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Research

Genome-wide identification of touch- and darkness-regulated Arabidopsis genes: a focus on calmodulin-like and *XTH* genes

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Summary

• We sought to gain insight into functions potentially altered by mechanostimulation and investigate the relationship between touch and darkness responses.

• Microarrays and quantitative RT-PCR were conducted to identify genes and analyze behaviors of calmodulin-like (*CML*) and xyloglucan endotransglucosylase/hydrolase (*XTH*) genes.

• Strikingly, 589 genes had touch-inducible expression; 171 had reduced expression. Darkness increased expression of 461 genes and decreased expression of 72 genes. Over half of the touch-inducible genes resembled the *TCH* genes in that they were also up-regulated by darkness; 67% of those darkness-inducible were also touch inducible. Expression of 12 *CMLs* and four *XTHs* was elevated by touch; three *XTHs* had reduced expression. In darkness-treated plants, 10 *CMLs* and nine *XTHs* had increased expression and one *XTH* was repressed.

• Over 2.5% of total genes were touch-inducible. Many were also darkness up-regulated, consistent with the hypothesis that these stimuli have partially overlapping signal transduction pathways. Regulated gene identities suggest that calcium and kinase signaling, wall modification, disease resistance and downstream transcriptional responses may be altered in response to mechanostimulation or darkness.

Key words: abiotic stress, calcium, calmodulin, darkness, mechanical stress, microarray, touch, xyloglucan endotransglucosylase/ hydrolase.

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Introduction

Plants are highly sensitive and responsive to environmental stimuli. Indeed, even the seemingly innocuous stimulus of touch can elicit significant responses in plants. In addition to the rapid touch-induced movements of specialized plants, such as Venus' Fly Trap and *Mimosa pudica*, most, if not all, plants sense and respond to touch. Darwin noted that plant roots turn and grow away from points of contact (Darwin, 1880). Jaffe coined the term thigmomorphogenesis to describe the touch-induced decreased elongation and enhanced radial expansion of plant shoots (Jaffe, 1973).

Touch stimulation can also rapidly alter gene expression regulation. The *Arabidopsis thaliana TCH* genes were discovered to be up-regulated in plants perturbed by touch or wind (Braam & Davis, 1990). *TCH1* encodes one of the Arabidopsis calmodulins (CaM2) (Braam & Davis, 1990), *TCH2* and *TCH3* encode CaM-like proteins (CML24 and CML12, respectively) (Braam & Davis, 1990; Sistrunk *et al.*, 1994; Khan *et al.*, 1997; McCormack & Braam, 2003) and TCH4 is a xyloglucan endotransglucosylase/hydrolase (XTH; formerly abbreviated XET) (Xu *et al.*, 1995; Purugganan *et al.*, 1997; Campbell & Braam, 1998; Rose *et al.*, 2002). Subsequently, a number of genes have been found to have mechano-sensitive expression regulation, including those encoding other CaMs (Ling *et al.*, 1991; Perera & Zielinski, 1992; Gawienowski *et al.*, 1993; Botella & Arteca, 1994; Botella *et al.*, 1996; Oh *et al.*, 1996), protein kinases (Botella

et al., 1996; Mizoguchi *et al.*, 1996), a retrotransposon element (Royo *et al.*, 1996), a lipoxygenase (Mauch *et al.*, 1997), an isoflavone reductase-like protein (Eldick *et al.*, 1997), ACC synthases (Arteca & Arteca, 1999; Tatsuki & Mori, 1999), a 12-oxophytodienoate reductase 3 (Müssig *et al.*, 2000), transcription factors (Gilmour *et al.*, 1998), extensins (Shirsat *et al.*, 1996; Hirsinger *et al.*, 1999), a H^+-ATPase (Oufattole *et al.*, 2000), and a cytosolic ascorbate peroxidase (Gadea *et al.*, 1999). The prevalence of touch inducibility among plant genes, however, is not known.

Furthermore, it is unknown whether other touch-induced genes show expression characteristics shared by the *TCH* genes. *TCH* expression is not only regulated by mechanical stimuli, but also by a variety of environmental, hormonal and developmental stimuli (Braam & Davis, 1990; Braam, 1992; Sistrunk *et al.*, 1994; Antosiewicz *et al.*, 1995; Xu *et al.*, 1995; Polisensky & Braam, 1996). It is possible that these different stimuli use distinct signal transduction pathways to regulate *TCH* expression. Alternatively, these stimuli may cause mechanical perturbations that then lead to *TCH* expression regulation (Braam, 2000). If the latter hypothesis were true, we would expect that all touch-inducible genes would also be inducible by the other *TCH*-responsive stimuli.

The same 102 base-pair 5′ untranscribed *TCH4* region is capable of conferring regulation of expression to touch, dark, cold, heat and brassinosteroids (Iliev *et al.*, 2002). A similar sequence is an important touch and cold regulatory site for *CBF2* expression (Zarka *et al.*, 2003). As more touch-inducible genes are identified, the motif responsible for touch-inducible regulation may be defined.

Here, we conduct a search for the full suite of touch-inducible genes to gain insight into both the cellular functions that may be altered by mechanical perturbation and the shared regulatory properties of these genes. We find that 589 genes are up-regulated in response to touch; just over half of these are also up-regulated by darkness, a characteristic shared by the *TCH* genes. Genes encoding calcium-binding, cell wall modifying, disease resistance, kinase and transcription factor proteins represent the majority of the touch-inducible genes in the Arabidopsis genome. We verify the expression behavior of 15 members each of the *CML* and *XTH* gene families using quantitative real-time RT-PCR (Q-PCR).

Materials and Methods

Plant growth and treatments

For growth in soil, 4″ pots were filled with Bacto Professional potting mixture (Michigan Peat, Houston, TX, USA) and prewetted with a solution of fertilizer (1/4 tsp Peters Professional All Purpose Plant Food, United Industries, St. Louis, MO, USA per gallon water). *Arabidopsis thaliana*, Columbia (Col-0) accession, seeds were sown on the surface of the soil. The pots were covered with plastic wrap and placed at 4°C for 1–3 d for seed stratification. Plants were grown under constant light (*c.* 70 μmol m^{-2} s^{-1}) between 20 and 22°C. Plastic wrap was removed 2 d after germination.

For touch treatment, rosette leaves were gently bent back and forth manually. Aerial portions of the plants were harvested 30 min later and immediately submerged in liquid nitrogen. For darkness treatment, large pots covered with aluminum foil were inverted over the plants for 30 min. Aerial portions of the plants were harvested and immediately submerged in liquid nitrogen. Control plants were unstimulated but harvested simultaneously with the touch- and darkness-treated plants.

RNA manipulations

Frozen tissue was ground using a mortar and pestle, and total RNA was isolated as described (Verwoerd *et al.*, 1989), except that phenol was used at 24°C. RNA was quantified with 260 nm and 280 nm readings and integrity was verified by ethidium bromide-stained formaldehyde gel analysis.

Quantitative real-time RT-PCR

DNA primers were designed to hybridize over *XTH* exon junctions to prevent interaction with genomic DNA. Because most of the *CMLs* lack introns, the RNA template used for reverse transcription was first DNAse treated, as described below, to prevent genomic DNA-derived PCR products. Primer sequences are listed in Table 6. DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). Ten μg of RNA was reverse transcribed at 42°C in a 100 μl reaction using 7.5 μl of 100 μM oligo-dT (15-mer, Integrated DNA Technologies) as a primer with 20 μl of dNTP (2.5 mM each, Bioline USA, Randolph, MA, USA) and 5 μl of M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA). The enzyme was heat inactivated by incubation at 70°C for 10 min, and the reaction mixture diluted with four equivalent volumes of water. When necessary, the RNA template was first treated with DNAse (Roche Diagnostics, Indianapolis, IN, USA) for 1 h at 37°C before being reverse transcribed.

