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Journal of Pharmacology & Clinical Toxicology

Research Article

Identification of Sublethal Changes in *Arabidopsis thaliana* Exposed to Li^+ Over Multiple Generations Using Gene and Gene Isoform Level Changes

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Submitted: 16 January 2019
Accepted: 25 January 2019
Published: 30 January 2019
ISSN: 2333-7079

Keywords
- *Arabidopsis thaliana*
- Next generation sequencing
- Lithium exposure
- Soil
- Transgenerational

Abstract

Two generations of terrestrial plants were exposed to non-lethal levels of lithium chloride (LiCl) and the mRNA transcript abundance of the matured and seeding plants were analyzed using next generational sequencing. *Arabidopsis thaliana* plants received 0 or 0.05 mM LiCl for their F0 and F1 generations with cumulative Li exposures of 1.80 to 3.2 mg Li kg^{-1} dry weight soil. Gene and gene isoform changes relative to the controls were obtained for LiCl-treated and control plants in the F0 and F1 generations. Sublethal effects in the F0 and F1 plants exposed to LiCl were a decrease in photosynthetic genes and a significant increase in up-regulated stress and defense genes that were associated with water stress or dehydration. Gene isoforms changes were mainly observed with lower abundance genes that were up-regulated. They included genes for calcium and calmodulin binding: ACA2, ACA8, CEN2, and CP1. In general, chronic Li^+ exposure produced down-regulation of several ATP-binding and photosynthesis related gene isoforms of: ACA1, ACA4, ADK1, BAM1, EPR1, ER,FLS2, HSL1, HST, PGM, and SEP1. No phenotypic or seed viability differences occurred other than yellowing of leaves in the LiCl-treated plants near the end of the life cycle.

INTRODUCTION

Lithium (Li) is a soft, silver-white metal belonging to the alkali metal group of chemical elements. It is sometimes used in mineral form, but largely as a processed chemicalprepared from these minerals or brine deposits. Two Li-rich minerals, spodumene ($LiAlSi_2O_6$) and petalite ($LiAlSi_4O_{10}$) are used in ceramics and glass because they serve as a flux or glaze to lower the thermal expansion of the pyroceramics. Lithium is used in greases and lubricants to expand the temperature range, in aluminum (Al) production where it lowers the temperature and viscosity of the cryolite bath, in synthetic rubbers where it increases resistance to abrasion, and in commercial air conditioners as a dehumidifier [1]. Lithium is successfully used for the long-term treatment of patients with recurrent unipolar and bipolar affective disorder. A side effect from chronic Li use is nepropathy (kidney damage) which is displayed as impaired glomerular function in patients and a sodium and water imbalance that presents as dehydration [2,3].

Currently Li^+ is the staple for the battery market. It is used for powering laptop computers, cordless heavy-duty power tools, hand-held electronic devices, and sensors, electronic and medical devices and is beingutilized in the increasingly popular field of battery-powered vehicles [4]. There is disagreement on whether the global supply of Li is adequate to support the future of global electric vehicles. One projection by Gaines and Nelson [5], says the U.S. auto industry could potentially use as much as 22,000 tons of Li by 2030 and 54,000 tons by 2050. Gruber et al.[6], investigated the 103 deposits worldwide and estimated that the global Li resource is about 39 million tons and the highest demand scenario does not exceed 20 million tons from 2010 to 2100.

Lithium metal is the 33rd most abundant element on the planet. It is found in trace amounts in most rocks, soils (brine salt flats and clay deposits) and natural waters. Large deposits can be found in pegmatite minerals (*i.e.*, spodumene and petalite) and in brine lake deposits or salt flats in Argentina, Australia, Canada, Chile, China, Portugal, Zimbabwe, and the US [4]. Brine deposits are typically found in arid regions where the surrounding ecosystem is highly dependent on local water resources. In many cases, the water from these areas is used for Li extraction, the irrigation of local crops and as drinking water for humans and livestock. Freshwater Li^+ levels are usually <0.04 ppm (~0.006 mM) as reviewed by Kszos and Stewart [7], although some natural waters can have higher levels. Irrigation with Li^+-rich waters can have adverse effects on crops (such as with *Citrus*) and have negative impacts the agricultural industry [8,9].

Lithium is generally thought to have no known biological use and it does not appear to be an essential element for life in humans, animals or plants [10,11]. However, there is evidence that some levels of Li^+ are essential [11]. Lithium-depleted diets will alter reproductive success in rats and goats [9,12-16]. Lithium is used in the medical field to treat bipolar disorder and related mental diseases such as depression and schizoaffective disorder. Several mechanisms have been proposed for its action. Although research continues, the mechanism for how Li^+ helps treat mental illnesses remains unknown [17]. High levels of Li^+ produce toxicity in patients [16], and may be teratogenic in the early developmental stages [18,19], but Li^+ is not mutagenic nor does it cause chromosomal damage (breaking) [20].

As the interest and use of Li continues to grow, the potential for Li^+ to be introduced into the environment increases. Releases from Li^+ extraction and mining effluents (sludges) and the disposition of Li^+ batteries into landfills may result in contaminated surface and groundwaters. Additionally, the potential environmental and human health impacts of Li^+ batteries in the electronic waste stream are not well understood due to insufficient toxicity data [21].

Gene expression measurements have the potential to detect low level responses that are not phenotypically evident. Fedorenkova and co-workers [22], reviewed the gene expression as well as the toxicity data for cadmium (Cd) exposure in aquatic organisms. They determined that gene expression changes (referred to as the lowest observable effect concentration or LOEC) occurred at four times the no observable effects concentration (NOEC) for Cd and that the median lethal effect concentration or LC_{50} exceeded the concentration in which changes in gene expression were observed by 11-fold. This study concluded that gene expression should be examined at concentrations that are much lower than immediately toxic or reproductively limiting levels [23]. They concluded more testing at low exposure concentrations is needed if gene expression were to be used to indicate a no observed transcriptional effect level or "NOTEL" (proposed by Lobenhofer et al. [24]), for metals and other contaminants in environmental systems. Ankley et al. [25], stated that transcriptome and related data could provide information on a class of chemical stressors, even when information on chemicals is unavailable. They also note that small sample quantities are required and they can be taken through non-lethal methods, which make gene expression measurements useful in stress detection with rare or endangered species.

In this paper we investigate transgenerational effects in chronically-exposed *Arabidopsis thaliana(At)* by measuring changes in overall gene expression levels as well as in gene isoforms. Gene isoforms result from alternative splicing pathways, which lead to changes in protein localization and protein function. Such information can be used to address abiotic and biotic stresses as well as observe normal growth and development related changes. The use of gene isoform information is still new, but it is a growing field given the advances in RNA-seq technology. However, the use of the information is limited given the complexity of transcriptome information and read assignment uncertainty [26].

MATERIALS AND METHODS

Soil-based Growth of At

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), 500µL TritonTM 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. 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. 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 were maintained at ambient conditions under lighting of 9 h light: 15 h dark for the entire study.

Treatment with LiCl for multiple generations

F0 and F1 plants received 25 mL of 1/64 HP media to each quadrant of the pot (100 mL total volume) every 3 to 4 d containing either 0 or 0.05mM of LiCl. Treatments for the F0 plants began at d 28 and plants were harvested on d 92. F1 plants were treated starting on d 6 and were harvested on d 109. Time for harvest was determined based on the plant. Rosette leaves were taken when the plant had fully bolted, produced flowers and silques were beginning to dry.

