, 21 encoded hypothetical or putative proteins. By comparison, 39 of the 43 transcripts repressed by trehalose encoded known functional or putative functional proteins.

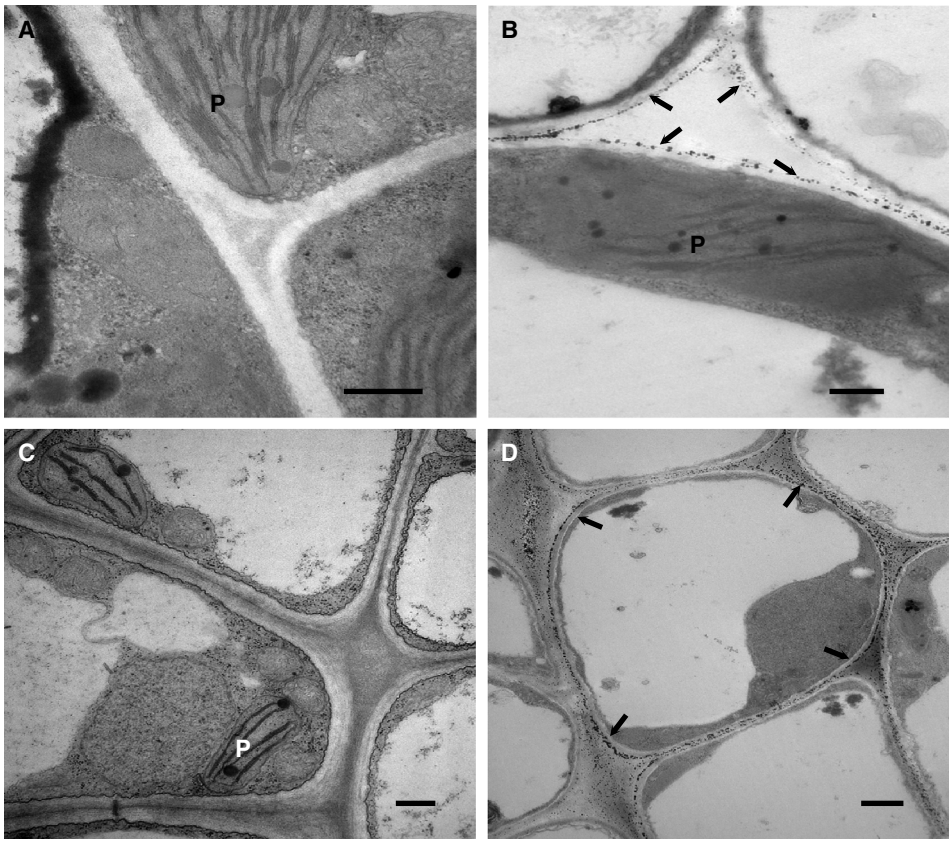

Fig. 1. Transmission electron micrographs showing ultrastructural changes to roots and cotyledons of *Arabidopsis thaliana* seedlings treated with exogenous trehalose. Seeds were germinated on agar plates either in the absence (A, C) or presence (B, D) of 30 m*M* trehalose. Micrographs are for cotyledons (B, C) or roots (D, E) from 2-week-old seedlings. Arrows indicate the presence of densely stained particles in the extracellular regions of treated tissues. Horizontal bars indicate a scale of 500 nm, and plastids (P) are shown.

Table 2. DNA microarray analysis of *Arabidopsis thaliana* transcripts that were increased or decreased by 1–6 h of treatment with 30 m*M* exogenous trehalose. Gene annotation and categorization were determined by The Institute of Genomic Research and The Arabidopsis Information Resource. Ratio is the average of three independent replications. Minus (–) ratio means repression of gene expression. SD, standard deviation; AGI, *Arabidopsis* Genome Initiative.