For each Q-PCR reaction, a volume of the cDNA mixture equivalent to 100 ng of RNA (5 μl) was added to 12.5 μl of qPCR Mastermix Plus for SYBR Green I (VWR International, West Chester, PA, USA), 1 μl each of gene-specific primer (10 μM), and 5.5 μl of water. Thermal cycling and fluorescence detection were done in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's universal cycling conditions.

A threshold level of fluorescence was selected (consistent for each gene within one Q-PCR replicate) and the cycle number (Ct) at that threshold was obtained. The expression of each gene was calculated relative to the expression of *TUB4*

(encoding β-tubulin) by subtracting the Ct of the gene of interest from the Ct of *TUB4* to give ΔCt. Expression was calculated as $2^{\Delta Ct}$, which gives a value of gene expression normalized to the expression of *TUB4*. Expression values derived from stimulated plants were compared with expression values derived from control plants to arrive at ratios of induction or repression.

Microarray analyses

Twenty μg of RNA for each sample was sent along with nine Arabidopsis ATH1 Genome Arrays (Affymetrix, Santa Clara, CA, USA) to the Texas A & M Affymetrix Microarray Facility (College Station, TX, USA) for analysis. Image files were analyzed and quantified in the Microarray Analysis Suite (Affymetrix, Santa Clara, CA, USA). Numerical data were manipulated and analyzed in R (http://www.r-project.org) and Excel (Microsoft, Redmond, WA, USA).

Results

We sought to determine the prevalence of touch-inducible genes in the Arabidopsis genome. In addition, we sought to assess whether all touch-inducible genes are similar to the *TCH* genes in that they are also inducible by other stimuli, or whether there are genes that are responsive only to touch. Furthermore, we wanted to gain insight into the physiological relevance of touch-induced gene expression. Identification of all touch-regulated genes should shed light on the types of cellular processes that may be altered in response to mechanical stress perturbations.

We chose to use microarray analysis to screen the 22 810 genes represented on the Affymetrix Arabidopsis gene chip for inducibility of expression. Three-week-old, soil-grown plants were left alone as controls or mechanically stimulated by gently touching the rosette leaves and bending them back and forth 10 times. We chose to use darkness as the second stimulus because it appears to be very different from direct mechanical perturbation and can be delivered to plants uniformly by simply removing light. The likelihood of accidentally perturbing the plants in a mechanical manner while giving them a darkness treatment is low. Three separate sets of plants were harvested for each stimulus; RNA was isolated and used to generate probe independently for the resultant nine tissue samples. Hybridization and detection was carried out by the Texas A & M (College Station, TX, USA) Affymetrix Microarray Facility.

Quality analyses of chip data

Affymetrix chips use 11 probe pairs per gene distributed spatially across the chip to determine gene expression levels. One member of each probe pair is designed to hybridize with the cDNA derived from the corresponding RNA transcript; the other differs by one base in the center of the oligonucleotide. Interaction with only the former is evidence of hybridization specificity. The software can be instructed to omit probe pairs from the analysis if spatial anomalies of hybridization are detected. To verify that all nine chips hybridized uniformly, we visually analyzed the overall hybridization patterns and found that all appeared to show relatively uniform signal across the entire surface (data not shown). However, because individual cells can have drastically different intensity values than their immediate neighbors, the appearance is punctate, which can make it difficult to visualize potential spatial anomalies. To solve this problem, we created a short software utility that averages intensity values from each cell with its neighbors. This spatial 'smoothing out' of intensities allows for an easier visual analysis of hybridization patterns. The source code to this software utility is available to the community as supplementary online material (see Appendix S1). Using this utility, we found uniformity of hybridization across all nine chips, indicating that all of the probe sets and chips were usable.

Fig. 1 shows pair-wise comparisons of the relative expression levels from a total of four chips. Each gene is represented by a circle. Placement relative to the *x*-axis is determined by the expression level detected on one chip; placement relative to the *y*-axis is determined by expression level detected on a second chip. Expression level intensities are transformed by $\log_2$ to illustrate a greater dynamic range. The upper and lower red lines on each graph represent twofold higher or lower expression boundaries, respectively. Points above the upper red line represent genes whose expression is increased more than twofold in the sample plotted along the *y*-axis compared with the control sample represented on the *x*-axis, and points that are below the lower red line represent genes whose expression is reduced more than twofold relative to the control sample. Fig. 1(a) plots the expression levels of each gene from two biological replicates of untreated control plants to evaluate reproducibility between replicates. 83.2% of the genes fall within the two red lines; most outlying points represent genes with low expression levels. Similarly, pair-wise comparisons of the other control chip data show 82.9% and 83.8% of the genes falling within the twofold range with greatest variation among genes with low expression (data not shown). Variability of data for genes with low expression levels is likely due to reduced reliability of detection at low levels of hybridization signal intensity.

The Affymetrix Microarray Suite software takes into account the reduced reliability of data at low intensity values by calculating an intensity of hybridization for each probe pair and indicating a statistical call of confidence of whether RNA is present, absent, or marginal, that is, present at a low level of detection. Nearly half of the genes are marked absent or marginal on at least one of the nine chips in our study.

Fig. 1(b) shows the expression levels of genes from the same two control chips as in Fig. 1(a), but omits the genes marked

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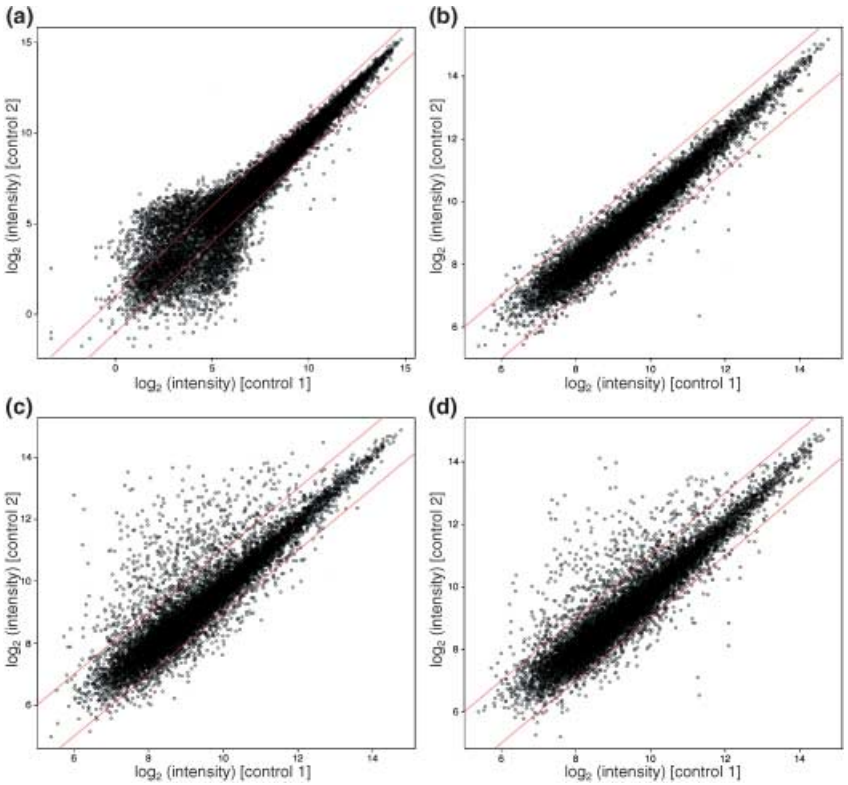