Collection and processing of at tissue for RNA analysis

Using sterile conditions to limit RNAase contamination, rosette leaves were collected from each plant. A total of 150 mg of fresh weight biomass was harvested for each replicate sample, flash frozen in liquid nitrogen, 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 a RNA Integrity Number (RIN) on the bioanalyzer exceeding 7) were used for the construction of cDNA libraries based on TruSeq RNA sample preparation protocol (Illumina Inc., San Diego, CA) and analyzed with Next Generation Sequencing (NGS) using a HiSeq 2500 (Illumina Inc., San Diego, CA).

RNA analysis

The NGS data were de-multiplexed using CASAVA 1.8.2 software by aligning reads to the *At*genome (TAIR 10) using TopHat 2.0.8. Runs were set as 50 cycle paired-end reads. Sequences were compared to three publicly-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 2.0.2 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. Differentially-regulated genes were identified by ranking the fold-change (in log base 2, sometimes referred to as "log 2") to a specific "cut-off" value at *p*-value of 0.05 (Student's *t*-test). A recent review of NGS technique and data analysis which is used widely in this report can be obtained from Van Verk et al. (2013) [27]. For the NGS data from the soil study, the amount of data was massive and for simplification, the discussion was often limited to the 100 most abundant transcripts that met the *p*-value cut-off and were up- or down-regulated.

RESULTS AND DISCUSSION

Observation of Plants

This study is somewhat unique in that plants were allowed to grow through their entire life cycle, from seed to young seedlings through vegetative growth and into reproductive growth nearing senescent phases of the adult plants. A F0 generation of plants was started and treated with either 0 or 0.05 mM LiCl twice weekly for 58 d and 103 d for the F1 generation (Table 1). During the treatment, visual observations were made of the plants every 3 to 4 d during watering events. General observations were made of the plants' appearance (*e.g.,* leaf coloration, morphology and size) and progress toward reaching reproductive status. Seeds were collected from the F0 control and F0 treatment plants and used to start the F1 generations. No phenotypic differences or delays to reproduction were observed between the treatment and control plants with the exception of yellowing in the oldest leaves of the most mature Li^+-exposed plants. No measurable changes in the seed viability between seeds collected from F0 untreated and F0 treated plants were observed.

Evaluation of trans-generational genomic effects in F0 and F1 untreated Plants

The F0 and F1 control plants were phenotypically similar and showed no signs of stress. Evaluation of genes related to photosynthesis and photosystem I and II (PS I and PS II) showed that LHCA1, LHCA2, LHCA3, LHCA4, LHCB2.1, LHCB2.2, LHCB3, LHCB4.1, LHCB.4.2, LCHB5 and LHCB6 were among the 100 most abundant genes and that abundance levels were higher in the older aged plants. Similar trends have been observed in the proteomics of aging *At* (Kuhne et al., 2014, in prep). However, significant differences in gene expression levels measured between the F0 and F1 plants were often related to the biological process and reproductive status at the time of harvest. One method used to evaluate possible trans-generational effects in the untreated plants was to compare the 25 genes significant genes for up- and down-regulation based on fold-change (Table 2).

Up-regulated gene expression in F0 vs. F1 untreated Plants: Genes that were significantly up-regulated in the F1 plants were associated with normal growth- and aging-related processes. Several of these genes were associated with delayed flowering (At1g12610), stigma development (At3g48520), pollen development, flowering and inflorescence (At1g54890) and circadian rhythm (At1g07050). Several genes related to cell wall modifications and loosening (At1g10550, At1g11370, and At3g45970) were also up-regulated. It is estimated that plants devote about 10% of their genome to the construction and modification or rearrangement of their cell walls during growth [28].

Several genes were up-regulated for Ca^{2+}-dependent lipid-binding. The role of these Ca^{2+} lipid-binding genes has been generally associated with the plants ability to respond to environment cues including stress. Four of the five most up-regulated genes were associated with stress response: Ca^{2+}-dependent lipid binding (At3g61030, At3g60950), enzyme inhibitor activity (At5g62360), and ethylene and jasmonate responsive plant defensin (At2g34600 and At5g44420, respectively). Additional defense- and stress-related genes were At5g47260, a plant thionin member that is predicted to encode a pathogenesis-related (PR) protein (At5g36910) and a low temperature induced 30 (LTI30 or At3g50970), which is a water deprivation response gene. Additional stress response genes includedgalactinol synthase 3 (GOLS3) and a rubisco subunit (RBCX1) (At1g09350 and At4g04330, respectively).

The remaining up-regulated genes had functions associated with DNA binding (At5g52020), Zn ion binding (At5g50450), copper (Cu) ion binding (At2g15780), nitrate reductase 1 (At1g77760), ascorbate activity (At1g19550) or were unknown (At3g28320 and At3g61920).

Down-regulated gene expression in F0 vs. F1 untreated

Table 1: Treatment and harvest conditions for F0 and F1 generation plants.

Generation	Age at First Treatment (d)	Harvest Age (d)	Treatment Period (d)	Number of Treatments	Total Li Concentration Received (mg Li kg^{-1} soil)
F0	28	86	58	17	1.8
F1	6	109	103	30	3.2

plants: Down-regulated genes were associated with circadian rhythm regulation (At1g01060, At5g37260, At3g20810 or JMJD5), reproductive organ development genes for petal differentiation (At5g06520) and late stage flowering (At2g26400), pollen tube growth (At3g09590, At5g55020 and At5g15950) and regulation of transcription (At3g61950 and At1g01520). Several genes associated with defense response (At1g58170, At2g39310 and At5g40990), transport (At5g37990 and At3g09450) and binding associated with nucleotide (At1g18830), Zn (At5g47610) and DNA (At1g66390) were also down-regulated. Gene At4g05540 is a member of the P-loop containing nucleoside triphosphate hydrolase superfamily. It was down-regulated along with a gene for proteolysis (At2g28040) and several genes with unknown function (At4g13680,At5g40790, At4g26950, At3g48240and At5g40800).

Evaluation of 50 most abundant genes in treated and untreated F0 and F1 Plants

Genes associated with normal growth, primarily related to pollen tube development, and aging and senescence due to the age of the plants were among the 50 most abundant genes across all treatments. Typical aging-related genes are those associated with ubiquitin 3,10 and xyloglucan endotransglucosylase/hydrolase 24 (XTH24) and the senescence-related genes (At5g60630, At1g20620, and At1g29330). The presence of these genes among treatments was not significant.

Several genes were associated with defense and stress response, particularly related to reactive oxygen species and hydrogen peroxide (H_2O_2) scavenging and removal (At1g23130, At3g14420, At4g16190, At3g4426). Given the advanced age of the plants, which were in the final stages of reproductive growth (by bolting, flowering and producing silques and seeds), genes associated with pollen tube growth were not unexpected [i.e., translationally controlled tumor protein (TCTP), arabinogalactan protein 15 (AGP15), and At5g19240 as listed in Table 3**]**.

Li^+ Effects on photosynthesis genes: The genes associated with photosynthesis and defense/stress related responses showed the greatest difference between the treatment and control plants. For the F0 and F1 control plants, there were 19 and 26 (respectively) genes that were associated with PS I and II among the 50 most abundant. However with Li^+ treatment, the abundance of these genes was severely reduced with only nine occurring in the F0 and one in the F1 plants (Figure 1). In the

Table 2: The 25 most abundant up- and down-regulated genes compared between F0 and F1 untreated plants. Pink color is up-regulation and green color is down-regulated.