AGI No.	Fold change	SD	Gene annotation	Molecular function
At4g10470	2.8	0.1	Expressed protein	Unknown
At5g61300	2.4	0.4	Expressed protein	Unknown
At1g80550	2.2	0.1	Pentatricopeptide repeat-containing protein	Unknown
At1g21920	2.1	0.2	Membrane occupation and recognition nexus repeat-containing protein/phosphatidylinositol-4-phosphate 5-kinase related	Unknown
At5g65170	2.1	0.1	No match	Unknown
At4g35320	2.1	0.1	No match	Unknown
At4g00380	2.1	0.0	Similar to XH/XS domain-containing protein/XS zinc-finger domain-containing protein	Unknown
At5g37300	2.1	0.1	No match	Unknown
At5g06440	2.1	0.2	No match	Unknown
At2g35320	2.1	0.2	No match	Unknown
At5g38880	2.1	0.0	No match	Unknown
At1g04780	2.1	0.0	No match	Unknown
At4g32440	2.1	0.0	Agenet domain-containing protein	Unknown
Rice gene	1.9	0.2	Similar to integral membrane family protein	Unknown
At3g04860	1.9	0.1	Expressed protein	Unknown
At2g30280	1.8	0.2	Expressed protein	Unknown
At3g53210	2.0	0.1	Nodulin MtN21 family protein	Unknown
At4g02200	2.1	0.1	Drought-responsive family protein	Unknown
At2g21100	1.9	0.1	Disease resistance-responsive protein-related/dirigent protein related	Unknown
At2g26920	2.1	0.0	Ubiquitin-associated/TS-N domain-containing protein	Unknown
At5g66920	1.9	0.1	Multi-copper oxidase type I family protein/similar to pollen-specific BP10 protein	Unknown
At1g05380	2.4	0.2	Putative Plant Homeodomain (PHD) finger transcription factor	Binding activity
At2g33610	2.2	0.2	SWIRM domain-containing protein/DNA-binding family protein contains similarity to SWI/SNF complex 170 KDa subunit [*Homo sapiens*]	Binding activity
At4g38340	2.4	0.0	RWP-RK domain-containing protein/similar to nodule inception protein (*Lotus japonicus*)	Binding activity
At1g51140	2.2	0.0	Basic helix-loop-helix (bHLH) family protein	Binding activity
At1g70000	2.1	0.0	DNA-binding family protein	Binding activity
At1g51640	2.0	0.1	Exocyst subunit EXO70 family protein	Binding activity
At1g62300	2.1	0.0	Transcription factor WRKY6	Binding activity
At4g08150	2.0	0.1	Homeobox protein knotted-1 like 1 (KNAT1)	Binding activity
At3g02310	1.9	0.1	Developmental protein SEPALLATA2/floral homeotic protein (AGL4) (SEP2)	Binding activity
At1g14290	2.1	0.1	Putative acid phosphatase	Catalytic activity
At3g54180	2.1	0.2	Cell division control protein 2 homolog B (CDC2B)	Catalytic activity
At3g09150	2.8	0.5	Phytochromobilin synthase (HY2)	Catalytic activity
At1g08210	2.4	0.1	Aspartyl protease family protein	Catalytic activity
At5g50850	2.1	0.3	Pyruvate dehydrogenase E1 component beta subunit, mitochondrial precursor (PDHE1-B)	Catalytic activity
At1g68040	2.1	0.1	*S*-adenosyl-L-methionine:carboxyl methyltransferase family protein	Catalytic activity
At5g53580	1.9	0.1	Aldo-keto reductase family protein	Catalytic activity
At1g58080	1.9	0.1	Adenosine triphosphate (ATP)-dependent phosphoribosyl transferase 1 (ATP-PRT1)	Catalytic activity
At1g68470	2.0	0.1	Exostosin family protein	Catalytic activity
At1g74420	2.1	0.0	Putative xyloglucan fucosyltransferase (FUT3)	Catalytic activity
At1g71380	2.0	0.1	Glycosyl hydrolase family 9 protein (endo-1,4-β-D-glucanase)	Catalytic activity
At2g38240	2.0	0.0	Anthocyanidin synthase, oxidoreductase, 2OG-Fe(II) oxygenase family protein	Catalytic activity
At3g18000	1.9	0.1	Phosphoethanolamine N-methyltransferase 1	Catalytic activity
At1g09350	2.1	0.1	Putative galactinol synthase	Catalytic activity
At5g03430	2.0	0.1	Phosphoadenosine phosphosulphate (PAPS) reductase family protein	Catalytic activity
At1g17580	2.1	0.1	Putative myosin	Motor activity
At3g09030	2.2	0.1	Potassium channel tetramerization domain-containing protein	Transporter activity
At4g09160	2.2	0.0	SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein	Transporter activity
At2g27860	−2.2	0.1	Expressed protein	Unknown

Table 2. Continued.

