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mRNA Transcript Abundance during Plant Growth and the Influence of Li^+ Exposure

M.C. Duff[1]*, W.W. Kuhne[1], N.V. Halverson[1], C-S. Chang[2], E. Kitamura[2], L. Hawthorn[2], N.E. Martinez[1,3], C. Stafford[1,4], C.E. Milliken[1], E.F. Caldwell[1], and E. Stieve-Caldwell[1]

[1] Savannah River National Laboratory, Aiken, SC 29808.
[2] Integrated Genomics Core, Georgia Regents University Cancer Center, Augusta, GA 30912.
[3] Dept. Env. and Radiological Health Sciences, Colorado State University, Ft. Collins, CO 80523.
[4] University of South Carolina Medical School, Columbia, SC 29208.

*Corresponding Author:

Dr. Martine C. Duff, Savannah River National Laboratory, Aiken SC 29808

Phone: 803-725-2054

Email: Martine.Duff@srnl.doe.gov

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Abstract

Lithium (Li) toxicity in plants is, at a minimum, a function of Li^+ concentration, exposure time, species and growth conditions. Most plant studies with Li^+ focus on short-term acute exposures. This study examines short- and long-term effects of Li^+ exposure in *Arabidopsis* with Li^+ uptake studies and measured shoot mRNA transcript abundance levels in treated and control plants. Stress, pathogen-response and arabinogalactan protein genes were typically more up-regulated in older (chronic, low level) Li^+-treatment plants and in the much younger plants from acute high-level exposures. The gene regulation behavior of high-level Li^+ resembled prior studies due to its influence on: inositol synthesis, 1-aminocyclopropane-1-carboxylate synthases and membrane ion transport. In contrast, chronically-exposed plants had gene regulation responses that were indicative of pathogen, cold, and heavy-metal stress, cell wall degradation, ethylene production, signal transduction, and calcium-release modulation. Acute Li^+ exposure phenocopies magnesium-deficiency symptoms and is associated with elevated expression of stress response genes that could lead to consumption of metabolic and transcriptional energy reserves and the dedication of more resources to cell development. In contrast, chronic Li^+ exposure increases expression signal transduction genes. The identification of new Li^+-sensitive genes and a gene-based "response plan" for acute and chronic Li^+ exposure are delineated.

1. Introduction

Lithium (Li) is a naturally abundant element in the earth's crust and it is found in relatively concentrated forms in igneous rocks, continental, oilfield, and geothermal brines, hectorite (aluminosilicate) clays, and ocean water. Chile is the major Li producer followed by Argentina, China, the US, and Russia [1]. It is used in ceramics, batteries, pharmaceuticals, and high energy propellants and for the production of tritium. As the uses for Li expand, environmental contamination from production and disposition processes becomes more important to monitor. Significant waste sources are the large-scale disposal of Li batteries, mining effluents and sludges [2] as well as Li isotopic separations facilities [3].

Within biological systems, Li is considered a non-essential nutrient although there is some evidence that it may be essential [4]. Lithium-depleted diets will alter reproductive success in rats and goats [5-10]. Lithium is used in the medical field to treat bipolar disorder and related mental diseases such as depression and schizoaffective disorder. It may have potential in the prophylaxis of Alzheimer's disease [11]. Several mechanisms have been proposed for its action. Although research continues, the mechanism for how Li helps treat mental illnesses remains unknown [4]. High levels of Li cause signs of toxicity in patients [9], may be teratogenic in the early developmental stages [12, 13] but Li is not mutagenic nor does it cause chromosomal damage (breaking) [2]. Lithium normally occurs in freshwater at levels below 0.04 ppm (~0.006 mM) as reviewed by Kszos and Stewart [3] although some natural waters can have higher levels. For plants, when Li-rich waters are used for irrigation, adverse effects on crops have been observed which negatively impact the agricultural industry [10, 14].

Lithium ions can modify biochemical processes through direct interaction with proteins and through the modification of gene expression in plants. The most thoroughly-described example in the literature is the mechanism by which Li^+ interacts with the inositol triphosphate second messenger system. The Li ion is an uncompetitive, rapid, and reversible inhibitor of *myo*-inositol-1-phosphatase (IMP or IMPase), an enzyme involved in a rate-limiting step of inositol second messenger recycling and the hydrolysis of *myo*-inositol phosphate. The Li^+ reduces the rate of hydrolysis of *myo*-inositol-1-phosphate without altering the binding of the substrate or the essential magnesium ion (Mg^{2+}) to IMPase according to research studies with animal systems [15]. For patients with mood disorders that have elevated levels of brain *myo*-inositol and respond to Li treatment, Li^+ may inhibit *myo*-inositol hydrolysis but Li^+ may exert a therapeutic influence by acting on secondary signaling pathways, such as that of protein kinase C [16]. In plants, Li^+ has been shown to inhibit IMPase but no verification that the plant uses a non-competitive mechanism has been conducted on a purified plant enzyme. Recently conducted nuclear magnetic resonance (NMR) studies with bovine IMPase [17] and binding site modeling studies [18] that calculated Gibbs free energy values for Mg^{2+} and Li^+ binding sites on various IMPases and glycogen synthase kinase 3β (GSK-3β) reveal that Li^+ can compete with Mg^{2+} when certain conditions permit its physical accessibility to these proteins. Similar observations have been made by Srinivasan *et al.* [19] with guanine-nucleotide binding proteins (G-proteins). It should be noted that Li^+ competition with Mg^{2+} is not thought to be universal with all proteins [18]. Lithium ion is unable to compete with Mg^{2+} for binding sites with other cell-essential mammalian proteins (that are unassociated with bipolar disorder) because those proteins do not have binding sites that are accessible to Li^+. The formation of inositol by IMPase from *myo*-inositol-1-phosphate drives a release of intracellular calcium (Ca^{2+}) from the endoplasmic reticulum (ER), which serves as a signal for many mammalian cellular processes [20] but this has not been shown in plants. In addition to effects on enzyme activity, Li^+ also modifies mRNA expression of IMPase in plants [21-23]. In yeast exposed to 0.2 mM Li^+, Li^+ causes the inhibition of mitochondrial RNA processing endoribonuclease as observed by Dichtl *et al.* [24].

Lithium also has variable effects on the 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) family proteins as noted by Liang *et al*. [25]. ACC synthase catalyzes the synthesis of ACC, a precursor for ethylene, from *S*-adenosyl methionine (SAM). This reaction is a rate-limiting step for the synthesis of the important plant hormone, ethylene. Lithium modifies mRNA expression of SAM [26-28] and an array of other

proteins including the potentially important pathogenesis-related (PR) protein family [29, 30]. Finally, LiCl solutions are occasionally used in the place of NaCl solutions in plant salinity studies. Although LiCl can provide control over the ionic strength of the solution there may be specific ion effects of Li^+ that do not emulate that of Na^+. The specific ion effect of Li^+ on plant enzyme activity and gene expression is relevant to the biochemical and molecular processes in cells and it may improve the understanding of Li^+ effects in mammals as well as plants.

Since there is a growing interest in investigating differences in plants at both the phenotypic and genomic levels under native conditions (i.e., in soil) [31] we utilized both growing platforms, hydroponic solutions (HP) and soil. In this paper we discuss the effect of control and Li^+-exposed (0.05 to 1 mM LiCl) plants that were grown in soil (as mainly chronic Li^+-exposure) and in HP solution (for acute Li^+-exposure) in relation to their controls. We measured the Li content of plant shoots from our HP studies to determine how rapid Li^+ uptake could occur and analyzed the transcriptome of the controls and the Li^+-exposed plants. We discuss the gene regulation for shoot tissue from soil-grown plants over a 5-wk period in the controls and discuss the impact of chronic low-level Li^+ on the gene regulation. We also report on the effects of acute Li^+ exposure in HP studies and how that compares and contrasts to the long-term effects of chronic low-level Li^+ exposure.

2. MATERIALS AND METHODS

Arabidopsis thaliana seeds of the Columbia (Col-0) wild type (Lehle Seeds, Inc., Round Rock, TX) were sterilized by soaking in sterilization solution [22.1 mL deionized water (DI), 95% ethanol (EtOH), 500 µL Triton™ X-100 (Sigma-Aldrich, St. Louis, MO)] for 5 min. followed by an immediate rinsing with 95% EtOH. Once the 95% EtOH had evaporated and the seeds were dried they were kept at 4°C for 3 d. Seeds were then exposed to a red light for 30 min. to synchronize germination.

2.1 Hydroponic Growth of At

On average five seeds were placed onto a rockwool plug (Grodan 1.5" Miniblocks™, Netherlands) that had been pre-soaked in HP media [0.14 g L^{-1} 1/32 Murashige and Skoog Basal Salt Mixture or "MS salt" (Sigma-Aldrich, St. Louis, MO), with 0.25 g L^{-1} MES hydrate (Sigma-Aldrich) in DI water, pH balanced to 5.7 with potassium hydroxide in a HP growth tray]. HP growth trays are Pyrex glass containers (2.6 L) that were covered with aluminum foil and covered with a dark blue lid. For seeding and germination purposes a lid with 46 holes for holding individual rockwool plugs was used. Plugs were pre-soaked in HP media. Trays were initially covered with a perforated plastic wrap to promote germination and eliminate condensation buildup and maintained in growth chambers (Percival Scientific, Inc., Model AR66L3C8, Perry, IA) under conditions of 9 h light:15 h dark, 22 to 25°C, 50% humidity and light intensity of 250 µmoles m^{-2} s^{-1} at a shelf height of 0.3 m. Seeds were germinated and kept in HP media for 14 d at which time the plastic wrap was removed and an airline added. Plants were maintained until d 21 at which time the plants were culled to one plant per plug and roots had fully emerged from the rockwool but were not becoming entangled with one another. At 24 d (in age), the plants were moved to a new glass tray containing two glass replicate boats with the roots from eight plants emerged in 300 mL of HP media within each boat. A new lid design was used to accommodate two additional airlines per boat. Plants at 26 d of age were then exposed to LiCl- or NaCl-spiked HP media as well as control HP media solutions (**Table 1**). Plants were exposed to 1 mM LiCl, 1 mM NaCl or the control HP media for periods of 1 or 7 d. The plants were exposed hydroponically, via their roots which were submerged in the treatment solutions.

2.2 Lithium Uptake Studies with HP Plants

Kinetic uptake studies were conducted in duplicate with HP-grown, 46-day old *At* plants that were exposed to a concentration of 0.1 mM LiCl for periods of up to 1 to 96 h. The tissues were placed into paper bags and dried in an oven for 12 h at 50°C. Samples were then ground into a fine powder using liquid nitrogen (N_2)

with porcelain mortar and pestle and dried for an additional 48 h at 50°C. Lithium levels in the nitric acid-digested plants and HP test solution were determined quantitatively using inductively coupled plasma mass spectrometry (ICP-MS). A minimum of 0.5 g (dry weight or dw or 35 g wet weight) plant material was required for ICP-MS which was conducted in duplicate.

2.3 Soil-based Growth of At

Seeds were sown in large grow pots (Kordlok SQL0450, with 4:1 Promix PGX (Premier Horticulture, Quebec, Canada): Hoffman Horticultural Perlite (Good Earth, Lancaster, NY)). Prior to seeding, the pots were soaked in DI water for at least 3 d to pre-moisten soil for planting. Seeds were sown as 3 sets of 5 seeds per pot. Pots were kept at ambient temperature under short d light cycles of 9 h light: 15 h dark. Plants were allowed to grow for 14 d at which time plants were culled to only 3 plants per pot (1 plant per section). Plants received 25 mL of 1/64 HP media to each quadrant of the pot (100 mL total volume) every 3 to 4 d.

Table 1. List of experimental conditions used with plants in this study. The media in the HP studies was not changed during the exposure period.

Medium	Treatment and Associated Concentration	Exposure Periods	Age at Treatment Start (d)	RNA Analysis Method with Shoots*
HP	0 (control) and 1 mM LiCl in 1/32 strength MS media	1 and 7 d	26	MA
HP	1 mM NaCl in 1/32 strength MS media	1 and 7 d	26	MA
HP	0.1 mM LiCl kinetic study	1 to 96 h	43	Not applicable
Soil in square pot	0 (control) and 0.05 mM LiCl in 1/64 strength MS media, 100 mL per watering with 25 mL per pot quadrant twice weekly	Four wks plus 2 wks depuration using 1/64 HP twice weekly	37	NGS. Plants sampled after wk 1, 2, 3, 4, and 5 of LiCl exposure

*MA: MicroArray; NGS: Next Generation Sequencing.

2.4 Collection and Processing of At Tissue for RNA Analysis

Using sterile conditions to limit RNAse contamination, shoot tissues were collected from each plant. A total of 150 mg of fresh weight biomass was harvested for each replicate sample, flash frozen in liquid N_2, ground to a fine powder using a porcelain mortar and pestle, and kept at -80°C until RNA extraction. Total RNA was extracted from shoot samples with the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Extractions were conducted following the manufacturer's instructions and the quality and quantity of the extracted RNA was checked with the NanoDrop 2000C Spectrophotometer (Thermo Scientific®, Waltham, MA) and the Agilent Bioanalyzer 2100 (Agilent Technol., Inc., Santa Clara, CA). Only RNA that passed a defined series of quality control metrics (e.g., having a NanoDrop 260/280 ratio >1.8 and RIN or RNA Integrity Number on the bioanalyzer >7) were analyzed with Next Generation Sequencing (NGS) using a HiSeq 2500 (Illumina Inc., San Diego, CA).