Accession No.	Gene Name*	Fold-change	*p*-value	Accession No.	Gene Name*	Fold-change	*p*-value
At3g61030	Ca^{2+}-dependent lipid binding	9.5	0.0000273	At3g09590	CAP s.f.p.	-6.2	0.0180000
At5g38700	Unknown	9.2	0.0065720	At4g13680	DUF295	-5.6	0.0060000
At5g62360	Plant invertase/ pectin methylesterase inhibitor s.f.p.	8.3	0.0000000	At1g01060	LHY1	-5.4	0.0000000
At3g60950	C2 Ca^{2+}/lipid-binding endo/exo nuclease/ phosphatase	7.9	0.0000000	At2g26400	ATARD3	-5.4	0.0180000
At5g44420	PDF1.2A	7.6	0.0000159	At5g15950	Adenosylmethionine decarboxylase f.p.	-5.3	0.0000000
At1g12610	DDF1	7.3	0.0000000	At5g37990	*S*-adenosyl-*L*-methion-ine-dependent methyl-transferases s.f.p.	-4.7	0.0430000
At3g28320	DUF677	7.2	0.0030460	At5g40790	Unknown	-4.6	0.0000000
At3g61920	Unknown	7.0	0.0391990	At1g58170	Disease resistance-responsive f.p.	-4.5	0.0490000
At5g47260	ATP/GTP binding	6.9	0.0000259	At5g06520	SWAP	-4.4	0.0000000
At5g52020	Encodes member of DREB subf. A-4 of ERF/AP2 transcription factor f.	6.7	0.0000000	At1g18830	Transducin/WD40 repeat-like s.f.p.	-4.3	0.0040000
At3g48520	CYP94B3	6.6	0.0000000	At4g26950	DUF584	-4.2	0.0000000
At5g36910	THI2.2	6.5	0.0006490	At5g55020	MYB120	-4.1	0.0350000
At1g07050	CCT motif f.p.	6.5	0.0003930	At4g05540	P-loop containing nucleoside triphosphate hydrolases s.f.p.	-4.0	0.0160000
At3g50970	LTI30	6.3	0.0000000	At2g28040	Eukaryotic aspartyl protease f.p.	-4.0	0.0200000
At1g77760	NR1	6.3	0.0000000	At1g01520	ASG4	-3.9	0.0000016
At5g50450	HCP-like s.f.p. with MYND-type zinc finger	6.2	0.0000000	At5g37260	RVE1	-3.9	0.0000000
At1g19550	GST f.p.	6.2	0.0000000	At2g39310	JAL22	-3.8	0.0060000

At2g15780	Cupredoxin s.f.p	6.2	0.0189930	At5g40990	GLIP1	-3.8	0.0080000
At1g10550	XTH33	6.2	0.0014780	At3g20810	JMJD5	-3.8	0.0000086
At1g54890	LEA p.-related	6.1	0.0168930	At5g47610	RING U-box s.f.p.	-3.8	0.0000000
At2g34600	Jasmonate-zim-domain p. 7 (JAZ7)	6.0	0.0000000	At1g66390	PAP2	-3.7	0.0000000
At1g11370	Pectin lyase-like s.f.p.	5.9	0.0179180	At3g48240	Octicosapeptide/phox/Bem1p f.p.	-3.7	0.0000941
At1g09350	GOLS3	5.9	0.0000000	At3g09450	Fusaric acid resistance p.	-3.7	0.0000002
At3g45970	EXPL1	5.8	0.0000074	At5g40800	Unknown	-3.6	0.0000029
At4g04330	RBCX1	5.7	0.0000273	At3g61950	bHLH	-3.6	0.0020000

*s.f.p.: super family protein; p.: protein; f.p.: family protein.

Table 3: The 50 most abundant statistically-significant ($p \leq 0.05$) genes in the F0 and F1 0 and 0.05mM LiCl treated plants. Pink color is up-regulated and green color is down-regulated.

F0 Control		F0 Treatment		F1 Control		F1 Treatment	
Accession No.	Avg. FPKM Abundance	Accession No.	Avg. FPKM Abund.	Accession No.	Avg. FPKM Abund.	Accession No.	Avg. FPKM Abund.
ALP	1123	AGT	1055	AGP15	1964	AGP15	5055
ARFA1E	2062	AILP1	1489	AILP1	2171	AILP1	1669
At1g23130	1162	ARFA1E	1049	At1g54410	2152	AOC1	1464
At1g24880	1252	At1g23130	1513	At2g33830	1702	AOC2	1602
At1g29330	8521	At1g29330	3579	At2g45180	2777	AOS	1140
At2g01021	4968	At1g32920	1871	At3g08520	1677	At1g23130	1222
At2g25510	1588	At3g08520	1669	At3g16640	3701	At1g29330	2443
At3g08520	3925	At3g14420	1218	At5g38410	1902	At1g32920	1180
At3g14420	1434	At3g44260	1395	CAB2	3116	At1g61890	1877
At4g16190	1392	At4g16190	1170	CAB3	5814	At1g67865	1285
At5g21020	1572	At4g27280	1004	CAT3	4866	At2g01021	1309
At5g42530	1864	At4g29780	1072	CCL	2679	At2g15960	1771
CAB2	1741	At4g32020	1282	CCR2	1924	At2g23120	2131
CAB3	2579	At5g19240	1152	COR47	1718	At2g45820	1749
CAT3	3437	At5g54940	2172	CP12-1	1748	At4g30530	1986
CP12-1	1686	CAB2	1017	DRT112	1879	At5g21020	1161
CP5	1201	CAB3	1544	ECS1	1679	At5g54940	3272
DI19	1743	CAT3	3025	ERD15	2421	BGLU18	1587
ECS1	2371	CML38	1164	GRP-3	1889	CAT3	5628
ESM1	1471	CP12-1	1010	KIN2	1691	CCR2	5872
GRP-3	2031	DI19	1587	LHB1B2	8063	COR47	3594
GRP3S	1736	ERD14	1434	LHCA1	3602	CYP83B1	1379
HIS1-3	1109	ERD15	1503	LHCA2	2547	DI19	1899
LHB1B2	3275	GGT1	1036	LHCA3	3921	ERD10	2343
LHCA2	1595	GRP3S	1012	LHCA4	5711	ERD14	4035
LHCA3	1558	HSPRO2	2371	LHCB2.1	1776	ERD15	2527
LHCA4	2044	LHB1B2	2063	LHCB2.2	1868	ERD7	2983
LHCB2.1	1716	LHCA4	1353	LHCB3	4248	GRP-3	1520
LHCB3	1218	LHCB2.1	1126	LHCB4.1	1991	GSTU5	1699
LHCB4.1	1247	LHCB5	1026	LHCB4.2	1708	HSC70-1	1699
LHCB5	1547	MT2A	966	LHCB5	4518	JAZ6	1326
MT2B	1706	MT2B	2233	LHCB6	2111	LEA14	1137
MT3	6776	MT3	5975	MT3	7153	MT2A	2143
NIT1	2093	NIT1	1358	PSAD-1	1893	MT2B	1873
PSAD-1	1219	NUDT21	1242	PSAE-1	1694	MT3	7221
PSAG	1632	PRXR1	1006	PSAF	2132	OPR3	1746
PSAL	1542	PSAL	946	PSAG	2731	PCAP1	1295
PSAO	1320	PSBO1	1460	PSAL	2640	PEN3	1611

PSBO1	2830	PSBR	2426	PSAO	3403	PSBR	1648
PSBP-1	1445	RBCS1A	2834	PSBO1	3634	RCA	1284
PSBQ-2	1658	RCA	4403	PSBP-1	2752	SAG14	2820
PSBR	3837	SRC2	1642	PSBQ-2	2363	SRC2	1594
PSBX	1275	STZ	1486	PSBR	4787	TCH3	1568
RBCS1A	3537	SZF1	1136	PSBW	1970	TCH4	1161
RCA	2945	TCH3	980	PSBX	2636	TCTP	5092
TCTP	6904	TCTP	4375	RBCS1A	5649	TIFY7	1256
THI1	1084	THI1	1234	RCA	4724	UBQ10	2009
TRX3	1256	UBQ10	1993	TCH4	1715	UBQ11	1557
UBQ10	1519	UBQ11	1197	THI1	1859	WRKY18	1518
UBQ3	1157	VTC2	931	UBQ10	1977	XTH24	1200

Li^+-treated plants, the defense and stress response genes were more numerous in abundance relative to the control plants and relative to the photosynthesis genes (Figure 1).