AGI No.	Fold change	SD	Gene annotation	Molecular function
At2g26660	−2.1	0.1	SPX (SYG1/Pho81/XPR1) domain-containing protein	Unknown
At4g01050	−1.9	0.1	Hydroxyproline-rich glycoprotein family protein	Unknown
At1g04430	−1.8	0.1	Dehydration-responsive protein related	Unknown
At1g08510	−2.1	0.2	Acyl-[acyl carrier protein] thioesterase	Binding activity
At1g78900	−2.3	0.2	Vacuolar ATP synthase catalytic subunit A	Binding activity
At3g08580	−2.1	0.0	Mitochondrial adenosine diphosphate, ATP carrier protein 1	Binding activity
At5g01600	−1.9	0.0	Ferritin 1 (FER1)	Binding activity
AT5g54900	−2.1	0.2	Putative RNA-binding protein 45 (RBP45)	Binding activity
At3g45140	−1.8	0.1	Lipoxygenase (LOX2)	Catalytic activity
At1g09780	−2.0	0.1	Putative 2,3-biphosphoglycerate-independent phosphoglycerate mutase	Catalytic activity
At3g01120	−1.9	0.1	Chloroplast cystathionine γ-synthase (CGS)	Catalytic activity
At3g17240	−1.9	0.1	Mitochondrial dihydrolipoamide dehydrogenase 2 (MTLPD2)	Catalytic activity
At5g35630	−2.0	0.2	Glutamine synthetase (GS2)	Catalytic activity
At3g04120	−2.1	0.0	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC)	Catalytic activity
At4g37930	−2.0	0.1	Glycine hydroxymethyltransferase/serine hydroxymethyltransferase (SHMT)	Catalytic activity
At1g37130	−2.0	0.1	Nitrate reductase 2 (NR2)	Catalytic activity
At1g76080	−2.0	0.2	Thioredoxin family protein	Catalytic activity
At3g16800	−2.5	0.1	Putative protein phosphatase 2C	Catalytic activity
At4g04610	−2.2	0.3	5′-adenylylsulphate reductase (APR1)	Catalytic activity
AT4g19410	−2.5	0.6	Putative pectinacetylesterase (PAE)	Catalytic activity
At1g05850	−1.9	0.0	Chitinase-like protein 1 (CTL1)	Catalytic activity
At3g61440	−2.2	0.2	Putative cysteine synthase	Catalytic activity
At5g60360	−1.9	0.2	Putative cysteine proteinase	Catalytic activity
At1g47128	−2.3	0.2	Cysteine proteinase (RD21A)	Catalytic activity
At3g57270	−2.3	0.1	Glycosyl hydrolase family 17 protein (endo-1,3-β-D-glucanase, BG1)	Catalytic activity
At3g22425	−2.5	0.4	Imidazoleglycerol-phosphate dehydratase 1 (IGPD1)	Catalytic activity
At3g01420	−2.8	0.5	Putative pathogen-responsive α-dioxygenase	Catalytic activity
At3g01420	−2.4	0.5	Putative pathogen-responsive α-dioxygenase	Catalytic activity
At3g44310	−2.5	0.6	Nitrilase 1 (NIT1)	Catalytic activity
At3g44300	−2.5	0.4	Nitrilase 2 (NIT2)	Catalytic activity
At5g22300	−2.5	0.0	Nitrilase 4 (NIT4)	Catalytic activity
At3g14310	−2.2	0.2	Pectin methylesterase (PME) family protein	Catalytic activity
At4g37520	−2.7	0.6	Peroxidase 50 (PRXR2)	Catalytic activity
At3g15730	−2.3	0.2	Phospholipase D α 1 (PLD α1)	Catalytic activity
At5g13420	−1.9	0.0	Putative transaldolase	Catalytic activity
At3g12500	−2.4	0.0	Basic endochitinase	Catalytic activity
At5g60360	−2.0	0.1	Putative cysteine proteinase	Catalytic activity
At2g44100	−2.0	0.0	Rab GDP dissociation inhibitor (GDI1)	Enzyme regulator activity
At2g37620	−2.1	0.1	Actin 1 (ACT1)	Structural molecular activity
At4g20890	−2.1	0.2	Tubulin β-9 chain (TUB9)	Structural molecular activity
At1g76030	−1.9	0.1	Vacuolar ATP synthase subunit B/V-ATPase B subunit	Transporter activity
At1g01620	−2.0	0.1	Plasma membrane intrinsic protein 1C (PIP1C)	Transporter activity