Fig. 1 Pair-wise gene expression level comparisons show that genes with low absolute values of expression have increased experimental variability and that the majority of genes are not altered in expression. Log_2 expression intensities on one chip are plotted against the expression intensities of the same genes on another chip. The upper red lines in each graph are determined by $y = x + 1$ where y is the log_2 value of expression on the test chip and x is the log_2 value of expression on the control chip; the lower red lines are determined by $y = x - 1$. (a) Intensities from the second control chip vs the first control chip; (b) intensities from the second control chip vs the first control chip omitting all genes that are marked marginal or absent on any chip; (c) intensities from the first touch chip vs the first control chip omitting all genes that are marked marginal or absent on any chip; (d) intensities from the first darkness chip vs the first control chip omitting all genes that are marked marginal or absent on any chip.

absent or marginal on any of our nine chips. 11 520 genes remain, and only 1.2% of the genes fall outside the red lines indicating a high level of consistency between biological replicates. The other control chip pair-wise comparisons are similar, with only 0.5% and 0.8% of genes falling outside the red lines (data not shown). Many of the outlying points are positioned close to the red lines, indicating only small changes in expression detection. Based on this analysis, we chose to define a twofold expression change as significant and limit our analyses in this report to genes marked present on all nine chips. These criteria should increase the reliability of our data and limit the number of false positives. As a consequence, however, genes are omitted from analysis that have detectable expression only after stimulation of plants or have undetectable expression in stimulated plants.

Fig. 1(c) is a gene expression level comparison from touched plants ('touch chip') vs the expression levels in control plants ('control chip'). 8.6% of genes have at least twofold expression level differences in touched plant samples compared with control plant samples. Many ratios of differential expression are relatively high and lie further from the red lines than those in Fig. 1(b) indicating a greater magnitude of expression level differences than detected as variation in biological replicates of control plants. The other eight pair-wise comparisons between touch and control chips are similar with 7.6% to 9.1% of the genes laying outside the twofold expression change range (data not shown).

Similarly, most genes have less than a twofold change in expression in plants subjected to a 30-min darkness treatment. Fig. 1(d) shows gene expression levels detected on a chip analyzed with probes from darkness-treated plants vs that from the control plants. 6.1% of genes have altered expression levels in response to darkness. The other eight pair-wise comparisons between darkness-treated plants and controls show similar results with 5.7% to 7.0% of the genes showing greater than twofold differences in expression (data not shown).

Touch and darkness inducible genes

The availability of data in triplicate enables us to average the different expression ratios from the nine pair-wise comparisons of control vs touch data and the nine pair-wise comparisons between control and darkness data. In this way, we identify those genes whose mRNA levels are consistently altered at least twofold in the three biological replicates of plants subjected to touch or darkness. The most strongly up regulated genes by touch and darkness are listed in Table 1 and Table 2, respectively; the full data are available as supplementary material (Appendix S2). We define 589 genes with increased and 171 genes with decreased expression levels after touch (Appendix S3). Darkness results in increased expression of 461 genes and decreased expression of 72 genes (Appendix S4). Thus, nearly 7% of the genes analyzed are altered in expression by touch and 4.6% are altered in

Table 1 Genes with highest ratios of expression in touched vs. control Arabidopsis thaliana plants

Probe ID	ORF name	Gene name	Touch ratio	SD	*P*-value	Darkness ratio	SD	*P*-value
253643_at	At4g29780	expressed protein	72.73	17.26	0.0044	31.10	6.74	0.0002
252368_at	At3g48520	cytochrome P450 family	60.14	35.26	0.0055	1.42	1.03	0.9488
258947_at	At3g01830	calmodulin-related protein, putative (CML40)	56.93	9.85	0.0036	5.77	0.86	0.0000
261037_at	At1g17420	lipoxygenase (LOX), putative	46.08	5.29	0.0006	2.49	0.42	0.0135
254120_at	At4g24570	mitochondrial carrier protein family	43.45	6.35	0.0014	29.82	4.71	0.0031
261892_at	At1g80840	WRKY family transcription factor (WRKY40)	43.11	4.83	0.0008	12.99	2.31	0.0104
258792_at	At3g04640	glycine-rich protein	39.06	7.52	0.0051	31.78	5.97	0.0043
253830_at	At4g27652	expressed protein	33.57	4.27	0.0024	4.44	1.13	0.0360
248964_at	At5g45340	cytochrome P450 family	32.53	16.69	0.0166	52.49	24.87	0.0016
261648_at	At1g27730	salt-tolerance zinc finger protein	29.45	7.79	0.0053	23.59	5.77	0.0006
261033_at	At1g17380	expressed protein	26.32	6.36	0.0018	2.93	0.76	0.0037
251336_at	At3g61190	BON1-associated protein 1 (BAP1)	25.71	7.12	0.0014	7.48	2.21	0.0040
247655_at	At5g59820	zinc finger protein Zat12	22.58	3.37	0.0090	11.88	1.04	0.0022
263182_at	At1g05575	expressed protein	22.35	3.21	0.0059	8.77	0.90	0.0007
254158_at	At4g24380	expressed protein	22.04	1.29	0.0016	6.06	0.60	0.0062
259479_at	At1g19020	expressed protein	21.84	2.54	0.0011	7.05	0.80	0.0003
256526_at	At1g66090	disease resistance protein (TIR-NBS class), putative	21.72	1.76	0.0030	14.78	1.63	0.0059
247925_at	At5g57560	xyloglucan endotransglycosylase (TCH4)	21.17	8.21	0.0000	28.52	11.05	0.0000
254926_at	At4g11280	1-aminocyclopropane-1-carboxylate synthase 6	19.78	4.29	0.0060	10.93	2.66	0.0123
253485_at	At4g31800	WRKY family transcription factor (WRKY18)	19.56	1.72	0.0000	9.13	0.87	0.0005
263800_at	At2g24600	expressed protein	19.31	1.69	0.0011	8.11	0.64	0.0002
251745_at	At3g55980	putative protein zinc finger transcription factor (PEI1)	18.76	2.02	0.0030	18.21	1.47	0.0005
261443_at	At1g28480	glutaredoxin protein family	18.60	3.96	0.0057	1.87	0.40	0.0115
267357_at	At2g40000	nematode-resistance protein -related	18.50	2.64	0.0022	15.18	2.00	0.0007
256763_at	At3g16860	expressed protein	17.93	3.54	0.0045	3.66	0.75	0.0041
250781_at	At5g05410	DRE binding protein (DREB2A)	17.62	5.02	0.0102	3.85	0.96	0.0002
260656_at	At1g19380	expressed protein	16.76	2.66	0.0016	9.03	1.52	0.0031
260227_at	At1g74450	expressed protein	16.37	0.24	0.0000	2.65	0.15	0.0031
266834_s_at	At2g30020	protein phosphatase 2C (PP2C), putative	16.29	3.64	0.0093	3.41	0.81	0.0155
256017_at	At1g19180	expressed protein	15.89	2.91	0.0033	3.57	0.64	0.0011
258682_at	At3g08720	ribosomal-protein S6 kinase (ATPK19)-related	15.25	2.81	0.0002	5.31	1.00	0.0001
245777_at	At1g73540	MutT/nudix family protein	14.72	1.43	0.0030	7.33	0.51	0.0005
252679_at	At3g44260	CCR4-associated factor 1-related protein	14.27	3.17	0.0080	7.46	1.95	0.0191
266658_at	At2g25735	expressed protein	13.88	3.20	0.0033	7.98	1.70	0.0000
252474_at	At3g46620	expressed protein	13.37	3.39	0.0028	6.49	1.73	0.0053
247137_at	At5g66210	calcium-dependent protein kinase (CPK28)	13.08	2.95	0.0012	7.50	1.82	0.0046
255568_at	At4g01250	WRKY family transcription factor (WRKY22)	12.98	3.49	0.0283	7.37	0.89	0.0000
254300_at	At4g22780	uridylyltransferase-related	12.93	4.64	0.0042	2.84	1.00	0.0034
262360_at	At1g73080	leucine-rich repeat transmembrane kinase, putative	12.88	2.45	0.0040	2.31	0.39	0.0020
264289_at	At1g61890	MATE efflux protein family	12.73	2.04	0.0008	2.67	0.44	0.0005
253915_at	At4g27280	calcium-binding EF-hand family protein	12.69	1.54	0.0049	26.31	3.01	0.0038
267028_at	At2g38470	WRKY family transcription factor (WRKY33)	12.40	3.51	0.0042	11.20	3.00	0.0003
263972_at	At2g42760	expressed protein	12.38	3.01	0.0000	0.93	0.33	0.6204
251640_at	At3g57450	expressed protein	12.36	1.11	0.0005	7.80	0.93	0.0038
249264_s_at	At5g41740	disease resistance protein (TIR-NBS-LRR class)	12.30	2.11	0.0002	10.95	2.09	0.0036
256627_at	At3g19970	expressed protein	11.87	2.46	0.0000	3.04	0.74	0.0066
251636_at	At3g57530	calcium-dependent protein kinase (CPK32)	11.51	2.84	0.0012	6.87	2.06	0.0160
265184_at	At1g23710	expressed protein	11.36	4.04	0.0073	7.62	2.53	0.0001
254231_at	At4g23810	WRKY family transcription factor (WRKY53)	11.33	2.97	0.0151	5.22	1.02	0.0002
253140_at	At4g35480	RING-H2 finger protein RHA3b	11.18	1.31	0.0052	1.34	0.15	0.0353
252131_at	At3g50930	AAA-type ATPase family	10.69	3.25	0.0069	5.22	1.52	0.0012