Duff et al. [29], saw a similar trend with PS I and II genes being negatively-impacted by chronic low level Li^+ exposure. In our current study, chronic Li^+ treatment also had a negative impact on photosynthesis genes in the F0 and F1 generations. For the nine genes that were most abundant among the F0 control and treatment plants, the average FPKM abundance values for the F1 control plants were twice the values for the F0 control plants for the genes CAB2, CAB3, LHB1B2, LHCA4, LHCB5, PSAL and PSBO1. Abundance levels were equal for the gene LHCB2.1. For all the genes the abundance values were lower in the F0 treatment as compared to both the F0 and F1 controls. PSBR was the only gene found in all treatments that was among the 50 most abundant and its abundance was reduced in the F0 and F1 treatment plants relative to their corresponding controls (Figure 2).

Li^+ Promotes water stress and dehydration genes: As seen in Figure 1 there was a significant number of genes among the 50 most abundant in the F0 and F1 Li^+ treated plants associated with drought/water stress, cold and salt stress. The F0 Li^+-treated plants were abundant for the genes called DI19, SRC2, STZ and SZF1. The top 50 genes for the F1 Li^+-treated plants also had these four genes in addition to COR47 (cold regulated 47), ERD10 (early response to dehydration 10), ERD14, ERD15, ERD7, SRC2 (soybean gene regulated by cold-2) and At2g23120.

It is commonly known that plant water stress is often seen as a cellular water deficit occurring due to reduced soil water content. Many genes can be induced by water stress, including the ERD, COR, LTI (low-temperature induced) and KIN (a cold inducible gene). Genes COR47 and ERD15 are among the 50 most abundant genes in the F1 control plants, but not in the F0 plants. For the F1 Li^+-treated plants the genes ERD7, ERD10, ERD14 and ERD15 are among the most abundant. Only genes ERD14 and ERD15 are observed in the F0 treatment plants. The presence of the additional genes in the F1 treatment plants indicates a possible transgenerational effect.

Li^+ induces metallothionein (MT) genes: There was an up-regulation in the F0 and F1 Li^+-treated plants for the MTgenes,which function to detoxify the oxidative damage produced by Cu and other metals by binding to them. MT3 was the most abundant gene in the F0 and F1 Li^+-treated plants. Additional MT genes measured in the Li^+-treated plants included MT2A and MT2B.MT3 (At3g15353) was also one of the more abundant genes in the F0 and F1 control plants. Given the role of reducing oxidative damage, it is not completely surprising to see this gene abundant in these aging plants. This gene needs more evaluation to determine if it is a true Li^+ induced gene.

Li^+ induces allene oxide cyclase genes (AOS, AOC1 and AOC2): In the F1 treatment plants, genes in the Allene Oxide Cyclase (AOS, AOC1 and AOC2) family genes were among the 50 most abundant. These genes are part of the jasmonic acid biosynthesis process and are involved with ERD12. The gene expression is typically up-regulated during senescence phases

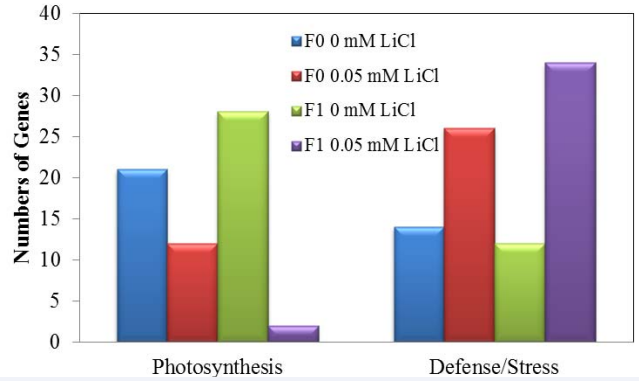

Figure 1 Relationship of statistically-significant ($p<0.05$) photosynthesis and defense/stress response genes in F0 and F1 untreated and treated plants.

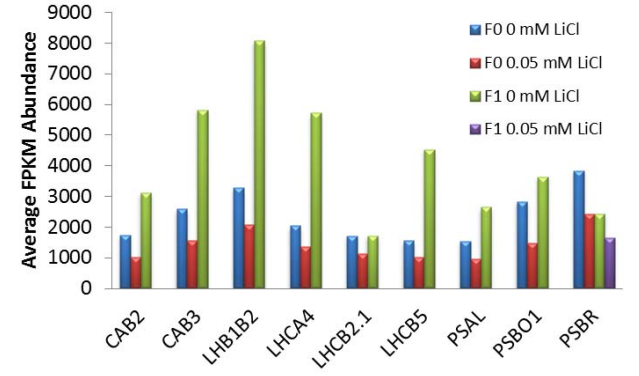

Figure 2 Average FPKM abundance levels for PS I and II genes.

of the plant life-cycle. Given the late age of the F1 plants it is unlikely that the expression of these genes is solely a Li^+ effect, but because it is not observed in the F1 control plants it cannot be ruled out. Additional senescence related genes observed in the F1 Li^+ treatment plants and not in the controls included senescence-associated gene 14(SAG14) and CAT3 (catalase 3, At1g20620), also known as senescence 2, which catalyzes the breakdown of H_2O_2 into water and oxygen.

Myo-Inositol-1-Phosphate Synthase (MIPS1, MIPS2, MIPS3): The most thoroughly-described mechanism found in the literature is by which Li^+ interacts with the inositol triphosphate second messenger system. The Li^+ ion is a non-competitive, rapid, and reversible inhibitor of *myo*-inositol-1-phosphatase (MIP), an enzyme involved in a rate-limiting step of the second messenger system. The Li^+ reduces the rate of hydrolysis of *myo*-inositol-1-phosphate without altering the binding of the substrate or the essential magnesium ion to MIP [30]. As seen in Duff et al. [29], chronic exposure of Li^+ causes down-regulation of MIPS1, MIPS2, and MIPS3 in this study (Table 4).

Indole-Acetic Acid (IAA): Duff et al.[29], investigated Li^+ exposure in soil-grown plants for 35 d and reported that the IAA genes were not greatly affected by chronic Li^+ exposure. However, several members of the IAA family were observed in this study, with IAA2 and IAA18 up-regulated in the F1 treatment plants. Down-regulation was observed for IAA1, IAA6, IAA8, IAA14, and IAA29 (Table 5).

Pathogenesis-Related Genes: Lithium has been shown to modify mRNA expression of members of the PR protein family [31,32]. Duff et al. [29], showed up-regulation of several PR genes (PR1, PR2 and PR5) relative to control plants watered with 0.05 mM LiCl in both hydroponic (1 d and 7 d exposures) and soil experiments lasting 35 d. In this experiment PR1 and PR5 were also up-regulated relative to the controls in the F1 generation plants (fold-change of 2.3, p=0.0000296 and fold-change of 1.8, p=0.0013, respectively).

New genes identified in both F0 and F1 Li^+-treated plants:

Table 4: Down-regulation of MIPS genes in Li^+-treated plants.

Accession No.	Gene ID	Fold-change	*p*-value
At4g39800	MIPS1	-2.9	0.0000000
At2g22240	MIPS2	-1.6	0.0004000
At5g10170	MIPS3	-1.8	0.0063000

Table 5: Up- and down-regulation of various IAA genes in Li^+-treated plants.