2.5 RNA Analysis

For the microarray (MA) analyses, Affymetrix GeneChips® (Affymetrix, Santa Clara, CA) were used according to the Affymetrix Expression Analysis Technical Manual for the *Arabidopsis* Genome ATH1 Array. Prior to the NGS, cDNA libraries were constructed of the Total RNA using TruSeq RNA Sample Prep. v.2 kits (Illumina). The libraries were checked for appropriate quantities and qualities of cDNA using a Qubit® 2.0

(Invitrogen™, Grand Island, NY) or qPCR (StepOnePlus, Applied Biosystems, Grand Island, NY) and an Agilent Bioanalyzer 2100. All MA and NGS samples were run in triplicate.

The NGS data were de-multiplexed using CASAVA 1.8.2 software by aligning reads to the *Arabidopsis* genome (TAIR 10) using TopHat 2.0.4. Runs were set as 50 cycle paired-end reads. Sequences were compared to three publically-available *At* databases: 1) NCBI *Arabidopsis thaliana* TAIR10, 2) Illumina/igenome.database/refgene.gtf, and 3) TAIR (http://www.arabidopsis.org/gene). The differential expression of the transcripts was determined using Cuffdiff/Cufflink 2.0.0 software. The software was used to detect raw mapped reads, normalize the data to "fragments per kilobase per million mapped reads" and compare the differential expression of the treatments versus the controls.

For the MA data, differentially-regulated genes were identified by ranking the fold-change (in log base 2, also referred to as "log 2") to a specific "cut-off" value at *p*-value of 0.05 (Student's *t*-test). The MA chips and that MA data are subject to problems as discussed by [32]. Therefore, the MA data were constricted to a conservative fold-change value of ±1.5 whereas the NGS data, which are more robust, quantitative and qualitative, were not restricted to a fold-change value. A recent review of NGS technique and data analysis which is used widely in this report can be obtained from Van Verk *et al*. [33]. For the NGS data from the soil study, the amount of data was massive and for simplification, the discussion was often limited to the top 100 most abundant transcripts that met the *p*-value cut-off and were up- or down-regulated. In some cases, all of the identified genes that met the *p*-value cut-off in the soil study were discussed as noted.

3. RESULTS AND DISCUSSION

3.1 Phenotypic Observations and Uptake Behavior in Li^+-exposed HP Plants

The HP plants that were exposed to 1 mM LiCl for 7-d exhibited chlorosis and shriveling, which were most evident in the older leaves (see image of 7-d exposure plants in **Fig. 1**). Root tissues in the HP plants that were exposed to Li^+ for 7 d were severely compromised and much of the root tissue biomass was lost relative to the controls. In contrast, phenotypic symptoms of plant stress in the 1-d HP exposure (with 1 mM NaCl or LiCl) and all of the soil treatments were not visible. **Figure 2** shows that the uptake of 0.1 mM Li^+ from HP media by *A. thaliana* shoots is rapid. The exposed plants are most likely undergoing processes to cope with the Li^+ influx, which involves gene expression changes.

Lithium sensitivity is plant species dependent; however, the level of Li^+ (0.05 mM, which equates to 0.347 ppm Li) that was used in the soil-based study was high enough to induce minor injury to some agricultural crops. Concentrations as high as 0.07 mM Li^+ are tolerated by some plants like irrigated corn as described by Bingham *et al*. [10]. Higher levels of Li^+ (12 ppm) produce chlorosis, leaf tip necrosis, interveinal necrosis and abnormal leaf development after a 7-d exposure with some symptoms being marginally-evident as early as 1 d for *Phaseolus vulgaris* (snap bean) plants that were treated with 8.0 and 12 ppm Li^+ [34]. Other studies performed by our laboratory [35, 36] have shown that upon 3 wks of exposure to 15 mM LiCl, soil-grown *At* leaves also experience chlorosis. But the chlorosis is most pronounced in the oldest leaves, which is somewhat evident after 1 wk of 1.0 mM LiCl exposure in HP-grown plants as in **Fig. 1**. This type of behavior is consistent with classic Mg^{2+} deficiency as described by [37] Taiz and Zeiger (2010) who note that Mg^{2+} deficiency in plants can influence the behavior of enzymes that are involved in photosynthesis, respiration, as well as RNA and DNA synthesis. Interveinal yellowing in the leaves was evident in these other studies, which is also consistent with the delayed effect of Mg^{2+} deficiency on the chlorophyll in the vascular bundles. Some interveinal yellowing is apparent in **Fig. 1** as well. Additional studies were recently performed by our laboratory [35, 36] with near IR reflectance measurements and quantification of extracted chlorophyll a+b (Chl a+b) levels of the *At* leaves in soil-grown plants treated with 15 mM LiCl. Upon 3 wks of exposure, *At* leaves produced vegetation indices (specifically the SIPI or structural independent pigment index) which were inversely-correlated with measured

Chl a+b levels and with RWC (relative water content) indices. Low values of the SIPI and RWC indices were observed in plants that were exposed to Li^+ for long periods of time (3 wks, relative to controls).

Figure 1. Images of control 33-d old plants that were given nutrient media (control, left image) and or 1 mM LiCl (right image) for 7 d. Chlorosis and shriveling are visible in the treatment plants.

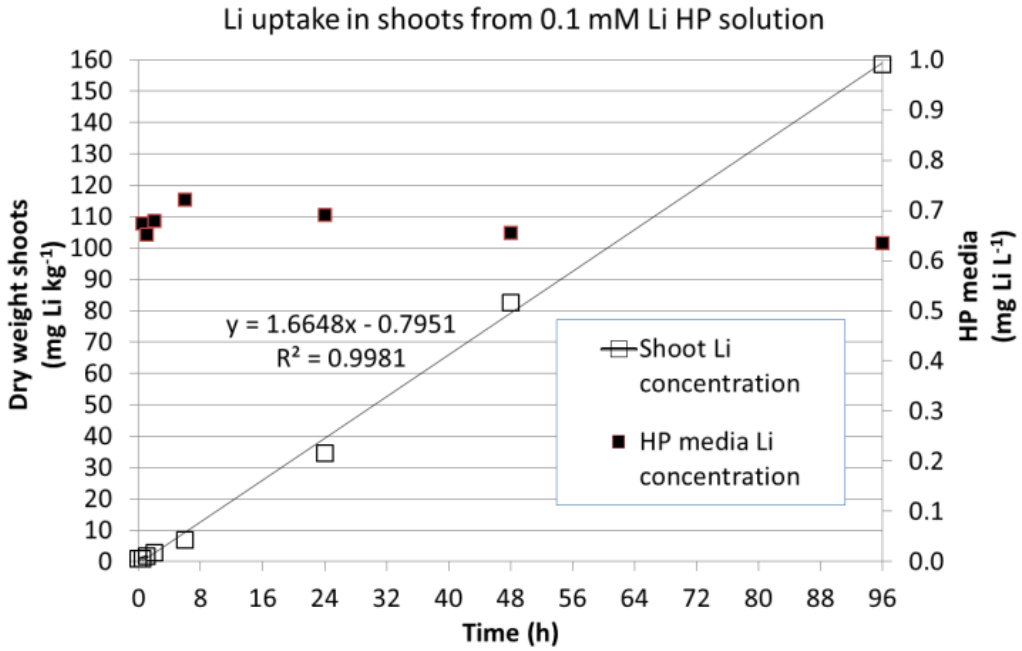

Figure 2. Plot of Li^+ uptake with respect to time [1.0 mM Li^+ is equal to 0.694 mg Li^+ L^{-1} of HP media.]

3.2 Analysis of Entire Genome in the Control Plants from the Multiweek Soil Studies

Analysis of the total RNA collected from plants over the 5 wks based on the cDNA fragments sequenced and mapped back to the genome show that the younger plants in wks 1, 2 and 3 had the highest numbers of genes that met the cut-off requirements (2463, 3006, and 3213 respectively). Older plants in wks 4 and 5 showed a decrease in the number of identified genes (622 and 744 respectively) but they had higher abundance levels of the identified transcripts.

The TAIR GO Functional Analysis search engine [38] was used to determine biological function of genes present by wk (**Table 2**). The levels of genes associated with cell organization and biogenesis (10.2%), developmental processes (8.2%), and electron transport or energy pathways (4.1%) dominated at wk 1. As the plants aged, the genes associated with above mentioned biological functions were reduced in number. Genes

associated with the biological functions of response to abiotic or biotic stimulus (31.4%), response to stress (32.3%), signal transduction (17.8%), and transport (21.0%) showed inverse responses with the lowest values being observed in younger plants (wks 1 and 2) and they increased to their maximum values by wk 5.

For the GO functional categories of other cellular processes and other metabolic processes the levels for all five wks averaged ~49.0% respectively just under the ~56 and 53% reported for the whole genome. The elevated levels of stress response genes in wks 4 and 5 could potentially be explained at least in part by the experimental design. The plants were sown 3 per pot for statistical replication and biomass purposes. Daily observation of these plants revealed no obvious signs of fungal disease, pest/insect infestation or discoloration of leaves. While no obvious signs of stress were present, the plants at 4 and 5 wks were quite large and could have been crowded or dehydrated. These conditions could be reflected in the genomic response, although these conditions are also likely to exist in nature—particularly as the plant prepares for senescence and seed production at the end of its growing cycle.

Table 2. TAIR GO functional analysis search engine for biological function of all genes that met the statistical cut-off. Whole genome information from TAIR is provided for reference.

Biological Function	Whole Genome N=24621[1]	Wk 1 (%) N=1216; Gene IDs: 2463; 199037 FPKM*	Wk 2 (%) N=1514; Gene IDs: 3006; 160797 FPKM	Wk 3 (%) N=1583; Gene IDs: 3213; 168729 FPKM	Wk 4 (%) N=292; Gene IDs: 622; 38709 FPKM	Wk 5 (%) N=338; Gene IDs: 744; 21304 FPKM
Cell organization and biogenesis	13.6	10.2	7.3	7.1	4.4	3.5
Developmental processes	15.6	8.2	7.5	7.2	6.5	5.6
DNA or RNA metabolism	3.7	0.6	1.3	1.6	0.3	0.0
Electron transport or energy pathways	2.8	4.1	1.1	1.1	0.7	0.6
Other biological processes	14.8	18.0	18.0	18.4	30.1	29.3
Other cellular processes	56.7	52.1	47.7	47.8	49.3	47.6
Other metabolic processes	53.6	52.0	46.3	46.8	48.3	47.3
Protein metabolism	21.3	16.4	17.5	17.3	13.0	13.3
Response to abiotic or biotic stimulus	15.3	15.3	19.2	19.2	31.5	31.4
Response to stress	16.6	18.4	20.7	21.3	35.6	37.3
Signal transduction	8.1	7.6	11.8	12.2	18.3	17.7
Transcription, DNA-dependent	10.6	6.5	6.0	6.4	4.8	4.7
Transport	14.3	13.6	13.5	13.6	19.5	21.0
Unknown biological function	32.9	38.5	42.6	42.6	36.3	37.0

* FPKM: Sum of Fragments Per Kilobase of transcript per Million mapped reads.

3.3 Analysis of the 100 Most Abundant Genes by Wk from the Control Plant in the Soil Studies

Analysis of the top 100 most abundant genes by wk showed that in comparison, the overall trend in gene expression was that of down-regulation in the control plants. In wks 1 and 2, 36% of the genes were up-regulated and related to photosynthesis processes (specifically, At1g23130, At1g51402, At1g74470, At3g14420, At4g32260, At5g38410, CA2, CA3, CAB2, DRT112, ESM1, FED A, GAPA, GGT1, LHCA1, LHCB2.1, LHCB2.2, LHCB3, LHCB4.2, LHCB6, PRK, PSAD-1, PSAD-2, PSAE-1, PSAG, PSAH2, PSAK, PSAN, PSAO, PSBQA, PSBTN, PSBW, PSBX, PSBY, RCA, TRX3). In tissues from wks 2 to 3, only 7% of genes were up-

[1] The age of the plant that contributed the whole genome information in TAIR was not known.

regulated and they were related to unknown functions, translation, dehydration response, lateral root development and cellular protein modification (At2g25510, At5g42530, At5g54940, ERD14, RGF9, and UBQ11).