Probe ID is the Affymetrix identifier, ORF name is the Arabidopsis Genome Initiative open reading frame name, gene name is the common abbreviation. Expression ratios are averaged differences between control and experimental chip expression data. *P*-values were calculated using a Student's *t*-test between the measurements from the control chips and the appropriate measurements from the stimulus chips. *P*-value = 0.0000 is equivalent to *P*-value ≤ 0.0001. (SD, standard deviations.)

Table 2 Genes with highest ratios of expression in darkness-treated vs. control *Arabidopsis thaliana* plants

Probe ID	ORF Name	Gene description	Touch ratio	SD	*P*-value	Darkness ratio	SD	*P*-value
248964_at	At5g45340	cytochrome P450 family	32.53	16.69	0.0166	52.49	24.87	0.0016
247543_at	At5g61600	AP2 domain transcription factor, putative	4.79	0.77	0.0060	36.53	5.68	0.0044
260856_at	At1g21910	transcription factor TINY family	4.08	1.18	0.0102	32.46	8.81	0.0075
258792_at	At3g04640	glycine-rich protein	39.06	7.52	0.0051	31.78	5.97	0.0043
253643_at	At4g29780	expressed protein	72.73	17.26	0.0044	31.10	6.74	0.0002
254120_at	At4g24570	mitochondrial carrier protein family	43.45	6.35	0.0014	29.82	4.71	0.0031
247925_at	At5g57560	xyloglucan endotransglycosylase (TCH4)	21.17	8.21	0.0000	28.52	11.05	0.0000
253915_at	At4g27280	calcium-binding EF-hand family protein	12.69	1.54	0.0049	26.31	3.01	0.0038
261648_at	At1g27730	salt-tolerance zinc finger protein	29.45	7.79	0.0053	23.59	5.77	0.0006
255064_at	At4g08950	phi-1 phosphate-induced protein-related	7.25	2.53	0.0007	21.79	7.46	0.0005
251745_at	At3g55980	putative protein zinc finger transcription factor (PEI1)	18.76	2.02	0.0030	18.21	1.47	0.0005
252193_at	At3g50060	myb DNA-binding protein (MYB77)	4.73	0.89	0.0185	16.99	1.84	0.0019
267357_at	At2g40000	nematode-resistance protein-related	18.50	2.64	0.0022	15.18	2.00	0.0007
255733_at	At1g25400	expressed protein	8.07	1.18	0.0023	14.95	2.07	0.0014
256526_at	At1g66090	disease resistance protein (TIR-NBS class), putative	21.72	1.76	0.0030	14.78	1.63	0.0059
262383_at	At1g72940	disease resistance protein (TIR-NBS class), putative	8.57	0.99	0.0045	14.66	2.95	0.0176
245755_at	At1g35210	expressed protein	8.04	2.01	0.0064	14.21	3.48	0.0057
261892_at	At1g80840	WRKY family transcription factor (WRKY40)	43.11	4.83	0.0008	12.99	2.31	0.0104
265387_at	At2g20670	expressed protein	0.95	0.24	0.6487	12.80	2.91	0.0074
247655_at	At5g59820	zinc finger protein Zat12	22.58	3.37	0.0090	11.88	1.04	0.0022
262382_at	At1g72920	disease resistance protein (TIR-NBS class), putative	5.08	1.44	0.0450	11.52	1.69	0.0062
260037_at	At1g68840	AP2 domain protein RAP2.8 (RAV2)	3.52	0.88	0.0017	11.46	2.88	0.0044
252563_at	At3g45970	expansin protein family (EXPL1)	6.31	2.40	0.0003	11.45	4.69	0.0091
267028_at	At2g38470	WRKY family transcription factor (WRKY33)	12.40	3.51	0.0042	11.20	3.00	0.0003
249264_s_at	At5g41740	disease resistance protein (TIR-NBS-LRR class)	12.30	2.11	0.0002	10.95	2.09	0.0036
254926_at	At4g11280	1-aminocyclopropane-1-carboxylate synthase 6	19.78	4.29	0.0060	10.93	2.66	0.0123
246777_at	At5g27420	RING-H2 zinc finger protein-related	9.15	2.18	0.0027	9.27	2.20	0.0025
246253_at	At4g37260	myb DNA-binding protein (AtMYB73)	4.67	0.44	0.0012	9.18	0.98	0.0030
253485_at	At4g31800	WRKY family transcription factor (WRKY18)	19.56	1.72	0.0000	9.13	0.87	0.0005
260915_at	At1g02660	lipase (class 3) family	10.47	1.58	0.0000	9.06	1.41	0.0002
260656_at	At1g19380	expressed protein	16.76	2.66	0.0016	9.03	1.52	0.0031
259364_at	At1g13260	AP2 domain transcription factor, putative (RAV1)	4.05	0.39	0.0001	9.01	0.94	0.0014
246200_at	At4g37240	expressed protein	1.48	0.38	0.0774	8.82	2.19	0.0010
255524_at	At4g02330	pectinesterase, putative (PME3)	7.48	1.57	0.0000	8.80	3.19	0.0450
263182_at	At1g05575	expressed protein	22.35	3.21	0.0059	8.77	0.90	0.0007
259979_at	At1g76600	expressed protein	10.51	0.93	0.0012	8.53	0.62	0.0000
248164_at	At5g54490	calcium-binding protein, putative (PBP1)	3.73	0.61	0.0166	8.32	0.94	0.0044
254707_at	At4g18010	inositol polyphosphate 5-phosphatase II (IP5PII)	6.72	0.62	0.0034	8.30	0.66	0.0020
257076_at	At3g19680	expressed protein	1.59	0.24	0.0072	8.18	1.12	0.0000
263800_at	At2g24600	expressed protein	19.31	1.69	0.0011	8.11	0.64	0.0002
251507_at	At3g59080	expressed protein	5.94	0.87	0.0013	8.04	1.71	0.0152
266658_at	At2g25735	expressed protein	13.88	3.20	0.0033	7.98	1.70	0.0000
251640_at	At3g57450	expressed protein	12.36	1.11	0.0005	7.80	0.93	0.0038
245866_s_at	At1g57990	purine permease-related	6.90	0.73	0.0035	7.79	0.60	0.0003
252053_at	At3g52400	syntaxin of plants SYP122	10.43	1.56	0.0022	7.68	1.18	0.0028
265184_at	At1g23710	expressed protein	11.36	4.04	0.0073	7.62	2.53	0.0001
250777_at	At5g05440	expressed protein	2.40	0.94	0.0793	7.57	2.30	0.0081
261193_at	At1g32920	expressed protein	8.55	2.08	0.0000	7.55	1.83	0.0000
247137_at	At5g66210	calcium-dependent protein kinase (CPK28)	13.08	2.95	0.0012	7.50	1.82	0.0046
251336_at	At3g61190	BON1-associated protein 1 (BAP1)	25.71	7.12	0.0014	7.48	2.21	0.0040
252679_at	At3g44260	CCR4-associated factor 1-related protein	14.27	3.17	0.0080	7.46	1.95	0.0191
255568_at	At4g01250	WRKY family transcription factor (WRKY22)	12.98	3.49	0.0283	7.37	0.89	0.0000