Accession No.	Gene ID	Fold-change	*p*-value
At3g23030	IAA2	1.2	0.0323000
At1g51950	IAA18	1.8	0.0000586
At4g14560	IAA1	-2.3	0.0000049
At1g52830	IAA6	-1.5	0.0372000
At2g22670	IAA8	-1.9	0.0000068
At4g14550	IAA14	-2.5	0.0000054
At4g32280	IAA29	-5.3	0.0003000

A comparison of genes that were up-and down-regulated in both the F0 and F1 generations revealed a new set of genes not yet described in the literature for Li^+ exposure. Late embryogenesis abundant genes (LEA) are primarily associated with seeds, but can be found in vegetative organs under stress conditions such as cold, drought, or salinity [33,34]. Three LEA (LEA, LEA18, LEA4-5) and one LEA-related protein (ECP63) were up-regulated with Li^+ ion exposure. Two of the four genes (LEA18 and LEA4-5) have been shown to be expressed under conditions of salt stress. LEA and embryonic cell protein (ECP63) are expressed in seeds [35].

TGG1 and TGG2 (β-thioglucoside glucohydrolase 1 and 2) are members of the myrosinase gene family and they are expressed primarily in aerial plant tissue. They function to create toxic degradation products when the leaf tissue is disrupted by chewing insects. This allows the glucosinolates to come into contact with myrosinase [36]. TGG1 has been shown to provide the most myrosinase activity and it is found in stomatal guard cells and phloeom cells [37]. Activity levels have been shown to vary with age of plant and stage of development, with highest activity levels in the mature rosette leaves and in flowers [38]. TGG1 and TGG2 function redundantly in the absicic acid (ABA) and methyl jasmonic acid (MeJA) signaling pathways and function upstream of cytosolic Ca^{2+} elevation in the ABA and MeJA signaling in the guard cells [39].

In higher plants the glycoside hydrolase family 1 beta-glycosidases have 47 members that share a common evolutionary origin and similar sequence identity. The beta-glycosidases are believed to participate in chemical defense against herbivory, lignification, hydrolysis of cell wall-derived oligosaccharides during germination and control of active phytohormone levels [40].

Beta-glucosidase 31 (BGLU31) is a major protein component of Endoplasmic Reticulum (ER) bodies in *At* [41]. Studies conducted by Nakano et al. [42], showed that ER bodies are rare in rosette leaves but wounding of the rosette leaves induces de novo formation of ER bodies, suggesting that these structures are associated with resistance against pathogens and/or herbivory. Additional they showed that ER bodies accumulate large amounts of beta-glucosidase which can produce substances to protect against invading pests.

At5g06740 is a member of the L-type lectin receptor kinases. They are considered to play crucial roles during development and in the adaptive response to various stimuli. This gene is a member of the singleton clade and is referred to as gene LecRK-S.5. The LecRK genes are differentially-expressed in various tissues during development and with exposure to hormone treatment, abiotic stress, elicitor treatment and pathogen infection. Differential expression of LecRK-s.5 has been shown in roots, young rosette leaves, and flowers and silques using microarray expression [43].

Several genes that were up-regulated in the treatment plants versus the controls have been normally associated with pollen and late stage flowering in *At* (NAC and ACA13). NAC is only expressed during pollen and late stage flowering 14 and 15. The Ca^{2+} transporting ATPase 13 (ACA13) along with ACA12 have very low expression levels in most cell types under basal condition, but their expression is dramatically induced upon exposure to

specific stresses such as pathogens or UVB light [44]. ACA13 is also been shown to be induced in papilla cells for the export of Ca^{2+} to pollen tubes, which promotes successful fertilization [45]. As seen in normal aging plants, as growth patterns change from vegetative growth to reproductive growth, leaf senescence occurs to promote the movement and support of nutrients and ions to developing flowers and silques. One gene significantly down-regulated in our study is a member of the CSLA genes, which are known for their role in biosynthesis of mannans and glucomannan cell wall polysaccharides [46]. CSLA09 was significantly down-regulated with a fold-change of -3.1 and -4.5 in both Li^+-treated plant generations.

Basic helix-loop-helix (bHLH) transcription factor genes, bHLH038, bHLH039 and bHLH100 have redundant functions in regulating iron-deficiency response and uptake in *At*. The three genes show different sensitivities to iron deficiency, indicating that they play a differently important role in the regulation of iron (Fe) homoeostasis [47]. Iron is a component of the photosystems and is essential for photosynthesis. In Fe-deficient environments, transcript expression of PSI subunits PSAF, PSAN and FED2 has been shown to be decreased together with those genes associated with light harvesting complexes and related proteins (such as LHCB6, LHCA3, LHCA2 and LHCB4.1). Additionally, a subset of seven unknown proteins (At1g47400, At2g14247, At1g13609, At1g47395, At3g56360, At2g30766, and At5g67370) has been shown to be strongly up-regulated in leaves along with bHLH38, bHLH39 and bHLH100 [48]. In the current study unknown proteins (At1g47395 and At1g47400), as well as bHLH38, bHLH39 and bHLH100, were strongly down-regulated in leaves. These observations indicate that Fe was not deficient and perhaps Li^+ competes with Fe or limits the availability of Fe to the leaves.

Genes with one or more significant gene isoforms

Gene isoforms, mRNAs that are produced from the same locus but are different in their transcription start sites (TSSs) and lead to greater functional diversity, were analyzed in F0 and F1 plants treated with Li^+. Several gene isoforms were identified for genes already known to be associated with Li^+ exposure (such as the MIPSand IAA genes), however additional isoforms were identified for genes affected in the photosynthetic and sugar pathways, endoplasmic reticulum (ER) stress pathway, and for new genes not yet identified as associated with Li^+ exposure.

Gene isoforms of known genes responding to Li^+ exposure: In the review of the current literature it is well known that MIPS and IAA genes respond to Li^+exposure. In this study gene isoforms were identified for genes MIPS2 and IAA8 (Table 7).

Photosynthesis-and sugar-related genes with significant gene isoforms: Sugars are the prime carbon and energy source to build and fuel cells, and are important in signaling pathways for controlling metabolism, stress resistance, growth and development [49]. Plants generate their own sugars through the process of photosynthesis, in which photosynthetic source tissues convert carbon dioxide (CO_2) and water to carbohydrates (sugar) and oxygen during the day, using sunlight as an energy source. Sucrose is transported to non-photosynthetic sink tissues. Sucrose is then converted to different hexoses by invertases and sucrose synthases or stored in vacuoles in amyloplasts as starch

Table 6: New genes identified as responding to Li^+ treatment in plants.c

Accession No.	Gene ID*	Fold-change F0 Control vs. Treatment	*p*-value	Fold-change F1 Control vs. Treatment	*p*-value
At3g12910	NAC	3.1	0.0002588	3.5	0.0002429
At3g22910	ACA13	3.2	0.0000086	2.2	0.0000015
At3g53040	LEA	5.7	0.0004389	3.3	0.0029689
At4g12400	HOP3	2.5	0.0000043	3.8	0.0000000
At5g06740	LecRK-s.5	3.1	0.0013176	2.7	0.0003529
At5g52720	Cu transport f.p.	6.0	0.0000573	4.8	0.0079204
At5g24540	BGLU31	5.6	0.0000017	4.2	0.0049098
At2g36640	ECP63	5.8	0.0001479	4.5	0.0081534
At2g35300	LEA18	5.8	0.0004636	4.2	0.0000437
At5g06760	LEA4-5	3.2	0.0009725	2.7	0.0006111
At1g47395	Unknown	-4.1	0.0000001	-8.0	0.0000000
At1g47400	Unknown	-3.2	0.0000372	-8.0	0.0000000
At5g03670	AtCSLA09	-3.1	0.0001500	-4.5	0.0000000
At3g56970	bHLH038	-7.1	0.0000000	-8.2	0.0000056
At3g56980	bHLH039	-7.3	0.0000001	-5.8	0.0000181
At2g41240	bHLH100	-8.2	0.0000002	>>-100	0.0002851
At5g26000	TGG1	-4.8	0.0000000	-4.0	0.0000000
At5g25980	TGG2	-6.5	0.0000000	-5.2	0.0000000

*f.p.: family protein.

for longer term storage [50]. In the dark the enzyme RuBisCO captures CO_2 from the atmosphere and in a process that requires NADPH, releases three-carbon sugars which are later combined to form sucrose and starch. The light-based reactions are associated with PS II and the dark reactions with PS I.