Transcripts induced by exogenous trehalose

The 27 annotated *Arabidopsis* transcripts induced by trehalose treatment were separated into functional categories using Gene Ontologies (Fig. 2A). These categories were binding activity (9 transcripts, 20%), transporter activity (2 transcripts, 4%), catalytic activity (15 transcripts, 31%), and motor activity (1 transcript, 2%).

Binding activity. Trehalose induced transcripts of several genes that encoded proteins that function by binding to DNA, other proteins, copper ions, and other biomolecules. Examples of induced transcripts with binding activity included transcription factors. WRKY6 belongs to a class of WRKY proteins that are zinc-finger-type plant-specific transcription factors. WRKY proteins are involved in early processes related to leaf senescence, plant defense, and wounding and are strongly expressed in roots. The WRKY6 protein interacts with protein kinases, and these activate early steps in senescence and defense-associated processes in plant cells (Robatzek and Somssich 2002). *AGL4* encodes a MADS

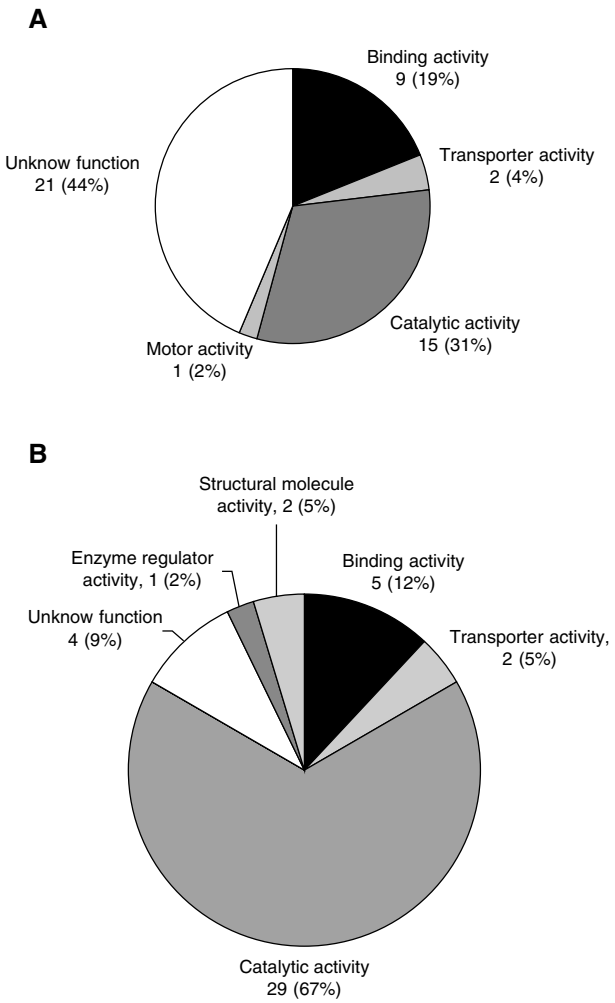

Fig. 2. Pie charts showing the functional distribution of genes that were induced (A) or repressed (B) by 1–6 h of treatment with 30 m*M* exogenous trehalose.