Probe ID is the Affymetrix identifier, ORF name is the Arabidopsis Genome Initiative open reading frame name, gene name is the common abbreviation. Expression ratios are averaged differences between control and experimental chip expression data. *P*-values were calculated using a Student's *t*-test between the measurements from the control chips and the appropriate measurements from the stimulus chips. *P*-value = 0.0000 is equivalent to *P*-value ≤ 0.0001. (SD, standard deviations.)

Table 3 Gene number corresponding to functional categories and behaviors detected by microarray

	Whole chip	Present on all	Touch (up)	Dark (up)	Touch and dark (up)	Touch (down)	Dark (down)	Touch and dark (down)
Array control	46	17	0	0	0	0	0	0
Calcium binding	133	79	15	12	10	0	0	0
Cell wall associated	324	151	22	25	14	4	3	2
Chaperone	165	112	2	2	1	0	1	0
Cytoskeleton/motility	175	91	2	1	1	0	0	0
Disease resistance	309	111	18	16	12	0	1	0
Kinase	1111	613	72	40	31	6	3	0
Metabolism	4731	2767	98	61	37	28	28	3
Phosphatase	186	125	8	5	3	1	0	0
Photosynthesis	77	60	1	2	1	0	0	0
Protease	168	76	0	0	0	0	1	0
Pseudogene	55	0	0	0	0	0	0	0
Signal transduction	160	96	3	4	3	0	0	0
Transcription factor	1601	634	66	67	48	33	6	5
Transcription/translation	178	128	2	0	0	0	0	0
Transporter	726	394	18	14	11	4	3	2
Ubiquitin/protein degradation	574	258	5	2	1	7	0	0
Unknown function, known response	108	78	4	2	2	0	0	0
Vesicle transport	91	67	4	3	3	0	0	0
Unknown function, unknown regulation	11892	5663	249	205	132	88	26	12
Total	22810	11520	589	461	310	171	72	24

response to darkness under our experimental conditions. The great majority of the expression differences are the result of up regulation in plants stimulated with touch or darkness (5.1% and 4.0%, respectively). Only 1.5% and 0.6% of genes, respectively, have decreased expression in touched or darkness-treated plants.

Many of the touch and darkness-induced genes behave like the original *TCH* genes in that they are increased in expression by both touch and darkness stimuli. Over 300 genes are up regulated in expression in both touch- and darkness-treated plants (Appendix S2), indicating that 53% of the touch-induced genes are also up regulated by darkness and 67% of the darkness-induced genes are also touch induced.

Functional classification of touch and darkness inducible genes

To gain insight into the types of cellular processes that may be affected by mechanical stimulation or darkness, we determined the functional classes of genes identified as expression-altered genes. We assigned putative functions to each of the 22 810 genes represented on the Affymetrix chip based on the limited annotations available from GenBank for each locus. In large part, assignments were made based on sequence similarities to known proteins. Therefore, these are, at best, putative functional assignments. For example, genes bearing sequence similarity to known kinases were assigned as potential kinases, and those containing MADS box domains were assigned as potential transcription factors. These categorizations of the full 22 810-gene complement are available as supplementary material for others to use and to refine as more functional information becomes available (Appendix S5).

The numbers of genes in each category and those altered in response to touch and darkness are listed in Table 3. To visualize the relative distribution of these functional gene classes, the percentage of genes with similar function are represented by a colored portion of a bar graph in Fig. 2. A total of 11 892 genes (52%) was defined as having no known or inferred function or regulatory behavior; these genes are not enriched in representation in the touch or darkness regulated genes and were not included in Fig. 2. The first column on the left (Fig. 2 'whole chip') represents all functional classes of genes on the Affymetrix chip (22 810 genes). The functional groups with the greatest number of members encode putative proteins involved in general metabolism (pale blue), transcription factors (pink), and kinases (purple).

The second column (Fig. 2 'present on all') represents the breakdown of function among only those genes whose expression is marked present on all nine chips. As expected, pseudogenes, which are thought to lack expression, are absent in this column. Overall, however, the subset of genes marked present on all chips has a similar functional distribution as the overall genome.

There is a relative enrichment in several categories and a concomitant decrease in other categories among the gene subsets that are up regulated at least twofold by touch, darkness, or both touch and darkness (Table 3, Fig. 2 'touch (up)', 'dark (up)', and 'touch and dark (up)', respectively). Enrichment in genes encoding five functional classes of proteins is apparent in the touch-inducible gene set (Fig. 2 'touch (up)'). Putative