As seen with the overall abundance values, the photosynthesis genes appeared to be impacted by Li^+ treatment, with fewer of the genes among the 50 most abundant. Of the genes impacted by Li^+ treatment, gene isoforms were identified for 11 genes associated with PS I and II (Table 8).

Other notable genes of interest compared between the F0 control and treatment and the F1 control and treatment were the sugar-related genes (Table 9). The genes At3g15450 and At4g26530 had fold-changes greater than 1.5, but were not consistent between the generations. MUR4 (high sugar response 8, At1g30620.1), was consistently up-regulated between the generations. High sugar concentration in the leaves has been shown to repress regulation of photosynthesis genes and photosynthesis machinery [51].

ER stress related: The ER is an organelle in which the synthesis of secretory proteins and the folding and assembly of new proteins occurs. When unfolded or misfolded proteins accumulate in the ER a stress condition is initiated. The ER also acts as an intracellular Ca repository and regulates calcium signaling. Depletion of Ca levels to the ER lumen can also induce ER stress. To overcome the stress, the ER will induce gene encoding chaperones and protein folding-related enzymes, attenuate translation events or even initiate ER degradation and cell death.

Plants differ from animals in that they produce several different types of ER-derived vesicles that are involved in the accumulation of proteins. Two types of bodies are the ER body and protein bodies. The ER body is thought to be involved in pest/pathogen response and response to wounding [52]. Protein bodies play an important role in protein transfer to seeds.

In this study several genes are up- and down-regulated in relation to the ER stress (Table 10). Three genes in particular are up-regulated; two provide assistance in protein folding (such as heat and H_2O_2 or "HOP_3"and heat shock protein 70 or "HSP70") and one responds to unfolded proteins (serine/threonine protein kinase or "S6K2"). HSP70 had two isoforms that were significant at the 1.5 fold-change level. Down-regulated genes included MEB1 (At4g27860) that had two isoforms that were down-regulated. MEB1 (membrane of ER body 1) has been shown to be mainly involved in ER body function rather than ER body formation [53].

Table 7: Gene isoform identified for MIPS2 and IAA8 for Li^+ exposure.

Accession No.	Gene ID	Locus	Fold-change	TSS	Isoform Length	*p*-value
At2g22240.1	MIPS2	2:9449983-9454094	-1.6	25937	1985	0.0000280
At2g22670.4	IAA8	2:9636379-9638745	-1.9	20770	1418	0.0000006

Table 8: Gene isoforms associated with PS I and II were identified in plants exposed to Li^+.

Accession No.	Gene ID	PS	Locus	Fold-change	TSS	Isoform Length	*p*-value
At3g54890.1	LHCA1	I	3:20339503-20341103	-2.8	15764	1109	0.0010890
At1g45474.1	LHCA5	I	1:17179301-17182957	-1.9	24799	928	0.0005160
At3g08940.2	LHCB4.2	II	3:2717674-2722624	-2.9	22019	1124	0.0000295
At1g64770.1	NDF2	I	1:24057062-24059561	-1.8	23309	1303	0.0000016
At1g55370.2	NDF5	I	1:20674782-20676449	-2.6	14857	1414	0.0000000
At1g18730.1	NDF6	I	1:6460597-6462224	-1.0	17577	672	0.0004240
At1g06680.1	PSBP-1	II	1:2047824-2049418	-2.5	17048	1139	0.0008580
At5g64040.1	PSAN	I	5:255628587-25629615	-2.3	22305	760	0.0000412
At4g21280.1	PSBQA	II	4:1134401-11335773	-2.6	15058	902	0.0000000
At2g46820.1	PSI-P	I	2:19243495-19245141	-2.3	13950	936	0.0000023
At3g61870	Unknown	II	3:22902637-22904070	-1.4	15145	1058	0.0001000

Table 9: Sugar related genes with significant gene isoforms in Li^+ exposed plants.

Accession No.	Gene ID	Locus	Fold-change Control vs. Treatment for F0 (*p*-value)	Fold-change Control vs. Treatment for F1 (*p*-value)	TSS	Isoform Length
At3g15450	Al-induced protein with YGL and LRDR motifs (fructose/sucrose)	3:5212983-5216261	2.8 (0.0000009)	-2.0 (0.0023330)	18224	1051
At4g26530	Fructose-bisphosphate adolase 5	4:13391292-13393107	2.7 (0.0000407)	-4.6 (0.0000000)	6026	1372
At1g30620	MUR4	1:10854548-10858244	1.8 (0.0004900)	1.5 (0.0000731)	31459	1925

Table 10: Gene isoforms associated with ER stress.

Accession No.	Gene ID	Locus	Fold-change Control vs. Treatment for F0 (*p*-value)	Fold-change Control vs. Treatment for F1 (*p*-value)	TSS	Isoform Length
At2g42380.2	BZIP34	2:17646899-1764-8945	Not observed	-3.4 (0.0001160)	26660	1556
At3g08720.1	S6K2	3:2648508-2651160	3.9 (0.0000118)	1.7 (0.0011190)	8165	1999
At3g09440	HSP70	3:2903197-2905729	Not observed	5.0 (0.0000127)	26576	2277
At3g09440	HSP70	3:2903197-2905729	Not observed	5.1 (0.0022600)	26578	2193
At3g12120.1	FAD2	3:3860284-3863050	-1.9 (0.0003220)	-1.7 (0.0013020)	23300	1635
At4g12400	HOP3	4:7338658-7341363	2.5 (0.0000076)	5.1 (0.0015750)	1260	2066
At4g27860	MEB1	4:13873707-13876528	Not observed	2.0 (0.0001290)	13116	2179
At4g27860	MEB1	4:13873707-13876528	Not observed	1.8 (0.0037030)	348	2433

Three basic leucine zipper (bZIP) transcription factors, bZIP17, bZIP28 and bZIP60 can mediate ER stress response [54-57]. In our study a new bZIP34 gene was identified with a significant gene isoform. The bZIP34 gene has been associated with pollen transcription and several metabolic pathways including lipid metabolism and/or cellular transport in developing pollen [58]. Fatty Acid Desaturases (FADs) play important roles in plant responses to abiotic stresses. FAD2, found in the ER, has recently been reported to mediate high-level vacuolar and plasma membrane fatty acid desaturation, which provides alt tolerance to seeds and seedlings during germination and early growth [59]. In our study this gene was down-regulated in both the F0 and F1 plants with Li^+ exposure.

Transgenerational gene isoforms: Additional gene isoforms identified in both F0 and F1 generations of plants exposed to Li^+ ions showed a wide range of functions and expression levels (Table 11). Six genes were up-regulated in both F0 and F1 generations, including At1g10040, and several genes related to stress response: At4g08290, At4g36010, CNGC10, RD26 and WRKY18. Three genes were down-regulated in both generations. They function in the Fe-sulfur cluster assembly (At5g22340), metabolism (At4g37550) and proteolysis (At1g20160). Differential expression between generations was measured for gene JMJD5 (which is associated with the regulation of circadian rhythm) and an unknown gene (At5g59050).