Control plant shoot tissues from wks 3 to 4 showed up-regulation of 15 out of the top 100 genes. Investigation of the function of these genes revealed that they were associated with root hair elongation (At4g05150 and BAL1) and response to other organisms (At1g75800). These genes could be associated with the fact that plants were crowded and in search of water although this may be a normal progression of plants that are ending their lifecycle. Other genes up-regulated from wk 3 to 4 show increased activity related to the gibberellic acid signaling pathway (At1g74670, XTH24), xyloglucan degradation (EXGT-A3) which has been described as an important step prior to cell death in aging leaves [39], and defense response (At1g75800 and At2g30930). From wks 4 to 5 the only up-regulated gene was At2g41090, which is associated with Ca^{2+}-binding, response to mechanical stimulus, and phosphate starvation. By wk 5 (relative to wk 4), there were 61 up-regulated genes (**Table 3**) relative to wk 4 in the top 100 most abundant genes for the control plants. Of these 2 were unknown, 36 genes are associated with pathogenesis-related response or defense response genes and 6 associated with programmed cell death or leaf senescence.

Table 3. List of the 61 genes that were up-regulated at wk 5 relative to wk 4 in the soil-grown control plants that met the *p*-value of ≤ 0.05.

Accession No.	Gene Name; Function
AAP2	Amino Acid transmembrane transporter
ACD6	Accelerated Cell Death 6
AOC1	P-P bond hydrolysis drive protein transmembrane transporter activity
AOC2	Allene Oxide Cyclase 2; senescence; defense response
ARR7	Response regulator 7
At1g27020	Unknown
At1g35710	Defense response
At1g55210	Disease resistance responsive; defense response
At1g58225	Unknown
At1g76960	Unknown
At2g18690	Defense response
At2g41090	Calcium-binding EF–hand f.p.
At2g43530	Defense response
At3g16530	Defense response
At3g22235	Pathogenesis-Related
At3g22600	Lipid transport; response to nitrate
At3g28220	Regulation of anthocyanin biosynthetic process; defense response
At3g28540	ATP binding
At3g47480	Calcium ion binding; defense response
At3g55240	Development
At3g60420	Defense response
At4g24920	SecE/sec61-gamma protein transport protein
At4g38840	Small auxin up-regulated RNA 14; defense response
At5g05600	Iron ion binding; jasmonic acid biosynthetic process; defense response
At5g50460	P-P bond hydrolysis drive protein transmembrane transporter activity
At5g55450	Lipid binding; defense response
AtERDJ3B	Defense response
AZI1	AZelaic acid Induced 1; pathogenesis-response
BGL2	PR protein 2

BIP1	Luminal binding protein 1
BIP2	Luminal binding protein 2
CHI	Chitin binding
CNX1	Defense response
CRK4	Pathogenesis-response; programmed cell death
CRK6	Protein phosphorylation
CRT1a	Ca^{2+} binding
CRT1b	Defense response; response to H_2O_2
CRT3	Calcium ion binding; defense response
DMR6	Defense response
DR4	Drought Repressed 4; water deprivation
GSR 1	*Arabidopsis* glutamine synthase 1
GSTF6	Glutathione *S*-transferase 6; defense response
GSTF7	Glutathione *S*-transferase 7; defense response
LURP1	PR protein
PCC1	Pathogen and Circadian Controlled 1
PCR1	Plant cadmium resistance 1
PDIL1-1	Protein disulfide isomerase 5; programmed cell death
PDIL1-2	Protein disulfide isomerase 6; defense response
pEARLI 1	Early Arabidopsis Aluminum Induced 1; defense response
PR1	PR gene 1
PR5	PR gene 5
PRXCB	Cell elongation; defense response to H_2O_2
RING1	PR protein; programmed cell death
SDF2	Stromal cell derived factor 2; defense response
SHD	Heat shock protein
SUC1	Sucrose transporter
TI1	Trypsin Inhibitor 1; defense response
WAK1	Cell wall associated kinase 1; defense response
YLS5	Defense response; leaf senescence

* f.p.: family protein.

A wk to wk comparison of the top 100 most abundant genes in the control plants shows that only 5 genes were found in at least four of the wks observed: At1g72430, At1g74670, At2g15960, BGAL1 and CCL. In wks 1 to 2, the following genes were down-regulated: At1g12440, At2g23120, At3g15450, At3g61260, At3g63160, At4g05150, At4g27280, At5g10860, At5g57655, ASN1, BGAL1, CCL, COR15A, DYL1, GASA1, KIN2, STP1, TCH4, and XTH4, which are associated with developmental processes and stress/defense response. Many of these genes were also down-regulated by wk 3. See **Table 4** for gene descriptions and direction of gene regulation.

When results from wks 3 and 4 were compared, there were several up-regulated genes for stress/defense response (At1g72430, At1g74670, At3g48740, At4g05150, At4g05150, CCL and ELP). Those genes that became down-regulated by wk 4 were primarily associated with growth and cellular differentiation (At3g63160, KIN2) and defense response (ACS6, AOC1, At1g32920, At1g72910, At1g74450, At2g30020, At2g43530, At3g22235, At3g28220, At5g02160, AOC2, BIP1, COR15A, DIC2, ERF104, GSTF6, GSTF7, PCC1, RAV2, PRXCB, and PORB). In wks 4 to 5, many of the 100 most abundant genes were associated with defense/stress/pathogenesis-related response. Genes that were down-regulated in wk 4 but trending upward at wk 5 were: AGP12, At1g22190, At1g23710, At1g32928, At3g57450, At1g18740, At3g19030, At3g46620, At4g27652, At4g27657, At4g29780, At5g22250, CML38, IAA7, NUDT21, NUDT4, ORA47, PUP18, TCH2, TGG1, TGG2, UCP5 and WRKY40. See **Table 4** for more information.

Table 4. List of genes with the top 100 most abundant transcripts in the soil-grown control plants that met the *p*-value of ≤0.05. Pink boxes represent up-regulation and green boxes represent down-regulation.

Accession No.	Gene Name*; Function	Wks 1 to 2	Wks 2 to 3	Wks 3 to 4	Wks 4 to 5
ACS6	1-aminocyclopropane-1-carboxylic acid; protein binding; stress response				
AGP12	Arabinogalactan protein 12; cell signaling				
AOC1	Allene Oxide Cyclase 1; early response to dehydration 12; senescence				
AOC2	Allene Oxide Cyclase 2; senescence				
ASN1	Glutamine-dependent asparagine synthase 1				
At1g07135	Response to wounding				
At1g12440	A20/N1-like zinc finger f.p.; DNA binding, zinc ion binding				
At1g18740	Flavonoid biosynthetic process; defense response				
At1g22190	Related to AP2 4; transcription factor, defense response				
At1g23710	Unknown function (DUF1645); defense response				
At1g32920	Response to wounding				
At1g32928	Unknown				
At1g72430	Small auxin up-regulated RNA (SAUR 78)				
At1g72910	Defense response; signal transduction				
At1g74450	Response to chitin, wounding				
At1g74670	Gibberellin-regulated f.p.				
At2g10940	Lipid binding and transport				
At2g15960	Unknown				
At2g23120	Late embryogenesis abundant protein group 6; stress response				
At2g30020	Defense and abscisic acid signaling pathway; mature pollen stages				
At2g41090	Calcium ion binding, signaling				
At2g43530	Defensin-like f.p.				
At3g15450	Aluminum induced protein with YGL				
At3g19030	Positive regulation of flavonoid biosynthetic processes				
At3g22235	Unknown				
At3g28220	Regulation of anthocyanin biosynthetic process				
At3g46620	Encodes ABA and drought-induced RING-DUF1117; defense response				
At3g48740	Encodes member of the SWEET sucrose efflux transporter f.p; cation homeostasis				
At3g57450	Unknown; defense response				
At3g61260	Remorin f.p.-binding				
At3g63160	Cell differentiation, leaf morphogenesis; transcription				
At4g05150	Octicosapeptide/Phox/Bem1p; protein autophosphorylation; root hair elongation				
At4g27280	Calcium ion binding; stress (wounding)				
At4g27652	Unknown				
At4g27657	Unknown				
At4g29780	Defense response				
At5g02160	Cell differentiation; cation homeostasis				
At5g10860	Cystathionine beta-synthase domain-containing protein; Modulates development by regulating thioredoxin system				

At5g17350	Unknown; N-terminal protein myristolation				
At5g22250	CCR4-associated factor; mRNA deadenylation activity; defense response				
At5g54940	SUI1 f.p.; translation initiation				
At5g57655	Xylose isomerase f.p., carbohydrate metabolism				
BGAL1	Carbohydrate binding				
BIP1	ER-associated protein catabolic process; mature and germinated pollen stage				
CA2	Carbonic anhydrase – zinc ion binding				
CCL	Circadian Clock Like				
CML38	Calmodulin like 38; defense response				
COR15A	Cold-regulated 15A; stress				
DELTA-TIP	Delta tonoplast intrinsic protein; transporter				
DIC2	Dicarboxylate carrier 2				
DYL1	Dormancy-associated protein				
ELP	Polysaccharide binding; stress				
ERD14	Early response to dehydration 14				
ERF104	Ethylene response factor 104; response to wounding, chitin, mechanical stimulus				
EXPA8	Alpha-EXPAnsin gene family; multidimensional cell growth				
FLA9	Fasciclin-Like Arabinogalactan 9				
GASA1	GA responsive GAST1 protein homolog; cell elongation				
GSTF6	Glutathione-*S*-Transferase; seed development stage				
GSTF7	Glutathione *S*-Transferase 11; defense response; glutathione binding				
IAA7	Indole-3-Acetic Acid 7; transcription regulator acting as repressor of auxin-inducible genes; regulation of growth; senescence				
KIN2	Encodes protein kinase APK2b				
NUDT21	Nudix hydrolase homolog 21; hydrolase activity				
NUDT4	Nudix hydrolase homolog 4; hydrolase activity				
ORA47	Defense response, transcription				
PCC1	Pathogen and Circadian Controlled 1				
PORB	Light dependent NADPH; protochlorophyllide oxidoreductase b; biosynthesis and growth				
PRXCB	Class III peroxidase; defense response; cell elongation				
PUP18	Purine transporter				
RAV2	Regulator of the H^+-ATPase; transcription				
STP1	Sugar TransPorter 1				
TCH2	Calmodulin-like 24; touch 2				
TCH4	Touch gene 4; environmental stimuli				
TGG1	*Beta* glucosidase 38; defense response				
TGG2	*Beta* glucosidase 37; defense response; metabolism				
UCP5	Mitochondrial dicarboxylate carrier 1				
WRKY40	WRKY DNA-binding 40; defense response				
XTH24	Involved in aging; encodes a endoxyloglucan transferase-like protein				
XTH4	Xyloglucan endoTransglucosylase/Hydrolase 4; growth				
XTH6	Xyloglucan endoTransglucosylase/Hydrolase 6				

* f.p.: family protein.

3.4 Numbers of Unique Genes by Wk from the Control Plants in the Soil Studies

The numbers of unique genes that were identified between plants of different ages varied with the youngest plants showing the largest numbers (i.e., 1559) and the older plants at wks 3 through 5 had ten-fold less gene diversity. The biological functions of the unique genes revealed that younger plants in wk 1 were often representative of cell organization and biogenesis and developmental processes, as well as electron transport or energy pathways. Plants in wk 2 showed no unique genes as compared to wks 1 and 3. The older plants in wks 3, 4 and 5 showed decreases in the number of genes that were associated with cell organization and biogenesis and developmental processes, but there were increases in genes associated with DNA/RNA metabolism, protein metabolism, and response to abiotic or biotic stimulus or stress.

3.5 Gene Comparisons with Li^+-treated Plants in the Soil Studies

The Li^+-treated plants from wks 1, 2 and 3 had the largest numbers of identified genes that met the cut-off: 2748, 2753 and 2824 as compared to the older Li^+-treated plants in wks 4 and 5 with 936 respectively. Unlike the control plants where there were also fewer genes identified in the later wks, the Li^+-treated plants from later time periods had lower overall transcript abundances with the exception of several genes that function in stress defense and signaling (to be discussed) by wk 5—see **Table 5** for more information.

Use of the TAIR GO Functional annotation search engine with gene ID data for Li^+-treated plants from wks 1 and 2 revealed genes that were mainly associated with cell organization and biogenesis and developmental processes. By wk 3, the plants had greater DNA and RNA metabolism as well as protein metabolism. Additionally those genes that were associated with response to abiotic and biotic stimulus, as well as general stress response genes, began to increase in number. By wks 4 and 5, those genes that were associated with DNA or RNA metabolism or protein metabolism had decreased while the stress response genes remained elevated. Those numbers of genes that were associated with signal transduction and transport also reached a maximum level during these final wks, in terms of their contribution to biological function.

3.6 Review of the Top 100 Most Abundant Genes by Wk for the Soil-grown Li^+-treated Plants

A review of the top 100 genes by transcript abundance revealed that only 56 genes were found in more than two of the sampled wks. According to the TAIR GO Biological Function search engine, these 56 genes were primarily associated with developmental processes, other biological processes, response to abiotic or biotic stimulus, response to stress, signal transduction and transport (**Table 6** and *Supplementary Table A*).

Table 5. TAIR GO biological functional annotation for all genes identified in the Li^+-treated plants that were grown in soil that met the *p*-value of ≤0.05. Whole genome information from TAIR is provided for reference.