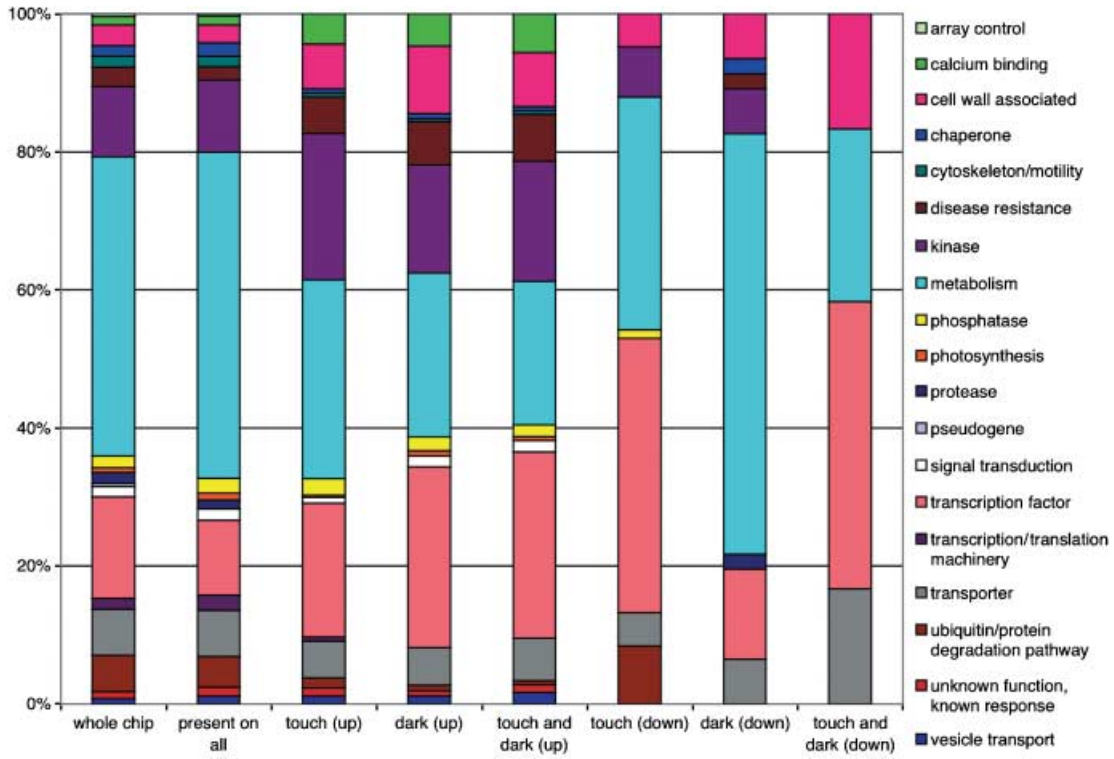

Fig. 2 Distribution of genes by functional category in gene sets defined by expression behavior. The functional categories are represented from top to bottom in each column according to the legend. Genes classified as unknown function or expression behavior have been omitted. The leftmost column represents the functional distribution of the genes represented on the Affymetrix Arabidopsis Gene Chip. The second column represents the subset of genes marked as present on all nine chips. The last six columns are subsets of the second column, with the following additional restrictions from left to right: at least twofold induction by touch, at least twofold induction by darkness, at least twofold induction by both touch and darkness, at least twofold reduction by touch, at least twofold reduction by darkness, at least twofold reduction by both touch and darkness. The numbers used to generate these percentages are listed in Table 3.

calcium-binding protein genes become enriched to the greatest degree with a 3.3-fold increase (from 1.4% of the total genes to 4.4% of the touch-inducible genes), disease resistance genes show a 2.8-fold enrichment (from 1.90% to 5.29%), cell wall associated protein genes increase 2.5 fold (from 2.6% to 6.5%), and genes encoding kinases and transcription factors show approximately twofold enrichment (from 10.5% to 21.1% and 10.9% to 19.4%, respectively). There is a relative loss of genes involved in general metabolism (47.2% to 28.8%) and putative ubiquitin/protein degradation pathway genes (4.4% to 1.5%) among the population of touch-inducible genes (Fig. 2 'touch (up)'), indicating that only a minority of genes in these categories are up regulated after touch.

The same functional classes are enriched and lost from the gene subset whose expression is regulated by darkness (Table 3, Fig. 2 'darkness (up)'). Genes encoding cell wall associated proteins show the greatest fold enrichment, 3.8 fold, from 2.6% to 9.8%, followed by genes encoding putative calcium-binding proteins (a 3.5-fold enrichment, 1.4% to 4.7%), disease resistance proteins (a 3.3-fold enrichment, 1.9% to 6.3%), transcription factors (a 2.5-fold enrichment, 10.8% to 26.2%) and kinases (a 1.5-fold enrichment, 10.5% to 15.6%). Genes encoding proteins involved in general metabolism and putative ubiquitin/protein degradation pathways decrease in representation from 47.2% to 23.8% and 4.4% to 0.8%, respectively (Fig. 2 'darkness (up)').

Indeed, touch and darkness appear to result in the coregulation of the same functional gene classes (Table 3, Fig. 2 'touch and dark (up)'). Genes that show increased expression in response to both touch and darkness encode putative calcium-binding proteins (1.4% to 5.6%), cell wall associated proteins (2.6% to 7.9%), disease resistance proteins (1.9% to 6.7%), kinases (10.5% to 17.4%), and transcription factors (10.8% to 27%); genes encoding proteins involved in general metabolism (47.2% to 21%) and putative ubiquitin/protein degradation pathways (4.4% to 0.6%) are decreased in representation.

Table 3 and Fig. 2 also show the relative enrichment of functional classes of genes found to have reduced expression in plants subjected to touch or darkness. Because the time interval from stimulation to harvest was short, only 30 min, one would expect to find only those genes whose transcripts are unstable in addition to having reduced transcription rates. Indeed, there are fewer genes identified: 171 touch-, 72 darkness-, and 24 both touch- and darkness-repressed genes (Table 3, Fig. 2 'touch (down)', 'dark (down)', and 'touch and

dark (down)', respectively). Genes encoding putative transcription factors and cell wall associated proteins are among those more significantly enriched in the pool of genes with decreased expression in touch- and/or darkness-stimulated plants (Table 3, Fig. 2 'touch (down)', and 'dark (down)').

Expression behavior of *CAM/CML* and *XTH* genes

Because calcium-binding protein genes and cell wall associated protein genes are among the most highly represented functional classes of touch- and/or darkness-regulated genes, we chose to focus further analyses on two gene families within these functional classes, the *CAMs/CMLs* and *XTHs*.

Six of the seven CaM-encoding genes are included on the microarrays and are marked as present on all nine chips (Table 4); *CAM6* (At5g21274) is missing. *TCH1* (*CAM2*) is the only CaM-encoding gene found to be up regulated by either touch or darkness through the microarray analysis (Table 4). The ability of the microarray to differentiate between the different *CAMs* is remarkable given that they share more than 84% nucleotide sequence identity over the coding regions.

Of the 50 *CML* genes, 48 are included on the chip and 21 are designated as having detectable expression on all nine chips (Table 4). Nine *CMLs* have a twofold or greater increase in expression levels in touched plants compared with control plants, including *TCH2* (*CML24*) and *TCH3* (*CML12*). All but two of the touch-inducible *CMLs* have a twofold or greater increase in expression in darkness-treated plants also. Thus, many *CMLs* behave like the original *TCH* genes in being regulated in expression by both touch and darkness. None of the *CAMs* or *CMLs* shows a twofold or greater reduction in expression levels in touch- or darkness-stimulated plants.

All 33 XTH-encoding genes of Arabidopsis are on the Affymetrix chip and 16 have detectable expression levels on all nine chips. Of these, two *XTHs*, in addition to *TCH4* (*XTH22*) show at least a twofold expression increase in touched plants and five have a twofold or greater expression increase by darkness (Table 5). The three *XTHs* with higher expression in touched plants, *XTH17*, *XTH22* and *XTH25*, also have increased expression in darkness-treated plants. Only *XTH31* exhibits a greater than twofold expression reduction to darkness, and none have a twofold or greater expression reduction in response to touch.

More than half of the *CML* and *XTH* genes had marginal or undetectable expression on at least one of the nine chips (Tables 4 and 5). Some of these may have very limited expression levels or may not be expressed in rosette leaves. A few have undetectable expression only under control conditions and thus may be inducible in expression; however, these data tend to have high standard deviations and, thus, are less reliable (Tables 4 and 5).