Newly identified genes responding to Li^+ based on evaluation of significant fold-change isoforms: Evaluating genes with gene isoforms with significant fold-change levels led to the identification of four new genes that were not previously associated with Li^+ ion exposure (Table 12). Three of the four genes had relationships to chloroplasts, whereas At3g47250 was an unknown protein.

LPXC2 (lipid A biosynthetic process or At1g24880) is a member of the UDP-3-O-[3-hydroxymyristoyl] N-acetyl-glucosamine deacetylase group. It functions in acyllipid metabolism, whereby the acyl lipids serve as a layer of cutin or cuticular waxes that is present on aerial surfaces of *At* and assists in preventing water loss. It is suggested that higher plants like *At* synthesize lipid A-like molecules. The physiological role of lipid A-like molecules in *At* is unclear. Work by Li et al. [60], has shown that lipid A precursors are synthesized in the mitochondria and may be transported to the chloroplasts. It is thought that the lipid A-like molecules may serve as structural components of the outer membranes of mitochondria and/or chloroplasts or may be involved in signal transduction or plant defense responses.

Lysine biosynthesis has gained recent agricultural interest within the development of novel herbicides and with the augmentation of crop nutritional value by increasing lysine production [61,62]. At3g59890 (dihydropicicolinate reductase or DHDPR) is understudied in plants, but it is localized in the chloroplast and has similar specificities for NADH and NADPH. NADPH is used as part of the light reactions and given the location of the DHDPR in the chloroplast, this indicates that NADPH might be the biologically-relevant cofactor for DHDPR [63] .

Chloroplasts and mitochondria communicate with the cellular nucleus for optimal plant function during normal and stress conditions. The communication between the organelle and the nucleus is known as retrograde signaling and the unknown At5g27290 gene has been shown to respond specifically to chloroplast perturbations but not those that are mitochondrial in nature [64].

Given the high current use of Li^+ as a source for batteries to power hand-held electronics, the growing interest for use in battery-powered vehicles, the continued mining and processing of Li^+ and the growing number of Li^+ batteries in municipal landfills, the potential for Li^+ release to the environment has substantially increased. There is limited information available on the toxicity of long-term chronic exposure to plants. This paper investigated the effects of long-term chronic exposure in a model plant system *A. thaliana* over multiple generations. The F0 and F1 *A. thaliana* plants were exposed to Li^+ for a time period representing their juvenile life-stage through adulthood and reproduction

In this study following plants from juvenile stages through adulthood and into reproduction, plants were ~92 and 109 d old at the time of leaf harvest. These plants had completed vegetative growth, were nearing the end of reproduction and beginning the early stages of senescence of rosette leaves. Down-regulated genes related to circadian rhythm (At1g01060, At5g37260, and At3g20810), normal growth and development (At5g06520, At5g55020, At5g15950), and regulation of transcription (At3g61950 and At1g01520) were observed in these plants, however no other differentially-regulated genes that were indicative of transgenerational effects in the 0 mM control plants were observed.

Table 11: Gene isoform changes in F0 and F1 generation plants exposed to Li^+ ions.

Accession No.	Gene ID*	Locus	Fold-change Control vs. Treatment for F0 (*p*-value)	Fold-change Control vs. Treatment for F1 (*p*-value)	TSS	Isoform Length
At1g10040	Alpha/beta hydrolases s.f.p.	1.3275890-3278697	2.1 (0.0007330)	2.6 (0.0000017)	3093	1689
At3g20810	JMJD5	3:7275673-7278379	-3.5 (0.0006420)	4.0 (0.0000000)	8522	1600
At4g08290	UMAMIT20 (usually multiple acids move in and out transporters 20)	4:5238905-5241034	1.8 (0.0004010)	1.0 (0.0053850)	15913	1510
At4g36010	PR-related thaumatin s.f.p. (response to other organisms)	4:17039062-17041141	3.5 (0.0001220)	1.8 (0.0035470)	22392	1287
At4g37550	Acetamidase/formamidase f.p.	4:17643569-17645830	-2.2 (0.0000060)	-2.0 (0.0000002)	17058	1574
At5g22340	Unknown - aromatic amino acid family biosynthetic process–Fe-sulfur cluster assembly	5:7394443-7396875	-1.7 (0.0007100)	-2.1 (0.0000000)	23981	1335
At5g59050	Unknown	5:23839156-23842677	3.9 (0.0000000)	-1.8 (0.0000148)	5215	1416
At1g20160.1	AtSBT5.2identical protein binding, serine-type endopeptidase activity	1:6990783-6993972	-2.5 (0.0000111)	-3.2 (0.0000001)	4268	2405
At1g01340.2	CNGC10	1:132327-135831	2.4 (0.0000180)	1.8 (0.0000294)	6604	2397
At4g27410.2	RD26	4:13707239-13709149	2.3 (0.0000181)	1.3 (0.0016030)	20255	1718
At4g31800.1	WRKY18	4:15383200-15385029	3.3 (0.0000000)	2.3 (0.0023030)	8255	1245

*s.f.p.: super family protein; f.p.: family protein

Table 12: New gene isoforms identified as responding to Li^+ ion exposure.

Accession No.	Gene ID	Locus	Fold-change	TSS	Isoform Length	*p*-value
At1g24880	LPXC2	1:8774996-8783775	-10.0	5394	717	0.0000001
At3g47250	DUF247	3:17399876-17401731	-8.9	8007	1750	0.0000000
At3g59890	Dihydropicicolinate reductase	3:22124197-22126630	-3.3	6366	1444	0.0000983
At5g27290	Unknown (divalent metal ion transport)	5:9617485-9620533	-3.2	19404	1284	0.0028410

Information from the current literature on chronic exposure to Li^+ is focused primarily on human effects in patients taking Li^+ as a medication to treat mental illness. A common side effect reported by patients is dry mouth or a perception of thirst [65]. In the F0 and F1 treatment plants genes associated with water stress or desiccation were among the 25 most abundant genes (namely: DI19, SRC2, STZ, SZF1, COR47, ERD10, ERD14, ERD15, ERD7, SRC2 and At2g23120). The gene ER (At2g26330) was significantly down-regulated in both the F0 and F1 treatment groups. The ER or ERECTA gene regulates transpiration efficiency (i.e., the ratio of carbon fixation to water loss) to sustain plant health [66]. Genes associated with water deprivation or water stress could be attributed to the plant age and potential of being slightly root-bound with aging; however, plants were watered the day before rosette leaves were harvested.

One potential hypothesis for the mechanism of Li^+ and the dehydration effects resides in the chemistry of the ion. Lithium is a member of the group IA of elements. It has an ionic radius of 0.60 Å and has a highly hydrated radius of 3.3 Å [67]. Its single valence electron is given up very easily to create bonds and form compounds, such as with water molecules. As the amount of Li^+ increases in the plant tissue, the potential for more of the plant's internal water to be bound to Li^+ increases, which results in less water available to the plant for biological purposes. Therefore a stress response to Li could be plant dehydration.

Transgenerational effects are of interest across several research and human health issues associated with exposure to metals, chemicals, pesticides and radiation. Investigations into potential transgenerational effects in patients to taking Li^+ to their offspring was investigated through the use of mammalian models. Lithium exposure in mammals has been shown to be toxic during developmental stages. Teixeira et al. (1995), investigated the toxicity of chronic exposure of Li^+ salts on Wistar rats and their F1 offspring. F0 males and females were administered 10 mM LiCl in their daily drinking water until pup weaning. A second treatment group had F0 males and females that were water restricted during pup weaning. Results indicated a decrease in the number of males produced, delay in eye opening and impaired performance of the righting reflex in both the Li^+-treated and water-starved groups [68]. Another study utilizing

rats showed increased levels of liver aldehyde dehydrogenase from maternal exposure and passing through milk [69].