domain protein, which are closely related proteins that serve as important developmental regulators in all eukaryotes (Fan et al. 1997). In *Arabidopsis*, *AGL4* is one of several MADS box transcription factors that function in floral morphogenesis. *AtKNAT1* is a *KNOX* gene that is active in apical tissue and inactive in leaves and may be involved in determining the fate of cells within meristems (Theodoris et al. 2003). *KNOX* genes also may regulate plant hormone levels and are active in mediating DNA binding (Frugis et al. 2001). Induced transcripts with DNA-binding activity were likely among the first to respond to exogenous trehalose treatment.

Cell wall, membrane, and motor activity. Exogenous trehalose treatment induced transcripts involved in cell wall modification. Endo-1,4-β-D-glucanase is a cellulase that hydrolyzes β-1,4 glucosidic linkages (Williamson et al. 2002). Cellulases are secreted into the apoplasm and are involved in weakening cell walls during dehiscence, abscission, and cellular expansion (Rose and Bennett 1999). A putative gene for xyloglucan fucosyltransferase (*FUT3*) was induced by trehalose and was likely involved in hemicellulose biosynthesis (Faik et al. 2000, Perrin et al. 1999).

Myosin is as a molecular motor and has an important role in transport processes associated with the cytoskeleton (Reddy and Day 2001). Myosin transports objects on actin filaments and may be involved in signaling, cell division, cytoplasmic streaming, plasmodesmatal opening, and morphogenesis (Baluška et al. 2001).

A transcript encoding a potassium channel tetramerization domain-containing protein also was induced by trehalose. This protein functions as a voltage-gated potassium channel that regulates the electrical properties of cells and opens potassium-selective transmembrane pores by sensing changes in membrane potential (Bixby et al. 1999).

A transcript for an exocyst subunit family protein, EXO70, was induced by trehalose. Exocyst proteins are multimeric complexes that determine where secretory vesicles dock and fuse with the plasma membrane (Lipschutz and Mostov 2002). Exocyst proteins are also involved in protein synthesis (Lipschutz et al. 2000), mRNA splicing (Awasthi et al. 2001), and cyst and tubule formation.

Stress-response activity. Trehalose treatment induced transcripts of several stress response genes. For example, anthocyanidin synthase converts leucoanthocyanidins to anthocyanidins (Wilmouth et al. 2002). Anthocyanins are secondary metabolites and are induced in response to stress, such as light and nutrient deficiency (reviewed by Winkel-Shirley 2002). Aldo-keto reductase reduces carbonyl groups, and the leaf-specific form of the enzyme is induced by various plant stresses (Gavidia et al. 2002). *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase (SAMT) catalyzes the formation of methylsalicylate and methyl benzoate, which are common floral scent compounds that accumulate in damaged tissue (Ross et al. 1999). SAMT was induced in snapdragon petal tissue by both salicylic and jasmonic acid applications (Negre et al. 2002).

Transcripts repressed by exogenous trehalose treatment

As above, annotated transcripts repressed by trehalose were separated into functional categories based on Gene Ontologies (Fig. 2B). These categories were enzyme regulator activity (1 transcript, 2%), structural molecule activity (2 transcripts, 5%), binding activity (5

transcripts, 12%), transporter activity (2 transcripts, 5%), and catalytic activity (29 transcripts, 66%).