To further verify expression behavior of these gene classes, we chose the 15 *CMLs* and 15 *XTHs* that had highest absolute expression levels under at least one of the three conditions (control, touch- or darkness-treated plants) to analyze with Q-PCR. Four of these, *CML37*, *CML38*, *XTH18* and *XTH23*, had absent or marginal expression on at least one control chip. Fig. 3 illustrates message levels of *CMLs* (Fig. 3a) and *XTHs* (Fig. 3b) in response to touch and darkness as determined by both the microarray analyses and Q-PCR. Each biological replicate of RNA used in the microarray experiment was independently reverse transcribed and analyzed using Q-PCR. The amplification efficiencies of all reactions were consistent and approached 100% (data not shown). Expression levels were normalized within replicates to the *TUB4* (encoding β-tubulin) mRNA levels. Induction or repression ratios were calculated by pair-wise comparisons of expression levels from treated and control replicates. These ratios were then averaged and transformed by $\log_2$.

The comparison analyses shown in Fig. 3 reinforce the finding that expression ratio changes of less than twofold determined by microarray analyses can be unreliable. For example, microarray data suggested that six *XTHs* (*XTH6*, *XTH7*, *XTH8*, *XTH15*, *XTH16*, and *XTH28*) had slightly reduced expression in touched plants (Table 5 and Fig. 3). However, Q-PCR indicates that these genes are not reproducibly down regulated in response to touch (Fig. 3). Similarly, for example, *CML10*, *CML27* and *CML42* had slightly altered expression levels in darkness-treated plants as detected by the microarrays (Table 4 and Fig. 3). By contrast, Q-PCR indicates that these genes are largely unaffected by a darkness treatment (Fig. 3). Four genes differ in magnitudes of expression differences between the two analyses; these include *CML12*, *CML38*, *CML40* and *XTH22*. For all but *CML40*, the fold induction levels are indicated to be greater using Q-PCR compared with the microarray.

Overall, however, the Q-PCR analyses show similar expression induction or repression by touch and darkness for all of the genes that were determined to have a greater than twofold change in expression by the array analysis (Tables 4 and 5, Fig. 3), indicating a high confidence level in these data. Consistent expression behavior is also true for four genes (*CML37*, *CML38*, *XTH18* and *XTH23*) that had marginal or undetectable expression under microarray control conditions, but high expression after stimulation (Tables 4 and 5, Fig. 3); Q-PCR demonstrates that these genes are indeed increased in expression in both touch- and darkness-stimulated plants. In summary, we find that 12 of the 15 *CMLs* and four of the 15 *XTHs* have increased expression and three *XTHs* have decreased expression in touch-stimulated plants. Expression of 10 *CMLs* and nine *XTHs* is increased in darkness-stimulated plants. Only *XTH32* is decreased in expression in darkness-treated plants.

Additionally, Q-PCR largely confirms the results of the microarray analyses. Thus, it is likely that the majority of

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Table 4 CAM and CML expression

Probe ID	ORF name	Gene name	Status	Touch ratio	SD	*P*-value	Darkness ratio	SD	*P*-value
249582_at	At5g37780	*CAM1*	P	0.93	0.10	0.3549	0.95	0.05	0.2393
267064_at	At2g41110	*CAM2/TCH1*	P	2.25	0.48	0.0194	1.58	0.38	0.0842
246290_at	At3g56800	*CAM3*	P	1.32	0.17	0.0368	1.07	0.12	0.5293
260138_at	At1g66410	*CAM4*	P	0.87	0.08	0.0819	0.92	0.13	0.4080
266317_at	At2g27030	*CAM5*	P	1.03	0.14	0.8400	1.05	0.18	0.7596
252713_at	At3g43810	*CAM7*	P	0.90	0.11	0.2706	0.89	0.06	0.1174
254782_at	At4g12860	*CML2*	N (C, D, T)	1.12	0.32	0.6389	1.25	0.73	0.6675
259064_at	At3g07490	*CML3*	N (C, D, T)	1.99	1.63	0.6402	0.86	0.94	0.4441
251488_at	At3g59440	*CML4*	N (C, D, T)	2.34	2.74	0.8544	3.03	2.73	0.4696
266447_at	At2g43290	*CML5/MSS3*	P	2.88	0.17	0.0002	4.46	0.42	0.0044
255423_at	At4g03290	*CML6*	N (C, D, T)	2.87	4.77	0.9512	2.09	2.47	0.7237
260960_at	At1g05990	*CML7*	N (C, D, T)	0.52	0.74	0.2537	0.55	0.47	0.2025
245257_at	At4g14640	*CML8/CAM8*	N (C, D, T)	1.93	1.36	0.7073	2.79	1.63	0.2536
252037_at	At3g51920	*CML9/CAM9*	P	1.98	0.18	0.0013	1.71	0.16	0.0033
267076_at	At2g41090	*CML10/CaBP22/CAM10*	P	0.88	0.11	0.1755	0.96	0.09	0.4899
256839_at	At3g22930	*CML11*	N (C, D, T)	15.79	11.25	0.0054	14.06	14.50	0.1751
267083_at	At2g41100	*CML12/TCH3/CAM12*	P	2.37	0.44	0.0028	2.55	0.46	0.0012
259538_at	At1g12310	*CML13/CAM13*	P	0.88	0.09	0.1465	0.93	0.10	0.3574
262639_at	At1g62820	*CML14/CAM14*	P	1.05	0.20	0.8033	1.07	0.15	0.5916
255772_at	At1g18530	*CML15*	N (C, D, T)	1.30	0.67	0.9395	0.81	0.53	0.4061
256755_at	At3g25600	*CML16*	P	4.24	0.31	0.0009	5.82	0.35	0.0000
260702_at	At1g32250	*CML17*	N (C, D, T)	1.02	0.23	0.9375	1.26	0.56	0.5637
258617_at	At3g03000	*CML18*	P	1.37	0.17	0.0332	1.62	0.27	0.0496
246197_at	At4g37010	*CML19*	N (C, D, T)	1.62	0.74	0.2152	1.14	0.50	0.9725
252206_at	At3g50360	*CML20/Centrin*	P	1.19	0.19	0.1957	1.21	0.20	0.1736
253963_at	At4g26470	*CML21*	N (C, D, T)	2.63	2.39	0.3280	1.97	2.00	0.5979
257245_at	At3g24110	*CML22*	N (C, T)	0.97	0.27	0.6704	1.11	0.32	0.8677
260135_at	At1g66400	*CML23*	N (C)	4.19	0.82	0.0089	4.88	1.13	0.0195
249583_at	At5g37770	*CML24/TCH2*	P	6.55	1.03	0.0053	6.70	1.58	0.0236
257405_at	At1g24620	*CML25*	N (C, D, T)	0.70	0.75	0.3212	2.66	3.02	0.6811
260076_at	At1g73630	*CML26*	P	0.76	0.08	0.0614	0.67	0.08	0.0281
256129_at	At1g18210	*CML27*	P	3.35	0.31	0.0006	1.41	0.11	0.0139
259044_at	At3g03430	*CML28*	N (C, D, T)	0.95	0.51	0.6249	0.73	0.62	0.4119
246431_at	At5g17480	*CML29/APC1*	N (C, D, T)	3.97	6.14	0.5190	2.00	1.98	0.6579
265494_at	At2g15680	*CML30*	P	0.88	0.27	0.4259	1.12	0.29	0.6806
263903_at	At2g36180	*CML31*	N (C, D, T)	3.00	3.67	0.7190	1.91	2.65	0.8268
246430_at	At5g17470	*CML32*	N (C, D, T)	2.18	3.11	0.8538	2.58	2.29	0.6226
259046_at	At3g03400	*CML33*	N (C, D, T)	0.37	0.23	0.0258	0.59	0.50	0.3111
259045_at	At3g03410	*CML34*	N (C, D, T)	0.81	0.52	0.3299	0.91	0.54	0.4294
266371_at	At2g41410	*CML35/PM129*	P	1.49	0.15	0.0187	2.19	0.17	0.0016
259143_at	At3g10190	*CML36*	N (D, T)	0.50	0.04	0.0005	1.38	0.15	0.0376
249197_at	At5g42380	*CML37*	N (C)	214.67	260.94	0.0042	26.18	32.00	0.0017
259879_at	At1g76650	*CML38*	N (C)	93.93	37.22	0.0012	32.35	13.30	0.0058
259866_at	At1g76640	*CML39*	N (C, D)	34.45	7.86	0.0035	0.93	0.34	0.6495
258947_at	At3g01830	*CML40*	P	56.93	9.85	0.0036	5.77	0.86	0.0000
252136_at	At3g50770	*CML41*	N (C)	1.73	0.26	0.0222	2.42	0.39	0.0158
254487_at	At4g20780	*CML42*	P	1.83	0.31	0.0060	1.55	0.37	0.0881
249055_at	At5g44460	*CML43*	N (C, D, T)	0.81	0.35	0.4869	1.14	0.42	0.6668
260881_at	At1g21550	*CML44*	P	4.61	1.05	0.0004	3.09	0.81	0.0090
249417_at	At5g39670	*CML46*	P	2.18	0.32	0.0135	2.86	0.54	0.0254
252417_at	At3g47480	*CML47*	P	0.63	0.19	0.0718	0.73	0.10	0.0238
265636_at	At2g27480	*CML48*	N (C, D, T)	0.96	0.14	0.6549	1.11	0.19	0.4513
259137_at	At3g10300	*CML49*	P	6.62	0.69	0.0001	1.80	0.27	0.0140
245694_at	At5g04170	*CML50*	P	1.12	0.25	0.5767	1.06	0.23	0.8944