In our current study a transgenerational effect on the number and abundance of photosynthesis genes in F0 and F1 Li^+- treated plants was different. The F1 plants showed fewer photosynthesis genes among the 50 most abundant genes and instead were dominated by defense and stress related genes. Given that the F1 plants were treated for a longer duration and with a slightly higher concentration of Li^+ as compared to the F0 generation, more studies would be required to confirm that the changes in the photosynthetic gene abundance occur from a more extensive Li^+ exposure. There were no differences observed in the genomes of the F0 and F1 control plants.

Lithium-treated plants showed decrease abundance of PS I and PS II genes as compared to the controls. In the control plants these genes were among the 50 most abundant genes. However, for the Li^+-treated plants these genes were replaced by those for defense and stress response. Lithium ion has been shown to compete with magnesium ion (Mg^{2+}), a possible mechanism for the effectiveness of Li^+ intreating bipolar disorder [67,70]. Research by Dudev and Lim [70], showed that competition between Mg^{2+} and Li^+ depends on the net charge of the metal complex, which is determined by the numbers of metal cations and negatively-charged ligands and the solvent exposure of the metal cavity. Their work revealed that Li^+ replaces Mg^{2+} in specific enzymes but not in all Mg-containing enzymes that are essential to all cells for biological processes. Both Li^+ and Mg^{2+} strongly bind weak acid anions, such as phosphate, pyrophosphate and ATP [67]. While it is unclear whether Li^+ and Mg^{2+} competition causes a decline in photosynthesis gene activity in Li^+-treated plants, some discussion of the important role of Mg^{2+} in photosynthesis is needed. Magnesium ion forms the central atom of chlorophyll and is a necessary activator for many critical enzymes including two carbon fixation enzymes, ribulosbiphosphate carboxylase (RuBisCO or RCA) and phosphoenolpyruvate carboxylase (PEPC). Comparison of RCA levels in control vs. treatment plants for both the F0 and F1 generations showed no significant differenceindicating that a plant Mg deficiency was not the primary cause. Other genes responding to Li^+ exposure included the previously described *myo*-inositol-1-phosphatase (MIPS). An overall trend of down-regulation was observed in the Li^+ exposed plants for MIPS1, MIPS2 and MIPS3.

Other gene families responding in this transgenerational study included genes for metallothionein, allene oxide cyclase, IAA, NAC, ACA and AtCSLA09. Up-regulation of metallothionein genes (MT2A, MT2B and MT3) and allene oxide cyclase genes (AOS, AOC1 and AOC2) was observed, but these genes need more evaultion to determine if the up-regulation was induced by the presence of oxidative stress due to the normal aging status of the plants or the Li^+ ion alone. Of the IAA gene family, genes IAA2 and IAA18 were up-regulated and IAA1, IAA6, IAA8, IAA14 and IAA29 were down-regulated. Indole-3-acetic acid is the most common plant auxin hormone. It is responsible for cell elongation and cell division for growth and development and is associated primarily with buds or developing young leaves. Genes NAC and ACA13 were up-regulated in the treatment plants versus the controls, which are normally associated with pollen and late stage flowering in *At*. The Ca^{2+} transporting ATPase 13 (ACA13) along with ACA12 had very low expression levels in most cell types under basal condition, but were dramatically induced upon exposure to specific stresses such as pathogens or UVB light [44]. ACA13 has also been shown to be induced in papilla cells for the export of Ca^{2+} to pollen tubes, which promotes successful fertilization [45]. As seen in normal aging plants, as growth patterns change from vegetative growth to reproductive growth leaf senescence occurs to promote the movement and support of nutrients and ions to developing flowers and silques. One gene significantly down-regulated in our study is a member of the CsLA genes, which are known for their role in biosynthesis of mannans and glucomannan cell wall polysaccharides [46]. AtCSLA09 was down-regulated by a fold-change of -3.1 and -4.5 in both Li^+ treated plant generations. More investigation is needed for all above mentioned genes to resolve if their response is due to normal aging processes or response to the Li^+ ion. New genes identified as responding to Li^+ ion exposure included those in the families LEA, LEA18, LEA4-5, TGG1 and TGG2, and At5g06740. All of these genes function in response to stress or plant defense or in adaptive response to a stimulus. The genes bHLH038, bHLH039 and bHLH100 play important roles in Fe homeostasis and were down-regulated in this study. Fe^+ plays an important role in photosynthesis machinery.

Plants develop ER-derived structures that function for protein storage in specific organs or during specific life stages. ER bodies in *At* and other plants in the order *Brassicales* have a unique rod shape and are typically found in roots and seedlings. However, wounding and jasmonic acid can induce *de novo* formation of ER bodies in rosette leaves, indicating that the ER bodies may play an important role in defense strategies against biotic stress [42]. The bGLU31 gene is associated with ER bodies that accumulate large amounts of *beta*-glucosidases in response to plant defense and was strongly up-regulated in both the F0 and F1 treatment plants (fold-changes of +6.0 at a *p*-value 0.0000573 and +4.8 at a *p*-value 0.0079204, respectively).

Gene isoforms are becoming more widely investigated to determine altered gene function in relation to contaminant exposure. Gene isoforms are mRNAs that are produced from the same locus but are different in their transcription start sites (TSSs), protein coding DNA sequences (CDSs) and/or untranslated regions (UTRs). This paper is the first to describe gene isoform changes for plants and chronic Li^+ ion exposure. The investigation of gene isoforms identified genes that were not among the top 50 most abundant but had highly significant fold-change levels. Gene isoforms responding with significant fold-change were related to ER stress, with genes both up- (HOP3, HSP70, MEB1 and S6K2) and down-regulated (bZIP17, bZIP28, bZIP60 and FAD2), or were photosynthesis- and sugar-related.

CONCLUSIONS

This paper represents the first investigation of transgenerational effects from chronic Li^+ exposure using genomic level changes as a biomarker of exposure response. The use of genomic investigations using NGS with *At* is a new and growing field that offers much promise in biomarker development. Chronic exposure to Li^+ during the course of the lifetime of *At* revealed no phenotypic changes to the plants (e.g. leaf size,

coloration, wilting, etc.) for the treatment vs. control or between generations. However, gene-level changes were measured specifically for genes related to water stress/dehydration, photosynthesis, ER stress, sugar, and circadian rhythm. Further investigation of some genes is required to resolve whether or not they are responding to normal aging-related changes or if the Li^+ ion effect alone is causing the gene expression change. While the *myo*-inositol-1-phosphatase mechanism is one of the most well-known pathways of Li^+ exposure response, this study provides evidence of other gene responses in plants, such as the potential mechanisms for Li^+ competition with Mg^{2+} and Fe that impact photosynthesis process and machinery. Additionally our studies have led to the discovery of possibly a new intracellular target of Li^+ injury in plants-the ER.

ACKNOWLEDGEMENTS

This project was conducted in conjunction with work accomplished under Contract No. DE-AC09-08SR22470 with the U.S. Dept. of Energy. This work was supported by U.S. DOE—National Nuclear Security Administration through the Office of Defense Nuclear Nonproliferation Research and Development—NA-22 (Grant No. DE-FG52-05NA27035).

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Cite this article

Kuhne WW, Duff MC, Halverson NV, Chang CS, Kitamura E, et al. (2019) Identification of Sublethal Changes in Arabidopsis thaliana Exposed to Li+ Over Multiple Generations Using Gene and Gene Isoform Level Changes. J Pharmacol Clin Toxicol 7(1):1134.