Nitrogen metabolism and amino acid biosynthesis activity. Three nitrilases were repressed by trehalose treatment. Nitrilase-4 (NIT4) is involved in cyanide detoxification, and nitrilases-1 and -2 (NIT1, NIT2) are thought to be involved in auxin metabolism. *Arabidopsis* encodes four nitrilases, which are enzymes that catalyze the hydrolysis of organic nitriles released by the breakdown of glucosinolates (Vorwerk et al. 2001). In *Arabidopsis,* the *NIT4* gene encodes β-cyano-L-alanine-hydratase/nitrilase, which may detoxify cyanide produced during ethylene biosynthesis (Piotrowski et al. 2001). Nitrilases 1 and 2 convert indole-3-acetonitrile to indole-3-acetic acid (Vorwerk et al. 2001). Nitrate reductase (NR2) was repressed by trehalose treatment. NR is a metalloflavoprotein with three functional domains that reduces nitrate to nitrite in the cytoplasm (Campbell 1999). The expression and activity of NR2 are tightly regulated by environmental and intracellular factors, such as levels of nitrate or ammonium (Daniel-Vedele et al. 1998; Raghuran and Sopory, 1999).

Trehalose treatment also repressed four transcripts of proteins involved in amino acid biosynthesis, such as cystathionine γ-synthase 1 (CGS1), glycine hydroxymethyltransferase/serine hydroxymethyltransferase (SHMT), imidazole glycerol-phosphate dehydratase (IGPD), and plastidic glutamine synthetase (GS-2). CGS1 is the first enzyme of the methionine biosynthesis pathway in higher plants (Chiba et al. 1999, Suzuki et al., 2001), SHMT catalyzes the interconversion of glycine and serine (Bauwe and Kolukisaoglu 2003), and IGPD is involved in histidine biosynthesis (Brilli and Fani 2004a,b). GS-2 is a highly regulated enzyme that converts glutamate into glutamine and facilitates ammonium recovery during photorespiration (Taira et al. 2004). The interaction of carbohydrate-regulated gene expression and nitrogen metabolism has not been examined closely in this study. However, we did not detect changes of glutamate or glutamine within 24 h after treatment with 30 m*M* exogenous trehalose was initiated (Bae et al. in press).

Plant defense and abiotic stress activity. Trehalose also repressed various plant defense, wound response, or pathogenesis-related (PR) genes. These included peroxidase-2 (PRXR2), basic endochitinase (PR), endo-1,3-β-D-glucanase (BGL1), lipoxygenase-2 (LOX2), and a chitinase-like protein 1 (CTL1). PRXR2 is a member of a large gene family of secretory plant peroxidases that are heme-containing and are involved in removing toxic hydroperoxides. Peroxidases also may function in lignin degradation, defense against microbes, and in auxin metabolism (Gaspar et al., 1991). Higher plant chitinases are a complex family of acidic and basic defense proteins that hydrolyse chitin, a cell wall constituent of fungi. ChiB is a basic chitinase that is constitutively expressed in roots, found in the vacuole or apoplasm, and induced by ethylene (Kawabe and Miyasgita 1999). BGL1 is a class-2 PR protein that hydrolyzes β-1,3-glucans and is induced by wounding or by elicitors such as jasmonic acid (Stotz et al. 2000). LOX2 from *Arabidopsis* oxidizes long-chain fatty acids, is likely localized in the chloroplast, and is involved in the synthesis of jasmonates (Bell and Mullet 1993, Jensen et al. 2002). Like BGL1, LOX2 is rapidly induced by wounding, herbivory, and positive feedback from methyl jasmonate applications (Stotz et al. 2000).

Trehalose treatment also repressed several transcripts known to be involved in abiotic stress. These included the vacuolar adenosine triphosphate (ATP) synthase catalytic subunit A, ferritin (*AtFER1*), cytosolic glyceraldehyde-3-phosphate dehydrogenase (*GAPDHc*), plasma membrane intrinsic protein (*PIP1C*), cysteine proteinase (*RD21A*), and 5′-adenylylsulphate reductase (*APR1*). Vacuolar H^{+}-ATPases are multimeric enzyme complexes that play a critical role in the maintenance of vacuolar homeostasis in plant cells during times of environmental stress including cold and salt stress (Barkla and Pantoja 1996, Magnotta and Gogarten 2002). Ferritins (FER1) are iron-storage proteins that regulate intracellular free-iron levels and are induced during age- and stress-related senescence (Briat and Lobreaux 1997, Harrison and Arosio 1996). GAPDHc catayzes a reversible oxidation and phosphorylation reaction in glycolytic metabolism, and protein levels of GAPDHc were increased by dehydration (Velasco et al. 1994), heat shock (Yang et al. 1993), and anaerobic stress (Chang et al. 2000). PIP1C is a member of the major intrinsic protein family. These proteins function as channels regulating the passage of water and solutes through the plasma membrane and are induced by drought or salt stress (Jang et al. 2004). RD21A is a vacuolar cysteine protease of the papain family that was strongly induced during leaf senescence (Gepstein et al. 2003) and under high-salt conditions (Koizumi et al. 1993). APR1 is a protein in the plant sulphate assimilation pathway leading to the synthesis of cysteine and glutathione (Leustek et al. 2000) in response to oxidative stress (Bick et al. 2001).