Biological Function	Whole Genome N=24621	Wk 1 (%) N=1375; Gene IDs: 2748; FPKM*: 146962	Wk 2 (%) N=1378; Gene IDs: 2753; FPKM: 293070	Wk 3 (%) N=1400; Gene IDs: 2824; FPKM: 116698	Wk 4 (%) N=422; Gene IDs: 936; FPKM: 29098	Wk 5 (%) N=422; Gene IDs: 936; FPKM: 32014
Cell organization and biogenesis	13.6	12.1	12.0	8.4	8.5	8.5
Developmental processes	15.6	10.5	10.4	8.4	10.7	10.7
DNA or RNA metabolism	3.7	1.5	1.5	3.4	0.9	0.9
Electron transport or energy pathways	2.8	4.4	4.4	1.4	1.6	1.6
Other biological processes	14.8	14.2	12.3	18.1	18.9	18.9
Other cellular processes	56.7	55.6	55.5	54.3	53.1	53.1
Other metabolic processes	53.6	55.5	55.4	53.9	52.4	52.4
Protein metabolism	21.3	18.2	18.2	22.1	17.3	17.3
Response to abiotic or biotic stimulus	15.3	14.7	14.7	17.3	23.7	23.7
Response to stress	16.6	15.9	15.9	19.3	24.9	24.9
Signal transduction	8.1	6.4	6.4	9.8	12.8	12.8
Transcription, DNA-dependent	10.6	6.8	6.7	6.4	4.5	4.5
Transport	14.3	16.4	16.3	13.4	17.8	17.8
Unknown biological function	32.9	38.8	38.7	37.8	35.3	35.3

* FPKM: Sum of Fragments Per Kilobase of transcript per Million mapped reads.

Table 6. Up- or down-regulation of the 56 most abundant genes found in more than two consecutive-wk comparisons of plant growth for the 0.05 mM Li^+-treated soil-grown plants. These genes were selected from a pool of the top 100 genes by transcript abundance that met the *p*-value value requirement of ≤0.05.

Accession No.	Gene Name; Function*	Wk 1 to 2	Wk 2 to 3	Wk 3 to 4	Wk 4 to 5
AGP12	Arabinogalactan protein 12; cell signaling				
AGP21	Arabinogalactan protein 21; cell signaling				
At1g21500	Unknown				
At1g29660	GDSL-like lipase/acylhydrolase sf.p; defense response				
At1g52190	Nitrate transporter				
At1g61740	Sulfite exporter TauE/SafE f.p.				
At1g62480	Vacuolar calcium-binding protein related; defense response				
At1g66760	MATE efflux f.p.; transporter and antiporter activity				
At1g74470	Geranylgeranyl reductase activity; photosynthesis				
At1g74670	Gibberellin-regulated f.p.				
At2g10940	2S albumin sf.p				
At2g15960	Unknown				
At3g06750	Hydroxyproline-rich glycoprotein f.p.; cell signaling				
At3g08030	Chlorophyll biosynthetic process; cell signaling				
At3g16370	GDSL-like lipase/alcylhydrolase sf.p				
At3g22060	Contains Pfam profile: PF01657 domain; defense response				
At3g22235	Unknown				

At3g63160	Outer Envelope Protein 6 (OEP6); growth and transcription				
At4g16980	Arabinoglactan-protein family; photosynthesis, defense response				
At5g08330	TCP family transcription factor				
At5g11420	Unknown				
At5g16030	Unknown				
At5g22580	Stress responsive A/B barrel domain				
At5g25460	DUF642 L-GALL responsive gene 2; root morphogenesis				
At5g39670	Calcium-binding EF-hand-f.p.				
At5g44020	HAD superfamily, subfamily IIIB acid phosphatase				
At5g51190	Ethylene Response Factor (ERF) subfamily B-3/AP2 transcription factor family				
At5g51720	NEET group protein				
At5g52760	Copper transport protein family				
CCL	Involved in cellular cation homeostasis; divalent metal ion transport				
COR15A	Cold-regulated 15A; defense response				
COR15B	Cold-regulated 15B; defense response				
COR413IM1	Cold regulated 314 inner membrane 1				
DELTA-TIP	Delta tonoplast intrinsic protein; transporter				
DYL1	Dormancy-associated protein 1; defense response				
ECS1	Encodes cell wall protein; defense response				
ELP	Chitinase-like protein 1; growth				
ERF104	Ethylene response factor 104				
EXPA1	Alpha-expansin gene family				
FLA9	Fasciclin-Like Arabinogalactan 9				
GAMMA-VPE	Gamma vacuolar processing enzyme; response to toxin-induced cell death				
GASA1	GA-responsive GAST-1 protein homolog				
IAA7	Indole-3-acetic acid 7; transcription regulator acting as repressor of auxin-inducible genes. Regulation of growth; senescence				
KIN2	Encodes protein kinase APK2b				
LTI78	Low-temperature cold induced 78; defense response				
MLP28	MLP–like protein 28; defense response				
MLP43	MLP–like protein 43; defense response				
PHI-1	Phosphate-induced 1; defense response				
PIP1C	Plasma membrane intrinsic protein 1C; transport				
PSBQ-2	Photosystem II subunit Q				
RTFL17	ROTUNDIFOLIA like 17; shoot system development				
TCH4	Touch gene 4; environmental stimuli				
XTH18	Xyloglucan endotransglucosylase/hydrolase 18				
XTH24	Xyloglucan endotransglucosylase/hydrolase 24; aging				
XTH4	Xyloglucan endotransglucosylase/hydrolase 4; growth				
XTH6	Xyloglucan endotransglucosylase/hydrolase 6; defense response				

*sf.p.: superfamily protein; f.p.: family protein.

The number of genes within the top 100 most abundant for the Li^+-treated plants that were found only in a single two consecutive-wk comparison was 86 for wks 1 vs 2, 80 for wks 2 vs 3 and 82 for wks 3 vs. 4. The biological functions of these unique genes by wk revealed that in wks 4 and 5 the plants had increased biological

function for cell organization and biogenesis and developmental process, along with electron transport and energy pathways, transport, response to abiotic and biotic stress, and signal transduction (*Supplementary Table B*).

3.7 Comparison of Treatment vs. Control on Gene Response in Soil-grown Plants

When all of the genes from wks 1 to 5 were input for the determination of GO biological functions in TAIR, the genes that represented cell organization and biogenesis, developmental processes, protein metabolism and transport were more elevated in the Li^+-treated plants relative to that of the controls (*Supplementary Table C*). Slightly lower values were measured in the Li^+-treated plants relative to the controls for response to abiotic or biotic stimulus, response to stress, signal transduction and transport.

However, the top 100 most abundant genes in Li^+-treated plants were associated with developmental processes, response to abiotic or biotic stimulus, and response to stress, signal transduction and transport (*Supplementary Table C*). Signal transduction increased with time while transport behaviors started high, decreased in wks 3 and 4 and increased towards initial values by wk 5.

In the Li^+-treated plants, several genes were measured in wks 4 and 5 that are commonly associated with the cold response pathway: CCL, COR15A COR15B, COR413IM1, DELTA-TIP, IAA7, KIN2, LTI78 and TCH4 (**Table 6**). Several of the COR genes also encode a Late Embryogenesis Abundant or "LEA" proteins as described by [40]. Many of these genes are also associated with drought or salt stress because they are responsive to cell dehydration. Additionally many of the cold response changes can respond to hydrogen peroxide formation. Treatment of plants with hydrogen peroxide has been shown to mimic cold acclimation [41, 42]. Cold response is also linked to circadian rhythms [43].

Knight *et al*. [44] used a transgenic 7-d *Arabidopsis* seedling to investigate cold Ca^{2+} signaling. Under normal conditions cold shock elicits an immediate rise in cytosolic free Ca^{2+} concentrations. This change in Ca^{2+} levels has been reported to be a necessary step in a temperature-sensing mechanism that enables the plant to withstand future cold stress better. Proteins enhanced by the cold-induced genes KIN1 and KIN2 may act as "antifreeze" for plants because they have a similar sequence to those found in fish. KIN2 is also associated with salt stress and drought/dehydration stress. Dehydration of cells is common to cold events.

In summary, of the up-regulated genes within the top 100 most abundant genes, those genes in the early wks of exposure (wks 1 vs 2) genes were associated mainly with cell organization and biogenesis, developmental processes, electron transport and energy pathways. This is consistent with normal growth and a requisite production of biomass of plants of this age and much like that in the controls. There were also several genes associated with protein metabolism, other metabolic processes and the stress response and response to abiotic and biotic stimulus genes.

In the later wks, wks 3 to 4 and 4 to 5, there was a change in the genome profile. The genes that were associated with cell organization and biogenesis remained similar in total number to the early wks (1 vs 2), while genes that were associated with electron transport and energy pathways and development became less abundant with time. By wks 3, 4 and 5, there was an increase in genes associated with response to abiotic and biotic stress, response to stress, signaling, transcription-DNA dependent, and transport.

Some but clearly not all of the identified genes and their expression behaviors that were observed in our study were unprecedented. These genes and their gene regulation responses are presented in several of the following examples and in **Tables 7** through **11** unless noted otherwise.

3.8 Inositol Monophosphate Gene Family Transcript Abundance Behavior in the HP vs. Soil Studies

In the HP 1-d and 7-d LiCl exposures, several genes that were related to the inositol synthesis and the phosphatidylinositol signaling pathway were differentially-expressed relative to the control and to equimolar NaCl. These included, but were not limited to, those that are listed in **Table 7**. The calculated fold-changes for the plant shoots from the HP studies are listed in **Table 8**. In the HP Li^+-treated plants, the MIPS2 gene was up-regulated after d 1 but by d 7, the MIPS1, MIPS2, the putative MIPS and inositol monophosphate genes were down-regulated. The putative trypsin inhibitor (TI1) was up-regulated in the Li^+-treated plants from the are involved with phosphatidyl-inositol phosphorylation, ER stress, and systemic acquired resistance) in the Li^+-exposed plants were up-regulated in the HP studies (**Table 7**). Some of these genes to a lesser extent were observed at the end of the soil-based studies but the level of up-regulation was not as pronounced.

Table 7. Listing of several genes that were highly regulated in the HP studies that met the *p*-value of $\leq$0.05.

Accession No.	Gene Name*
At4g39800	Inositol-3-phosphate synthase isozyme 1, MIPS1
At2g22240	Inositol-3-phosphate synthase isozyme 2, MIPS2
At5g54390	Inositol monophosphate f.p.
At5g10170	Inositol-3-phosphate synthase putative (put.)
At5g09290, At5g63990	3'(2'),5'-bisphosphate nucleotidase/inositol polyphosphate 1-phosphatase
At5g64000	3'(2'),5'-bisphosphate nucleotidase/inositol polyphosphate 1-phosphatase (SAL2)
At2g43510	Trypsin inhibitor put., TI1
At2g22810	1-aminocyclopropane-1-carboxylate synthase 4, ACS4
At4g26200	1-aminocyclopropane-1-carboxylate synthase 7, ACS7
At1g12010	1-aminocyclopropane-1-carboxylate oxidase, put.
At5g24240, At3g56600	Phosphatidylinositol (PI) 3- and 4-kinase f.p./ubiquitin protein
At1g21980	1-PhosphatidylInositol-4-Phosphate 5-kinase, put. (PIP) kinase, put.
At1g17860, At1g73260	Trypsin and protease inhibitor protein
At1g55780, At2g35730, At3g04900, At3g07600, At4g05030, At5g52720, At5g52740, At5g52760	Heavy-metal-associated domain (HMAP)-containing protein
At5g52770	HMAP-related
At1g22990	HMAP-related/copper chaperone (CCH) related
At1g17020, At3g13610, At4g10500, At4g54000	Oxidoreductase, 2OG-Fe(II) oxygenase f.p
At1g33880, At1g33950, At1g33930	Avirulence-responsive f.p, avirulence induced gene (AIG1) f.p.
At1g33960	AIG1
At1g05680	UDP-glucosyl transferase f.p. (UGT74E2)
At1g22400	UDP-glucosyl transferase f.p. (UGT85A1)
At2g30140	UDP-glucosyl transferase f.p. (UGT87A2)
At2g43820	UDP-glucosyl transferase f.p. (UGT74F2)
At3g11340	UDP-glucosyl transferase f.p. (UGT76B1)
At3g46660	UDP-glucosyl transferase f.p. (UGT76E12)
At3g53150	UDP-glucosyl transferase f.p. (UGT73D1)
At4g15280	UDP-glucosyl transferase f.p. (UGT71B5)
At4g34135	UDP-glucosyl transferase f.p. (UGT73B2)
At4g34138	UDP-glucosyl transferase f.p. (UGT73B1)
At1g01390, At2g36770, At3g46650, At3g46690, At4g15260, At5g17040, At5g49690, At2g36970, At3g02100	UDP-glucosyl transferase f.p
At1g53680	Glutathione S-Transferase 28 (GSTU28)