Cell wall and membrane activity. There are at least 12 pectin methylesterases (*PMEs*) or *PME*-related genes in the *Arabidopsis* genome that catalyze the demethylesterification of pectin (Micheli et al. 1998). This in turn makes the polysaccharide more accessible to pectin-degrading enzymes, such as pectate lyases (Davis et al.

1984). Phospholipase D (PLD) mediates plant responses to abscisic acid and is important in plant growth, development, and response to environmental stress, including salt and cold stress (Finkelstein et al. 2002, Zhang et al. 2004).

RNA blots of transcripts induced by trehalose treatment

Northern blots were successfully performed on nine identified transcripts with known functions that were altered in response to exogenous trehalose treatment (Fig. 3). Five *Arabidopsis* transcpripts (*HY2*, *ATP-PRT1*, *MYOSIN*, β-*1,4-GLU,* and *WRKY6*) were increased, and four (*NIT1, PRXR2, PIP1C,* and *PME*) were repressed by 30 m*M* exogenous trehalose between 0.5 and 12 h of treatment. These findings were in agreement with the DNA microarray results. Based on Northern blot analysis, transcripts of *PIP1C* and *PME* were transiently repressed by exogenous trehalose treatment. *WRKY6* transcripts were most abundant 30 min after the exogenous trehalose treatment was initiated.

Based on the Northern blot analyses, combining samples from 1- and 6-h treatments for the microarray study generally decreased the overall magnitude of transcript changes in response to exogenous trehalose. For example, in the Northern blot analysis, the transcript for myosin was maximally induced at 1 h, and reduced induction was observed at later timepoints. This suggested that the response of myosin in the microarray analysis showed decreased induction for the combined samples (2.1) compared with 1 h sample alone (>2.1).

Concluding remarks

In previous studies (Müller et al. 1998, Müller et al. 2000, Wingler et al. 2000), exogenous trehalose induced or repressed enzymes involved in carbohydrate metabolism, and these authors speculated that microsymbionts and plant pathogens released trehalose to redirect carbohydrate metabolism in host tissues. However, exogenous trehalose treatment did not alter expression levels of transcripts associated with carbohydrate metabolism in the current study. Schluepmann et al. (2004) concluded in a DNA microarray study that six stress response transcripts were induced in *Arabidopsis* by treatments, including trehalose feeding, that increased T6P levels. There was no direct overlap between this and the current study regarding specific transcripts affected by trehalose treatment. However, the stress-related transcripts that responded to trehalose treatment in the current study were primarily associated with abiotic rather than biotic stresses. In contrast to the findings of Schluepmann et al. (2004), the current transcription-profiling study showed that more plant defense and abiotic stress transcripts were repressed by trehalose treatment than were induced. Differences between the findings of Schluepmann et al. (2004) and the current study may be because partial genome DNA microarrays from different manufacturers were used. The current study used 30 m*M* of trehalose in the presence of 1% sucrose rather than 100 m*M* trehalose alone. Higher concentrations of trehalose would likely be more toxic to *Arabidopsis,* and sucrose potentially mitigated the effects of feeding exogenous trehalose (Schluepmann

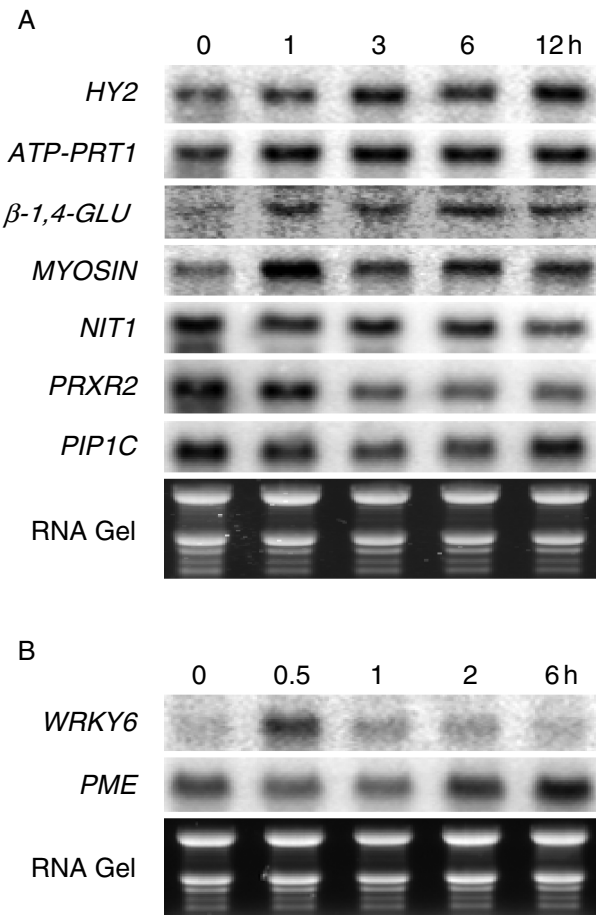

Fig. 3. RNA blots of *Arabidopsis* transcripts induced by exogenous trehalose treatment. Total RNA was isolated from samples that were harvested at indicated times. Seven micrograms of RNA was loaded per lane and was separated by denaturing gel electrophoresis. RNA blots were performed with ^{32}P-labeled gene-specific probes based on results in Table 2. *HY2* encoding phytochromobilin synthase; *ATP-PRT1* encoding ATP-dependent phosphoribosyl transferase 1; β-*1,4-GLU* encoding endo-1,4-β-D-glucanase (glycosyl hydrolase family 9 protein); *MYOSIN* encoding putative myosin; *NIT1* encoding nitrilase 1; *PRXR2* encoding peroxidase 50; *PIP1C* encoding plasma membrane intrinsic protein 1C; *WRKY6* encoding transcription factor WRKY6; *PME* encoding putative pectin methylesterase.

et al. 2004). Plants would commonly be expected to encounter trehalose produced by many different types of microorganisms in the natural environment. Therefore, the simultaneous induction and repression of both stress and plant defense genes potentially would have different consequences for microsymbionts and pathogens. Many of the abiotic stress and plant defense genes altered by trehalose treatment in the present study were associated with ethylene or methyl jasmonate. Thus, trehalose treatment may somehow be linked to the ethylene and jasmonate-signaling pathways.

Several cell wall- and plasma membrane-associated transcripts were affected by exogenous trehalose in this study. These included MYOSIN, BGL1, EXO70, PME, PLD, PIP, and a potassium channel protein. Plant cells are surrounded by cell wall and plasma membrane and a cytoskeleton, and trehalose-producing microorganisms would initially interact with these structures. This conclusion was confirmed by ultrastructural examination showing the presence of densely stained, granular particles in the extracellular space of trehalose-treated tissues. *Arabidopsis* seedlings exposed to exogenous 30 m*M* trehalose displayed severely diminished growth rates of roots and cotyledons. This could be explained in part by the repression of cell wall-loosening enzymes observed here. Taken together, these findings suggested that a primary response of plants to exogenous trehalose occurred in the extracellular space.

Lastly, the exogenous trehalose treatment repressed transcripts of three nitrilase genes and of four genes involved in amino acid synthesis. Taken together, the above findings are consistent with the suggestion that exogenous trehalose, or metabolites derived from exogenous trehalose, are important regulators of plant gene expression in plants.

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