At1g69930	GSTU11
At1g74590	GSTU10
At3g09270	GSTU8
At1g02820	Late Embryogenesis Abundant 3 f.p. (LEA3)
At5g06760	LEA4-5
At1g52690	LEA7
At2g35300	LEA18
At2g03850	LEA domain-containing protein
At3g22500	LEA protein (ECP31)
At3g15670, At3g53040, At2g46140	LEA domain-containing protein, put.
At2g44240	Glycine-rich protein 23 (GRP23); induced by 16-hydroxypalmitic acid (HPA)
At3g28510, At3g28540, At3g28580, At5g17760, At5g40010	AAA-type ATPase f.p.
At1g15520	ABC transporter f.p., Pleiotropic Drug Resistance 12 (PDR12)
At2g39350, At3g53510, At4g18050	ABC transporter f.p.
At3g22910	Ca^{2+}-transporting ATPase, plasma membrane-type, putative
At3g21180	Ca^{2+}-transporting ATPase, plasma membrane-type, putative
At1g73800, At1g73805	Calmodulin-binding protein
At5g26920	Calmodulin-binding 60-like-G (CBP60G) protein
At3g22930	Calmodulin-like 11 (CML11) protein
At5g42380	CML17
At4g14640	Calmodulin-8 (CAM8)
At4g14630	Germin-like protein (GLP9)
At2g22420	Peroxidase 27 (PER17)
At3g01190	PER27
At4g37530	PER50
At4g08770	PER37
At5g06720	PER53
At5g05340	PER52
At4g36430, At5g06730, At5g19880	Peroxidase super f.p.
At2g47340	Plant invertase/pectin methylesterase inhibitor f.p.
At1g11590, At5g20860	Plant invertase/pectin methylesterase inhibitor sf.p.
At1g48010	Invertase/pectin methylesterase inhibitor f.p.
At3g09540	Pectin lyase-like f,p.
At5g07410	Pectinesterase f.p./ Pectin lyase-like sf.p.
At1g14420	Pectin lyase f,p.
At2g45220	Pectinesterase f.p. (PME17)
At3g14310	Pectinesterase f.p. (PME3)
At4g26530	*At* fructose-bisphosphate aldolase 5 (AtFBA5) (requires Mg^{2+})

* f.p.: family protein, sf,p.: superfamily protein.

3.9 ACC Synthase Protein Gene Abundance Behavior in the HP vs. Soil Studies

The genes that are associated with 1-aminocyclopropane-1-carboxylate (**Table 7**) were typically down-regulated in the HP studies and they were not differentially-regulated and identified at the statistical cut-off in the soil studies (at a *p*-value of $\leq$0.05). High levels of auxin help to stimulate ACC synthase, which his induced by stress and it controls ethylene biosynthesis [47].

Table 8. Calculated fold-change and associated *t*-test *p*-values for the HP 1 mM LiCl, 1 mM NaCl and control exposures for MIPS, ACS, PR, heavy metal and AIG1 genes.

Accession No.*	**Fold-change****					
	1-d 1 mM Li^+ vs. Control	1-d 1 mM Li^+ vs. Na	1-d 1 mM Na^+ vs. Control	7-d 1 mM Li^+ vs. Control	7-d 1 mM Li^+ vs. Na^+	7-d 1 mM Na^+/Control
MIPS1	BD	BD	BD	BD	-2.94; p=0.001	BD
MIPS2	1.52; p=0.018	BD	BD	BD	-2.08; p=0.033	BD
At5g54390	BD	BD	BD	BD	-1.61, p=0.001	BD
At5g10170	BD	BD	BD	BD	-2.01; p=0.011	BD
At5g09290	BD	BD	BD	BD	2.78; p=0.029	BD
At5g63990	BD	BD	BD	BD	1.86: p=0.006	BD
SAL2	BD	BD	BD	BD	3.69; p=0.045	BD
TI1	2.31; p=0.002	BD	BD	3.82; p=0.042	3.29; p=0.051	BD
ACS4	BD	BD	BD	BD	-1.58; p=0.038	BD
ACS7	BD	BD	BD	BD	3.17; p=0.016	BD
At1g12010	BD	BD	BD	BD	-3.41; p=0.017	BD
At5g24240	BD	BD	BD	BD	7.29; p=0.021	BD
At3g56600	BD	-2.24; p=0.037	1.80; p=0.042	BD	BD	BD
At1g21980	BD	BD	BD	BD	-1.79; p=0.007	
At1g17860	BD	BD	BD	BD	1.54; p=0.013	BD
At1g73260	BD	BD	BD	BD	3.89; p=0.037	BD
PR1	BD	3.06; p=0.052	BD	6.36; p=0.013	5.98; p=0.019	BD
PR p. At2g14580	BD	BD	BD	4.30; p=0.044	BD	BD
PR p. At4g31470	BD	1.91; p=0.028	-2.08; p=0.002	BD	BD	BD
PR5	BD	BD	BD	4.16; p=0.050	BD	BD
At3g04900	1.80; p=0.020	BD	1.76; p=0.032	BD	BD	BD
At1g55780	BD	BD	BD	BD	4.88; p=0.022	BD
At2g35730	BD	BD	BD	BD	5.44; p=0.034	BD
At3g07600	BD	BD	BD	BD	2.82; p=0.007	BD
At4g05030	BD	BD	BD	BD	3.33; p=0.052	BD
At4g25780	BD	BD	BD	BD	-2.37; p=0.052	BD
At5g52720	BD	BD	BD	BD	5.26; p<0.000	BD
At5g52740	BD	BD	BD	3.98; p=0.013	BD	2.63; p=0.013
At5g52760	BD	BD	BD	BD	1.89; p=0.039	BD
At5g52770	BD	BD	BD	BD	3.10; p=0.041	BD
At1g22990	BD	BD	BD	BD	5.45; p=0.016	BD
At4g10500	BD	BD	BD	BD	5.79; p=0.022	BD
At1g33870	BD	3.04; p=0.023	BD	BD	BD	BD
At1g33880	BD	2.44; p=0.012	BD	BD	BD	BD
At1g33930	BD	BD	BD	BD	BD	-2.31; p=0.009
At1g33950	BD	BD	BD	BD	2.58; p=0.054	BD
AIG1 At1g33960	BD	BD	BD	6.93; p=0.025	BD	3.35; p=0.025
At1g33930	BD	BD	BD	-2.81; p=0.009	BD	-2.31; p=0.009
At2g44240	BD	BD	BD	7.83; p=0.010	8.99; p=0.005	BD

*p: putative; f.p.: family protein.

**BD: Below Detection. This is based on the fold-change cut-off of ±1.5 and a p-value of $\leq$0.05 for MA data.

3.10 Pathogenesis-related (PR) Protein Gene Abundance Behavior in the HP vs. Soil Studies

The plant shoots that were exposed to 1 mM LiCl in the 7-d HP tests demonstrated up-regulation of the PR proteins. These proteins were PR1, PR2 and putative PR genes (as discussed previously and as listed in previous tables). These genes had fold-change values ($p \leq 0.05$) from +4.16 to +6.36 for Li^+-treatment relative to the control for the 7-d exposures (**Table 8**). PR1 and another PR protein putative (At4g31470) were up-regulated when the gene expression of Li^+-exposure relative to Na^+-exposure was compared with fold-change values of +3.06 and +1.91 respectively. At4g10500 (an oxidoreductase) that is a predicted functional partner protein of PR1 was up-regulated as well in the HP studies. The 1- and 7-d 1 mM NaCl HP treatments did not exhibit the up-regulation response, suggesting that Li^+ has some unique effects on the transcriptome response. Predicted functional partners as found with STRING [45] revealed that PR1 is closely associated with other PR-type gene proteins. PR1 as well as PR5 are co-associated with the allergen V5/Tpx-1 f.p. (At5g66590, data not shown) and the PR protein putative (At4g25780), which were both down-regulated in the 7-d LiCl HP treatments (**Table 8**). Again, these proteins were not highly expressed the 1 mM NaCl HP treatments, suggesting that this is a Li^+-induced specific ion response as opposed to a Na^+- or Cl^--induced response.

In the soil studies (see specific genes as listed in **Table 11**) several wks of watering with 0.05 mM LiCl enhanced the up-regulation of the PR genes relative to that of the control. These included PR1 and PR5 which were in the top 20 most abundant transcripts at wk 5 of the Li^+-treatment series. Another identified gene which has a predicted functional partner of PR1 is BGL2 (beta-1,3-glucanase 2), which was up-regulated and the second most abundant transcript in the wk 5 LiCl treatments. Other transcripts that code for other predicted functional partner proteins and were in the top 100 most abundant transcripts for the Li^+-treatments at wk 5 were: At4g10500, LURP1 (late up-regulated in response to *Haloperonospora parasitica*), and SAG13 (At2g29350, alcohol dehydrogenase/oxidoreductase; senescence-associated gene). All of these high abundance genes were up-regulated relative to the control. None of these genes were expressed relative to the 1 mM Na^+ or 0 mM control in the HP studies.

In tobacco plants, treatment with high levels of Li^+ caused phenotypic responses (necrotic lesions, leaf curling), accumulation of ethylene, salicyclic acid, and gentisic acid and it induced the expression of the PR genes: PR-P, PR1, and PR5 [29]. With the exception of PR1, Li^+ produced the same effects in transgenic tobacco plants that do not accumulate salicylate due to an overexpression of *nahG* (the bacterial hydroxylase gene). Inhibition of ethylene biosynthesis with aminoethoxyvinylglycine (AVG) in these studies precluded any Li^+-induced cell death and PR5 expression. This suggests that Li^+ engages a hypersensitive plant response where ethylene production and enhanced signaling occur in the acute Li^+ exposures as well as the chronic, low-level Li^+ exposures.

3.11 Arabinogalactan Protein (AGP) Family Gene Abundance in HP

In the HP and the soil studies, the exposure of Li^+ produced up-regulation of the AGP family gene. **Table 6** lists several of the AGP genes from the soil studies. AGP2, AGP5, AGP10, and AGP24 were all up-regulated in the 7-d HP studies when Li^+ was compared to Na^+ AGP5 in particular was up-regulated by +4.69 (p=0.034) in the HP studies (not all data shown). These findings, which concur with that of Li *et al.* [30] suggest that Li^+ possesses a specific ion effect on AGP gene behavior. These proteins play an important role in plant cell structure but not all processes associated with AGP proteins are understood [46].

3.12 Heavy-Metal-Associated Domain-Containing Protein Gene Abundance in HP

In the 1- and 7-d LiCl exposures, several genes that were related to the heavy-metal protein response were differentially-expressed relative to the control and to equimolar NaCl. These included but were not limited to those listed in **Table 7**. The genes for these proteins were generally up-regulated in the HP studies (**Table 8**). This is indicative of a toxic ion response, much like that of other metals. The diversity of heavy-metal response genes was not observed in the soil studies, but some genes such as At5g52760 were highly up-regulated and also

high in transcript abundance (**Table 11**). Li *et al.* [30] observed the up-regulation of a couple of heavy-metal-associated genes but their gene accession numbers did not match those in our study and the levels of up-regulation after the acute exposure of high levels of Li^+ were not observed in the study by Li and researchers.

3.13 Avirulence-responsive Gene Abundance

The avirulence-responsive f.p. genes (AIG1 and AIG2) are often associated with system acquired resistance and general stress response in plants. They also influence endocytosis, salicylic acid biosynthetic process, and salicylic acid mediated signaling pathway. AIG1 genes were generally up-regulated in the HP studies with 7-d 1 mM HP LiCl exposures (**Table 8**). The AIG2 gene was not differentially expressed in the HP studies but it was with the soil-grown plant shoots and its highest abundance (up-regulation) occurring during wks 4 and 5 relative to the control (**Table 11**).

3.14 UDP-Glucosyl Transferase (UGT) and Glutathione S-Transferase (GST) Family Protein Gene Abundance

The UGT proteins respond to xenobiotic chemicals, are involved with the making of secondary metabolites and they facilitate the addition of a sugar groups to target molecules by the formation of a glycosidic bond. Their up-regulation in the acute exposures is to be expected. Several UGT f.p. genes were up-regulated in the acute 7-d 1 mM HP LiCl exposures although a few of the UGT genes were down-regulated as noted (**Table 9**). In one case for UGT73D1 in the 7-d exposures, a large fold-change of +6.02 (p=0.002) was observed when the LiCl treatments were compared to the NaCl treatments. This up-regulation behavior was observed by Li *et al.* [30] (within their supplementary data) who studied Li^+ exposure responses with *Brassica (Br.) carinata*.

The UGT genes were not highly abundant in the low level soil exposures. A few of them were significantly up- or down-regulated when the treatments were compared with the controls but there were few trends or similarities to that observed of the acute exposures. For example, UGT73B1 in the soil studies, for example was always down-regulated, which was unlike that of the acute treatments. This may be due to the hormesis effect, which may provoke a positive low level type response that is unlike that of an acute response. UGT73D1, which was highly up-regulated in the acute HP exposures was up-regulated in the week 5 treatment plants relative to the week 5 controls (+1.76, p=0.0002) and it also became increasingly up-regulated between wks 3 and 4 (+5.32, p=0.0002) and wks 4 and 5 (+2.97, p=0.00003) in the soil treatments but it was not differentially expressed in the soil-based controls from the same time periods.

The glutathione *S*-transferases also respond to xenobiotic chemicals (much like the UGT proteins) via conjugation with reduced glutathione. These enzymes are involved with the scavenging of reactive oxygen species (ROS) species which can form from drought, chilling, high ammonia and viral infection stresses. Like with the UGT protein genes, their up-regulation in the presence of substantial stress is to be expected, as it was observed in the acute (**Table 9**) and long-term chronic Li^+-exposures (*Supplementary Table B*).

3.15 Late Embryogenesis Abundant (LEA) Family Protein Gene Abundance

The LEA proteins often respond to stress, dehydration and low temperature exposure conditions [40]. Nearly all of the LEA genes were up-regulated in the acute 7-d 1 mM HP LiCl exposures relative to the NaCl (**Table 9**). The 7-d 1 mM NaCl treatment exhibited up-regulation of LEA3 but up-regulation of LEA3 in the equimolar LiCl treatments relative to Na^+ was not observed. Much like the UGT family genes, the LEA genes exhibited similar behavior in the soil study. LEA4-5 was down-regulated in wks 3 to 4 of the control as well as the LiCl soil-based treatments and up-regulated in wks 4 and 5 of the control and LiCl soil-based treatments. This up-regulation behavior after high level Li^+ exposure is consistent with the observations of Li *et al.* [30] with *Br. carinata*.

Table 9. Calculated fold-change and associated t-test *p*-values for the HP 1 mM LiCl, 1 mM NaCl and control exposures for UGT and LEA series genes.

Accession No.*	Fold-change**					
	1-d 1 mM Li^+ vs. Control	1-d 1 mM Li^+ vs. Na	1-d 1 mM Na^+ vs. Control	7-d 1 mM Li^+ vs. Control	7-d 1 mM Li^+ vs. Na^+	7-d 1 mM Na^+/Control
UGT74E2	BD	BD	BD	2.72; p=0.003	3.44; p=0.034	BD
UGT87A2	BD	BD	BD	BD	1.82; p=0.035	BD
UGT f.p. At2g36770	BD	BD	BD	BD	1.68; p=0.010	BD
UGT76B1	BD	BD	BD	BD	3.61; p=0.052	BD
UGT f.p. At3g46690	BD	BD	BD	BD	2.78; p=0.011	BD
UGT73D1	BD	BD	BD	BD	6.02; p=0.008	BD
UGT73B1	BD	BD	BD	BD	1.64; p=0.021	
UGT73B2	BD	2.75; p=0.011	BD	BD	3.51; p=0.005	BD
UGT85A1	BD	BD	BD	2.43; p=0.030	BD	BD
UGT f.p. At5g49690	BD	BD	BD	2.76; p=0.017	BD	BD
UGT76E12	3.28; p=0.031	BD	BD	BD	BD	BD
UGT74F2	1.66; p=0.004	BD	BD	BD	BD	BD
UGT f.p. At3g46650	-2.19; p=0.028	-2.58; p=0.024	BD	BD	BD	BD
UGT f.p. At5g17040	BD	BD	BD	BD	-2.31; p=0.011	BD
UGT f.p. At1g01390	BD	BD	BD	BD	-2.28; p=0.044	BD
UGT f.p. At4g15260	BD	BD	3.27; p=0.037	BD	BD	BD
UGT71B5	BD	BD	1.51; p=0.044	BD	BD	BD
UGT f.p. At2g36970	BD	BD	BD	1.81; p=0.040	BD	2.51; p=0.040
UGT f.p. At3g02100	3.05; p=0.015		3.66; p=0.026	BD	BD	BD
GSTU28	BD	BD	BD	BD	1.51; p=0.024	BD
GSTU11	BD	BD	BD	BD	5.15; p=0.029	BD
GSTU10	BD	BD	BD	BD	4.99; p=0.019	BD
GSTU8	BD	BD	BD	BD	5.91; p=0.004	BD
LEA3	BD	2.67; p=0.037	BD	3.99; p=0.010	BD	3.51; p=0.010
LEA f.p. At2g03850	BD	BD	BD	3.54; p=0.009	4.40; p=0.022	BD
LEA f.p. At3g15670	BD	BD	BD	3.21; p=0.023	1.99; p=0.038	BD
LEA f.p. At3g53040	BD	BD	BD	3.59; p=0.028	4.34; p=0.050	BD
LEA7	BD	BD	BD	BD	4.00; p=0.022	BD
LEA f.p. At2g46140	BD	BD	BD	BD	1.55; p=0.015	BD
LEA4-5	BD	BD	BD	BD	2.27; p=0.034	BD
LEA18	BD	BD	BD	BD	4.79; p=0.048	BD
ECP31	BD	BD	-2.11; p=0.037	BD	BD	BD

*p: putative; f.p.: family protein.
**BD: Below Detection. This is based on the fold-change cut-off of ±1.5 and a p-value of $\leq$0.05 for MA data.

3.16 Glycine-rich Protein Gene Abundance

One of the genes that was significantly up-regulated (+8.46, p=0.003) at in the acute 7-d 1 mM HP LiCl exposures relative to the NaCl was the gene for the glycine-rich protein 23 (**Table 10**). Park *et al.* [57] examined the expression of GRP23 and found that it can be induced by HPA which is a primary constituent of cutin. The PR-1 and PR-5 genes were also induced by HPA in this study. Park and researchers observed that the generation of cutin monomers may induce a pathogen response role as well as provoke the accumulation of peroxide. The addition of foliar abscisic acid as well as salicylic acid also caused the up-regulation of GRP3, GRP5, and GRP23.

3.17 AAA-type ATPase Family Protein Gene Abundance

The AAA-type ATPase f.p. are ubiquitous in nature and have diverse roles, such as DNA replication, DNA recombination, DNA repair, protein unfolding (sometimes termed as protein "re-modeling") as reviewed by Snide *et al.* [48]. The AAA-type ATPase family proteins in the acute HP Li^+ exposures were consistently up-regulated when the LiCl to NaCl treatments were compared (**Table 10**). In the soil exposures, three AAA-type ATPase f.p.s genes were up-regulated relative to the controls but the up-regulation was not restricted to the treatments only. This up-regulation behavior after high level Li^+ exposure is consistent with the observations of Li *et al.* [30] that examined *Br. carinata*. Up-regulation of At3g28580 in the soil studies was apparent during the last weeks of the study relative to the control (as noted in **Table 11**). Some down-regulation was also observed with time in the controls. It is evident that the low level exposures do not invoke the same responses as the acute high level exposures to Li^+.

3.18 ABC Transporter Family Protein Gene Abundance

The ABC (ATP-Binding Cassette) transporter genes are known to participate in ATP binding, ATPase activity, the coupling with the transmembrane movement of substances, the glucosinolate biosynthetic process, nucleoside-triphosphatase activity and nucleotide binding in plants. In the acute HP 7-d 1 mM Li^+ exposure plant shoots, these ABC transporter genes were up-regulated relative to equimolar 7-d Na^+ exposures (**Table 10**). In contrast, these genes did not exhibit differential regulation in the soil-based studies (data for soil-study not shown). This gene regulation behavior has not been attributed to Li^+ exposure in prior work with plants. The At1g15520 gene (PDR12) also responds to various stresses including dehydration, heavy-metal ion transport, fungal and bacterial defense, and it exhibits control over hydrogen peroxide metabolism and salicylic acid signaling mediation. Unlike in the HP studies, the PDR12 gene response in the chronic soil-based studies was the same for the control as in the treatment. The linkage of Li^+ exposure to ABC transporter genes has not been made with plants to our knowledge.

3.19 Calcium-ATPase and Calmodulin Gene Abundance

These genes regulate intracellular Ca^{2+} levels in cells, which is a major second messenger in cell signaling. The Ca^{2+}-ATPase genes code for proteins that limit Ca^{2+} levels so the up-regulation of these genes would suppress Ca^{2+} entry into cells. In the acute HP 7-d 1 mM LiCl exposure plant shoots, these genes were highly up-regulated relative to equimolar 7-d NaCl exposures (**Table 10**). This suggests that Li^+ may be responsible for some dysregulation of Ca^{2+} levels in the cell and these findings are consistent with that of Li *et al.* with *Br. carinata* and high levels of LiCl. In contrast, these genes did not exhibit differential regulation in the [30] soil-based studies. This gene regulation behavior has been examined in bipolar attributed to Li^+ exposure in prior work with plants. At3g22910 became slightly up-regulated in the low-level chronically-exposed Li^+-treated plants from the soil studies, but only between wks 4 and 5 (data for soil-study not shown).

3.20 Germin-like Protein Gene Abundance

A linkage between Li^+ response and GLPs has not been established in plants but GLP9 which had a fold-change of +9.00 (p=0.005) for the 7-d LiCl-treated plants relative to the 7-d NaCl-treated plants) was one of the most up-regulated genes that was measured in the entire study (see **Table 10**). GLP9 was differentially regulated in the controls and treatments from the soil study. After 4 wks, the soil-grown Li^+-exposed plants had a large fold-change of +6.24 (p=0.0000003) relative to the controls but similar up-regulation over time occurred in the treatments for wks 3 to 4 and in the controls for wks 4 and 5 so there must be other natural processes that promote significant up-regulation of this gene that are independent of Li^+ exposure (data for soil-study not shown). The differential regulation of germin-like proteins after high level Li^+ exposure was not observed by Li *et al.* [30]. Hurkman and Tanaka [59] examined the regulation of GLP genes in wheat after infection with powdery mildew. They observed that mRNA for GLP, which converts oxalate to CO_2 and H_2O_2 will become up-regulated to help

with the plant's response to various types of infections. The GLPs are defense response proteins. Three other germin-like genes were observed in the study by Li *et al.* [30] but they were predominantly down-regulated, which is in contrast to our present study which had highly up-regulated GLP9 upon acute Li^+ exposure.

Table 10. Calculated fold-change and associated t-test *p*-values for the HP 1 mM LiCl, 1 mM NaCl and control exposures for various proteins that were mostly up-regulated in the HP study.

Accession No.*	**Fold-change****					
	1-d 1 mM Li^+ vs. Control	1-d 1 mM Li^+ vs. Na	1-d 1 mM Na^+ vs. Control	7-d 1 mM Li^+ vs. Control	7-d 1 mM Li^+ vs. Na^+	7-d 1 mM Na^+/Control
GRP23	BD	BD	BD	7.89; p=0.010	8.46; p=0.003	BD
At3g28510	BD	BD	BD	BD	5.36; p=0.020	BD
At3g28540	BD	BD	BD	3.56; p=0.044	BD	2.38; p=0.044
At3g28580	BD	BD	BD	BD	2.39; p=0.021	BD
At5g17760	BD	BD	BD	BD	2.26; p=0.025	BD
At5g40010	BD	BD	BD	BD	3.63; p=0.026	BD
PDR12	BD	BD	BD	BD	4.01; p=0.029	4.43; p=0.001
At2g39350	BD	BD	BD	4.43; p=0.001	1.82; p=0.009	BD
At3g53510	1.64; p=0.004	BD	2.11; p=0.037	BD	1.93; p=0.045	BD
At4g18050	BD	BD	BD	BD	1.65; p=0.004	BD
At3g21180	-2.25; p=0.000	BD	BD	BD	BD	BD
At3g22910	BD	BD	BD	BD	4.08; p=0.013	BD
At1g73800	BD	BD	BD	BD	2.39; p=0.006	BD
At1g73805	BD	BD	BD	BD	1.97; p=0.011	BD
CBP60G	BD	BD	BD	BD	1.94; p=0.001	BD
CAM8	BD	BD	BD	2.50; p=0.048	3.92; p=0.039	BD
CML11	BD	BD	BD	BD	1.59; p=0.017	BD
CML17	BD	BD	BD	BD	3.28; p=0.014	BD
At1g67980	BD	BD	BD	BD	6.39; p=0.005	BD
GLP9	BD	BD	BD	BD	9.00; p=0.005	BD
PER17	1.95; p=0.024	BD	BD	BD	BD	BD
PER27	BD	BD	BD	BD	1.97; p=0.029	BD
PER50	BD	BD	BD	BD	2.61; p=0.007	BD
PER37	BD	BD	BD	BD	4.91; p=0.008	BD
PER53	BD	BD	BD	BD	3.49; p=0.0004	BD
PER52	1.85; p=0.043	BD	3.47; p=0.006	BD	BD	BD
At4g36430	1.92; p=0.012	BD	BD	BD	2.56; p=0.054	BD
At5g06730	2.44; p=0.004	BD	BD	BD	6.33; p=0.011	BD
At5g19880	2.71; p=0.021	BD	BD	5.79; p=0.013	5.85; p=0.014	BD
At2g47340	2.16; p=0.041	BD	BD	BD	BD	BD
At3g42570	BD	BD	BD	BD	-1.71; p=0.031	BD
At1g11590	BD	BD	BD	BD	-4.42; p=0.023	BD
At5g20860	-2.86; p=0.006	BD	BD	BD	BD	BD
At1g48010	BD	BD	BD	-2.41; p=0.053	BD	-1.56; p=0.053
At3g09540	BD	BD	BD	BD	-1.71; p=0.004	-1.92; p=0.031
At5g07410	-2.93; p=0.005	-1.58; p=0.039	BD	2.25; p=0.038	BD	3.14; p=0.038
At1g14420	2.18; p=0.050	BD	BD	BD	BD	BD
PME17	BD	BD	BD	BD	7.16; p=0.005	BD
PME3	BD	BD	BD	BD	-4.42; p=0.023	BD
AtFBA5	BD	BD	BD	BD	-2.31; p=0.019	BD

*p: putative; f.p.: family protein.

**BD: Below Detection. This is based on the fold-change cut-off of ±1.5 and a *p*-value of ≤0.05 for MA data.

3.21 Peroxidase Gene Abundance

It is well known that peroxidases will respond to oxidative stress in living organisms so it is somewhat expected that they will be differentially-expressed in plants after exposure to toxic ions. All of the peroxidases were up-regulated in the acute Li^+ exposure studies after a 7-d exposure (see **Table 10**). In contrast to the acute Li^+-exposure study with HP plants, the Li^+-exposed soil-grown plants did not exhibit significant up-regulation of the peroxidases. In one case, the control and the treatment had similar up-regulation behavior for PER50 with time and that suggests there was not a treatment effect of Li^+ on PER50 (data for soil-study not shown). Li *et al.* [30] (2009) reported the substantial up-regulation of one peroxidase (At4g23885) in their study but this particular gene was not highly differentially regulated in our study. However, they did see low-level down-regulation of At4g36430 and several other peroxidase genes as well as low-level up-regulation of PER50 and some other peroxidases.

3.22 Pectinase Gene Abundance

The high up-regulation of pectin-related genes due to Li^+ exposure is somewhat unprecedented as observed in our study but most of the pectin-related genes were down regulated in our study and that of Li *et al.* [30]. Such interactions with pectinesterase may be related to the influence of Li^+ on free *myo*-inositol as described by Loewus and Murthy [50]. Free *myo*-inositol metabolism has a role in pectin synthesis for making cell wall structure. However, the specific up-regulation of one of the pectinesterase genes and the down-regulation of another pectinesterase gene in addition to the down-regulation of pectin methylesterase inhibitor superfamily protein makes the data in this study difficult to interpret (see **Table 10**). It is possible that these pectinesterases are functioning in separate locations in the plants and that explains the inconsistent trend in regulation that is observed. It is possible that the structure of the ER may be influenced by acute high level Li^+ exposure due to its impact on the regulation of pectinesterase. Substantial up-regulation of pectinesterase (At2g45220 or PME17) has been observed by Li *et al.* [30].

3.23 Fructose-Bisphosphate Aldolase Gene Abundance

In the MA studies, the fructose-bisphosphate aldolase 5 (AtFBA5) gene was down-regulated (**Table 10**). This gene codes for a protein that is part of the Calvin cycle and gluconeogenesis. Lu *et al.* [51] examined the behavior of several of the AtFBA shoot genes to stress and identified AtFBA5 shoot genes to become significantly down-regulated in the presence of cadmium sulfate (heavy-metal stress) but up-regulated in the presence of salt, temperature, ethylene, absisic acid and other stresses. Li *et al.* [30] did not report data on the differential regulation of FBA in their study. FBA requires the incorporation of Mg^{2+} into its structure. It is possible that Li^+ may be able to occupy a similar location to that of the Mg^{2+} and decrease the function of this protein.

Table 11 shows data for AtFBA5 that indicates some up-regulation in the Li^+-treated soil-grown plants during wks 3 and 4, which is in contrast to the MA results. This up-regulation behavior in the soil-grown plants may be a hormesis effect but there are probably other explanations which could be pursued but are beyond the present study.

3.24 Photosystem I and II Gene Abundance

The MA analyses do not fully cover chloroplast mRNA abundance in depth but the NGS analyses capture much of the chloroplast genes. It is to be expected that young plants will be more photosynthetically-active during their younger days while they prepare for senescence. **Table 11** shows the effect of added Li^+ on the chloroplast transcripts of the soil-grown plants after 1 wk of 0.05 mM LiCl treatment caused a substantial decrease in the overall abundance of some photosystem II-related gene transcripts (down-regulation of the treatment relative to the control) such as: CAB3 (chlorophyll a/b-binding protein 3); PSAG (photosystem I

subunit G); LHCB3 (light-harvesting chlorophyll B-binding protein 3) and several other related proteins that comprise the light-driven reactions in the plant. In some cases, about 50% of the mRNA abundance was diminished in the Li^+ treatments relative to the wk 1 control but this varied with gene. However, by wk 2, the levels of transcript abundance of these photosystem family genes had nearly recovered to levels that were similar to that of the control in wk 2.

Table 11. Average transcript abundance values for specific identified genes from the soil-based studies. Values represent genes that met the cut-off requirements on a wk to wk per treatment (control or 0.05 mM Li^+) basis.

Accession No.	Wk 1 Li^+-treatment*	Wk 1 Control	Wk 2 Li^+-treatment	Wk 2 Control	Wk 3 Li^+-treatment	Wk 3 Control	Wk 4 Li^+-treatment	Wk 4 Control	Wk 5 Li^+-treatment	Wk 5 Control
SAL2	5	1	ND	6	64	ND	ND	ND	2	38
At3g28580	ND	ND	4	4	56	ND	ND	ND	ND	22
AtFBA5	ND	ND	ND	45	148	49	155	ND	ND	ND
BGL2	ND	ND	ND	ND	4	ND	61	2	814	594
At4g10500	ND	ND	ND	ND	ND	ND	7	ND	78	29
SAG13	10	ND	1	1	3	ND	22	2	102	40
LURP1	5	ND	1	ND	6	9	28	1	331	189
PR1	20	ND	ND	ND	ND	ND	38	ND	313	125
PR4	ND	39	11	15	54	ND	23	ND	105	ND
PR5	21	3	3	2	ND	ND	38	ND	594	405
At5g52760	ND	ND	13	55	52	ND	138	ND	346	ND
AIG1	ND	ND	ND	ND	3	ND	ND	ND	52	31
AIG2	ND	ND	29	20	101	72	66	30	138	ND
CAB2	1226	2324	6812	7941	ND	ND	ND	ND	ND	ND
LHCB3	646	1310	5586	5043	ND	ND	ND	ND	ND	ND
PSAG	1029	1741	4504	4657	ND	ND	ND	ND	ND	ND

*NG: Not Detected. These NGS data did not meet the *p*-value cut-off of $\leq$0.05.

3.25 IAA Gene Abundance

Lithium can inhibit Ca^{2+} channels and limits indole acetic acid (IAA)- and nitric oxide-induced adventitious root formation, which is a possible explanation for decreased root biomass after 7 d of 1 mM LiCl in the current HP study [52]. This would result in the down-regulation of IAA-type genes in Li^+-exposed *At* roots, but the down-regulation of IAA genes was not observed in the roots after 1 d of 1 mM LiCl exposure (unpublished data, not shown). The 1-d HP exposures to 1 mM LiCl did not result in differential regulation of IAA genes in the shoot tissues. After 7-d exposure, the shoots that were exposed to 1 mM LiCl showed some measureable changes in IAA gene with IAA3, which was down-regulated (fold-change for Li^+/control of -1.85, p=0.051) and with IAA17 (fold-change for Li^+/Na^+ of -1.87, p=0.027). The HP NaCl treatments did not exhibit differential regulation of IAA genes, regardless of exposure time.

As for the soil-grown 0.05 mM Li^+-exposed and control *At* shoots during the first few wks, the levels of mRNA transcripts for all identified IAA genes were similar throughout the 5 wk study (data not shown). There are some slight exceptions in that IAA7 transcripts were detected in the Li^+ treatment after wk 2 and wk 3 but not in the control, IAA9 was only observed in the control during wks 1 and 2, and IAA14 was only observed in the treatment in wks 1 and 2. However, when both treatment and control produced measureable transcripts of the same IAA gene, the transcript amounts were very similar. All of this suggests that the IAA genes were not greatly affected by Li^+ in the soil study, which had prolonged low-level Li^+ exposure.

3.26 Membrane Exchange and Antiporter Protein Gene Abundance

There are several cation and proton exchange proteins that are reported to be responsive to Li^+ as well as other ions (e.g., Zhao *et al*. [53]). These are the CHX (cation/H^+ exchanger), AtNHX (sodium$^+$/H^+ exchanger) and CAX (cation exchanger) gene series (all of which can be individually identified and sourced by searching for "lithium ion response" in TAIR). The HP studies with 1 mM LiCl showed the up-regulation of AtNHX6 (At1g79615, fold-change of +1.66 at p =0.027) and CAX7 (At5g17860, fold-change of +1.78 at p=0.027) in the 1-d treatments relative to the control. In the 7-d 1 mM LiCl exposure, CAX3 (At3g51860) was up-regulated relative to the 1 mM NaCl (fold-change of +2.35 at p=0.001) HP treatment. It should also be noted that the 1 mM Li^+ levels that were used were provided as 300 mL solutions so the available Li^+ pool was fairly large in Li by mass per plant (unlike the soil studies).

The 1 mM NaCl HP treatments did not exhibit similar gene expression behavior to the 1 mM LiCl HP treatments, regardless of exposure period. This is somewhat expected because the nutrient media for the control plants already has higher levels of NaCl than 1 mM NaCl so the slight increase in the 1 mM NaCl treatment is practically imperceptible to the plant. The soil-grown Li^+-exposed plants did not exhibit differential gene regulation of the membrane cation exchange and antiporter protein genes at the statistical cut-off (p-value) level.

Most cations are transported by proton coupled transporters, rather than primary ion pumps. Na/H antiporters fall into three families. The monovalent (H^+, Li^+, K^+) cation:proton antiporter-1 (CPA) family includes eight members, from AtNHX1 to AtNHX8. One recent study by An *et al.* [54] has identified AtNHX8 up-regulation as being unique to Li^+. This response triggers Li^+ efflux from the cell. Our study did not identify differential regulation of NHX8.

3.27 Acute vs. Chronic Li^+ Exposure Responses

Figure 3 depicts a plant's mRNA response plan for high-level acute and low-level chronic Li^+ exposure. Plant exposure responses to most toxic metals are generally concentration-dependent but the active growth of some plants and the total pool of bioavailable metal have to be considered. Therefore a plant's response is likely to be age-dependent where younger plants are more susceptible to Li^+ due to their low tissue biomass to Li^+ exposure level than older plants which have more biomass at the same Li^+ exposure level. Furthermore, another factor that may make older plants more tolerant to Li^+ exposure is the natural emergence of expressed genes for plant defense to predation as the plant ages. The planned emergence of these expressed genes in the older plants should help the plant respond to a toxin whereas younger plants that are putting their energy into growth will have to divert their growth activities to respond to Li^+ with the necessary expression, much like that of the younger HP plants that were exposed to high levels of Li^+. As an example, the soil-grown Li^+ treatment plants that exhibited similar changes in regulation of the PR protein genes in the HP study were mainly observed after several wks of chronic low level exposure. The older plants in the soil studies did not have differential-regulation of genes of the IMPase and ACC synthase gene families at the statistical cut-off level of $p \leq 0.05$. This may be due to the low Li^+ exposure levels and it may be correlated to the fact that the older plants are less sensitive on a per mass basis than the younger plants. The control plants in the soil systems were naturally increasing their levels of PR proteins to help avoid predation while preparing to flower and they may also be more equipped to contend with Li^+ exposure effects than the younger plants.

Uptake of Li^+ by plants has some stimulatory effect on plant growth [2]. This type of stimulatory effect may be one of hormesis, where low levels of a perceived toxin prove initially to be beneficial in some manner [55-57]. In the case of Li^+ and plants, such initial beneficial effects are most likely from the production of ethylene at low Li^+ concentrations.

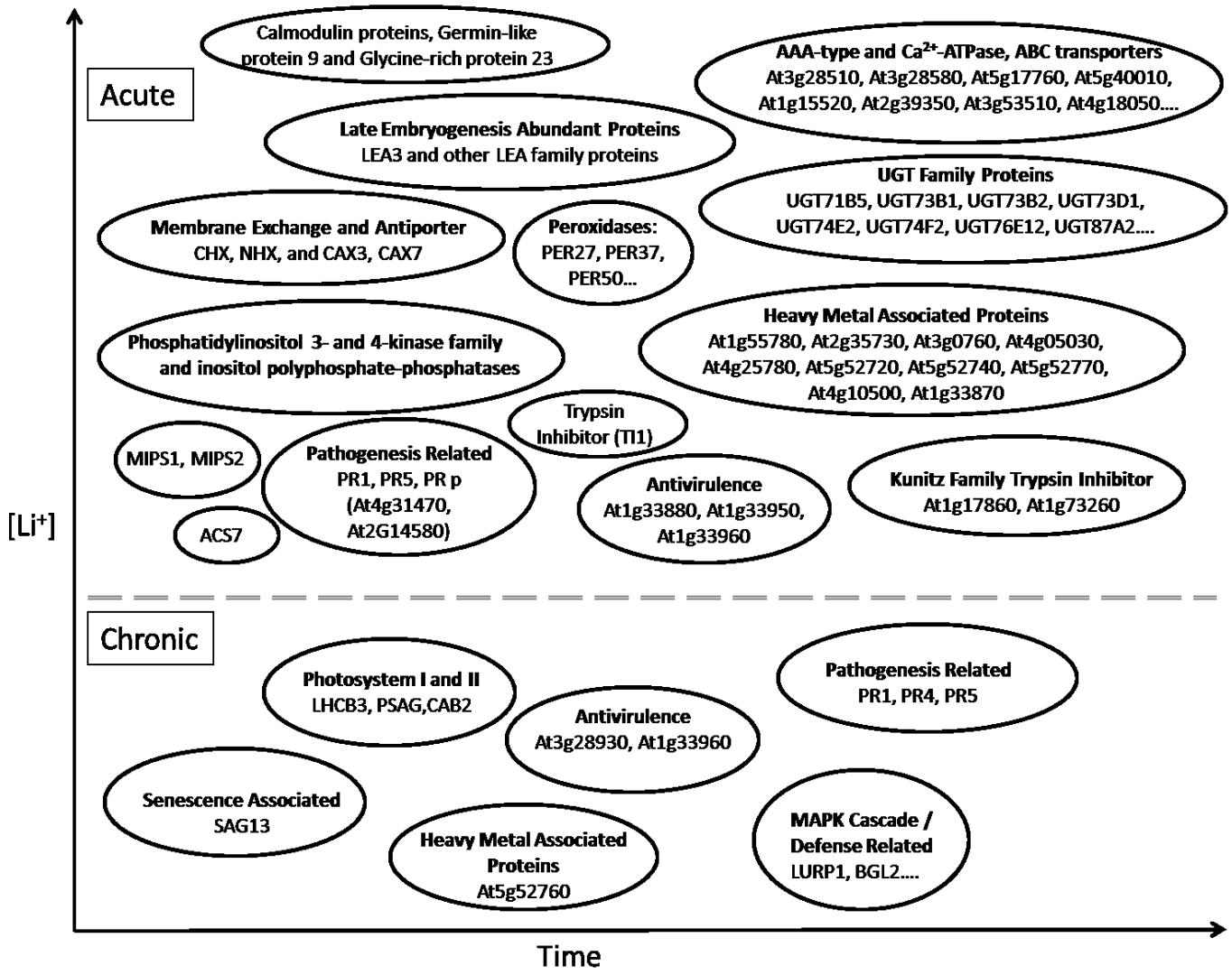

Figure 3. The plant's mRNA response plan for Li^+ exposure is likely to be related to the amount of available Li^+ as well as time. In actively-growing plants which have short lifetimes like *At*, the amount of plant biomass is likely to dictate the plant's type of response (acute or chronic).

At high concentrations, Li^+ has a substantial adverse effect on cellular processes and growth. Root and shoot biomass quantities, for instance, will become diminished after a 7-d (acute) exposure to Li^+ at 4 to 12 ppm [58] and at 1 mM LiCl (as in this study with HP growth conditions). High Li^+ concentrations cause excessive ethylene production as well as induction (up-regulation) of proton-coupled transporters and antiporters that will appear to mimic some salinity stress effects. Hawrylak-Nowak *et al*. [34] examined the effect of Li^+-induced lipid peroxidation and concluded that Li^+ peroxidation of cell membranes occurs and that excessive Li^+ will cause the accumulation of ROS in plants, much like it does in animals.

Our study findings with the acute HP-based Li^+-exposures on gene expression compare well with that of Bueso *et al*. [59] who examined catalase2 mutant responses to high levels of Li^+ and with those of Li *et al.* [30] who examined the effect of Li^+ on *Br. carinata* seedlings. Collectively, acute, high concentration Li^+-induced gene responses invoke differential regulation of trypsin, calmodulin, heavy-metal associated domain-containing proteins, beta-1,3, glucanase, PR proteins, and glutathione *S*-transferases. The uptake of Li^+ is primarily by the

hydrogen proton pump process. This mechanism is unique to monovalent cations including Na^+, Li^+ and K^+. Lithium and Na ions elicit similar homeostasis restoration responses. As such, Li^+ is often used experimentally to specifically examine a plant's stress signaling and response, unlike Na^+ which is used more often for salinity response.

Little is known about the receptors that sense stress but several downstream signaling cascades have been identified and studied. Stresses may lead to an increase in reactive oxygen species (ROS), glutamate transporters, cystosolic Ca^{2+}, ABC transporter gene up-regulation ([34] and in this study). Ultimately, many of these signaling cascades result in altered or increased expression of stress-responsive genes that are produced as part of the plant's normal growth processes—as noted in this study. Some of these encode proteins responsible for the biosynthesis of hormones, such as abscisic acid, which can act as signaling molecules that amplify and spread the initial stress signal.

4. Conclusions

With additional study, this information has the potential application of serving as a method of detection of damaging Li^+ (as opposed to other ions like Na^+) exposure in the agricultural and natural environment or for selecting more Li^+-tolerant plant varieties within the same plant species for cultivation—to make better use of Li-rich irrigation waters. Alternative methods of qualifying and quantifying Li^+ exposure in the environment are relevant to detecting contamination plumes and evaluation of environmental remediation approaches, decisions about agricultural irrigation water use, and the evaluation of crop health.

It should be noted that this study did not examine the effect of Li^+ on the plant proteome (including its substitution for structural Mg^{2+}) and it is possible that there are differences in what can be interpreted from the proteome vs. the transcriptome (such as noted by Ghazalpour *et al.* [60]). Our study elucidates the differences between high level acute Li^+ exposure and chronic low-level Li^+ exposure in plants. The effect that low level Li^+ has on greater cellular organization, increased metabolic processes, and enhanced ability to handle stress and terms of signaling may have relevance to mammalian systems. In our study, Li^+ exposure influenced a few genes that had not been identified to respond to Li^+ in prior studies, such as AtFBA5 and GLP9. Because AtFBA5 requires Mg^{2+} to function and was down-regulated with Li^+ exposure, the data suggest that that Li^+ may play a larger role in the inhibition of other Mg^{2+}-containing proteins in plant as well as mammalian systems. Up-regulation of the GLP9 gene suggests that cell wall structure becomes greatly modified upon Li^+ uptake, which is consistent with up-regulation response of other cell-wall related genes in this study. Furthermore, more support for the wide ranging effects of Li^+ on protein behavior is that a systemic Mg^{2+} deficiency also explains the observed changes in circadian rhythms in *Arabidopsis* [61]. Transcriptome profiling by Hermans and researchers [61] revealed several gene regulation similarities to that of the acute Li^+ toxicity responses in our current study, which further supports a Li^+-induced Mg^{2+} deficiency but Li probably has an effect on overall Ca^{2+} levels as well in plants and other organisms. Wallace [11] recently considered the possible link between Ca^{2+}-ATPase and Alzheimers Disease and suggests that Li^+ may be responsible for the intracellular control of Ca^{2+} levels in the cell to help preclude the progression of the disease. Khoury *et al.* [62] has observed such decreases in intracellular Ca^{2+} levels in bipolar patients upon the addition of Li-treatment in women. We suggest that the effect of Li^+ on the treatment of bipolar affective disorder and other neurological diseases may also be related to its ability to decrease the level and mobility of intracellular Mg^{2+} as well as Ca^{2+} levels.

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Supplementary Information

Table A. TAIR GO biological function annotation for the 56 genes found in more than two consecutive wk comparisons for the Li^+-treated plants. Only 26 of these had known GO annotations in TAIR.

Biological Function	**56 Genes in the Top 100 Most Abundant Found in More than Two Consecutive Wk Comparisons of Growth (%) N=26**
Cell organization and biogenesis	7.7
Developmental processes	30.8
DNA or RNA metabolism	0.0
Electron transport or energy pathways	7.7
Other biological processes	46.1
Other cellular processes	57.7
Other metabolic processes	50.0
Protein metabolism	11.5
Response to abiotic or biotic stimulus	42.3
Response to stress	30.8
Signal transduction	23.1
Transcription, DNA-dependent	15.4
Transport	42.3
Unknown biological function	38.4

Table B. TAIR GO biological function annotation for genes among the top 100 in abundance which were only in a single consecutive wk comparison of plant growth.

Biological Function	Wk 1 vs. 2 (%) N=20	Wk 2 vs. 3 (%) N=22	Wk 3 vs. 4 (%) N=33	Wk 4 vs. 5 (%) N=21
Cell organization and biogenesis	30.0	9.1	15.1	28.6
Developmental processes	10.0	13.4	9.1	14.3
DNA or RNA metabolism	0.0	0.0	9.1	0.0
Electron transport or energy pathways	15.0	4.5	0.0	14.3
Other biological processes	25.0	22.7	21.2	23.8
Other cellular processes	50.0	50.0	57.6	66.7
Other metabolic processes	40.0	54.5	48.5	57.1
Protein metabolism	20.0	18.2	9.1	14.3
Response to abiotic or biotic stimulus	30.0	31.8	27.3	28.6
Response to stress	35.0	31.8	30.3	42.3
Signal transduction	5.0	4.5	12.1	14.3
Transcription, DNA-dependent	0.0	4.5	12.1	9.5
Transport	25.0	18.2	12.1	23.8
Unknown biological function	35.0	50.0	30.3	33.3

Table C. Up-regulated genes within the top 100 most abundant transcripts for the Li^+-exposed soil plants. Whole genome information from TAIR is provided for reference.

Biological Function	Whole Genome (%) N=24622	Wk 1 vs 2 (%) N=6[1]	Wk 2 vs 3 (%) N=11[2]	Wk 3 vs 4 (%) N= 22[3]	Wk 4 vs 5 (%) N=8[4]
Cell organization and biogenesis	13.6	16.7	0.0	13.4	12.5
Developmental processes	15.6	16.7	0.0	13.4	0.0
DNA or RNA metabolism	3.7	0.0	0.0	0.0	0.0
Electron transport or energy pathways	2.8	33.3	27.3	0.0	0.0
Other biological processes	14.8	33.3	54.5	45.4	25.0
Other cellular processes	56.7	66.7	63.6	50.0	50.0
Other metabolic processes	53.6	50.0	36.4	36.4	25.0
Protein metabolism	21.3	33.3	45.4	13.4	12.5
Response to abiotic or biotic stimulus	15.3	50.0	45.4	36.4	50.0
Response to stress	16.6	33.3	45.4	36.4	62.5
Signal transduction	8.1	33.3	9.1	27.3	25.0
Transcription, DNA-dependent	10.6	0.0	0.0	9.1	12.5
Transport	14.3	16.7	9.1	36.4	50.0
Unknown biological function	32.9	33.3	45.4	36.4	50.0

[1] Within the top 100 most abundant genes, 48 up-regulated genes were common to wks 1 and 2: ATPC1, At1g74470, At1g74670, At1g78020, At3g14415, At4g01150, At4g32260, CAB2, CAB3, CRD1, CSD2, DRT112, ECS1, FED A, FBA2, GAPA, GGT1, LHCA1, LHCA3, LHCA4, LHCB2.2, LHCB2.1, LHCB3, LHCB4.2, LHCB5, LHCB6, PETC, PRK, PSAD-1, PSAD-2, PSAE-1, PSAE-2, PSAF, PSAG, PSAH-1, PSAH2, PSAK, PSAL, PSAN, PSBQ-2, PSBR, PSBTN, PSBX, PSBP-1, PSAO, PSBW, PSBY, and RBP31.

[2] Within the top 100 most abundant genes, 20 up-regulated genes were common to wks 2 and 3: At1g55450, At1g56045, At1g61890, At1g67430, At2g25510, At3g04640, At3g28940, At3g56360, At5g42530, At5g44580, At5g20290, ECS1, emb2386, JAZ1, KIN2, OPCL1, PEN3, RRTF1, SOUL-1, and UBQ1.

[3] Within the top 100 most abundant genes, 43 up-regulated genes were common to wks 3 and 4: AGP12, AGP21, At1g04040, At1g22890, At1g52190, At1g62480, At1g63860, At1g65845, At1g66760, At1g74670, At2g25460, At2g30930, At3g19680, At3g22060, At3g22235, At4g13340, At5g08330, At5g22580, At5g22690, At5g25440, At5g39670, At5g44020, At5g52760, At5g57655, At5g64870, DELTA-TIP, EXO, FLA9, GASA1, GRP5, LECRKA4.1, MLP28, MLP43, MPK11, NSP1, PCC1, PHI-1, STP1, TCH4, WRKY46, XTH18, XTH24, and XTH4.

[4] Within the top 100 most abundant genes, there were 22 up-regulated genes common to wks 4 and 5: AIG2, At1g24145, At1g27330, At2g18690, At3g22235, At4g24920, At5g25250, At5g39670, At5g52760, BI1, BIP1, CCL, CRT3, GAMMA-VPE, GSR 1, GSTF6, GSTF7, MBP1, PRXCB, RING1, SHD, and WRKY70.

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