Probe ID is the Affymetrix identifier, ORF name is the Arabidopsis Genome Initiative open reading frame name, gene name is the common abbreviation, and status indicates whether expression was detected (P, present on all nine chips; N, marginal or absent on at least one chip in the indicated sets (C, control; D, darkness; T, touch)). Expression ratios are averaged differences between control and experimental chip expression data. *P*-values were calculated using a Student's *t*-test between the measurements from the control chips and the appropriate measurements from the stimulus chips. *P*-value = 0.0000 is equivalent to *P*-value ≤ 0.0001. (SD, standard deviations.)

Table 5 XTH expression

Probe ID	ORF name	Gene name	Status	Touch ratio	SD	*P*-value	Darkness ratio	SD	*P*-value
254801_at	At4g13080	*XTH1/XTR22*	N (C, D, T)	1.97	0.82	0.1541	2.15	0.85	0.1092
254802_at	At4g13090	*XTH2/XTR23*	N (C, D, T)	0.68	0.73	0.2628	5.77	7.57	0.2953
257102_at	At3g25050	*XTH3*	N (C, D, T)	2.56	0.75	0.0148	1.21	1.50	0.8998
266215_at	At2g06850	*XTH4/EXGTA1*	P	1.04	0.12	0.7737	1.61	0.19	0.0076
250214_at	At5g13870	*XTH5/EXGTA4/XTR12*	N (C, D, T)	2.15	2.23	0.9583	3.62	3.56	0.2284
247162_at	At5g65730	*XTH6/XTR10*	P	0.54	0.11	0.0445	1.05	0.24	0.9104
253040_at	At4g37800	*XTH7/XTR15*	P	0.75	0.14	0.1116	0.82	0.17	0.1903
261825_at	At1g11545	*XTH8/XTR19*	P	0.73	0.17	0.1493	1.14	0.28	0.5555
255433_at	At4g03210	*XTH9/EXGTA6/XTR16*	P	0.60	0.16	0.0709	1.75	0.44	0.0184
266376_at	At2g14620	*XTH10/XTR14*	N (C, D, T)	1.23	0.78	0.9165	1.13	0.74	0.7569
252320_at	At3g48580	*XTH11/XTR24*	N (C, D, T)	2.43	1.36	0. 1851	2.50	1.34	0.1700
247871_at	At5g57530	*XTH12/XTR25*	N (C, D, T)	2.32	1.93	0.4083	0.71	0.20	0.1236
247914_at	At5g57540	*XTH13/XTR11*	N (C, D, T)	0.96	0.39	0.6113	0.87	0.68	0.5842
254044_at	At4g25820	*XTH14/XTR9*	N (C, D, T)	2.59	2.20	0.2249	1.77	1.94	0.8653
245325_at	At4g14130	*XTH15/XTR7*	P	0.75	0.21	0.1727	2.75	0.75	0.0047
257203_at	At3g23730	*XTH16*	P	0.75	0.16	0.1703	2.72	0.60	0.0011
264157_at	At1g65310	*XTH17/XTR1*	P	2.76	0.46	0.0258	3.61	0.61	0.0216
253628_at	At4g30280	*XTH18*	N (C)	72.45	29.59	0.0013	32.20	13.20	0.0012
253608_at	At4g30290	*XTH19*	N (C)	5.08	2.39	0.0883	3.78	1.00	0.0005
248732_at	At5g48070	*XTH20*	N (C)	1.63	0.38	0.0505	1.49	0.42	0.0996
266066_at	At2g18800	*XTH21/XTR17*	N (C, D, T)	3.89	3.34	0.3002	1.63	1.18	0.5180
247925_at	At5g57560	*XTH22/TCH4*	P	21.17	8.21	0.0000	28.52	11.00	0.0000
254042_at	At4g25810	*XTH23/XTR6*	N (C)	9.66	3.25	0.0117	21.68	8.60	0.0312
253666_at	At4g30270	*XTH24/MERI5*	P	1.20	0.16	0.2024	1.74	0.12	0.0061
247866_at	At5g57550	*XTH25/EXGTA5/XTR3*	P	2.22	0.60	0.0125	3.04	0.90	0.0073
253763_at	At4g28850	*XTH26/XTR18*	N (C, D, T)	0.62	0.41	0.3166	1.07	1.15	0.7788
263598_at	At2g01850	*XTH27/EXGTA3*	P	0.75	0.07	0.0425	1.59	0.15	0.0097
262842_at	At1g14720	*XTH28/EXGTA2/XTR2*	P	0.59	0.12	0.0175	0.86	0.17	0.2627
254598_at	At4g18990	*XTH29/XTR13*	N (C, D, T)	1.45	0.94	0.5947	1.62	0.72	0.2302
245794_at	At1g32170	*XTH30/XTR4*	P	1.35	0.35	0.2110	5.65	1.27	0.0011
252607_at	At3g44990	*XTH31/ATXG/XTR8*	P	0.61	0.17	0.0420	0.28	0.06	0.0190
263841_at	At2g36870	*XTH32/XTR20*	P	0.68	0.11	0.0475	0.80	0.24	0.2641
263207_at	At1g10550	*XTH33/XTR21*	N (T)	1.38	1.30	0.8626	4.79	3.02	0.0373

Probe ID is the Affymetrix identifier, ORF name is the Arabidopsis Genome Initiative open reading frame name, gene name is the common abbreviation, and status indicates whether expression was detected (P, present on all 9 chips; N, marginal or absent on at least one chip in the indicated sets (C, control; D, darkness; T, touch)). Expression ratios are averaged differences between control and experimental chip expression data. *P*-values were calculated using a Student's *t*-test between the measurements from the control chips and the appropriate measurements from the stimulus chips. *P*-value = 0.0000 is equivalent to *P*-value ≤ 0.0001. (SD, standard deviations.)

genes found to be altered in expression levels greater than twofold by the microarray experiments are truly altered in expression in response to touch and/or darkness.

Other touch- and darkness-regulated genes

Those genes with the highest averaged ratios of differential expression between control and touch- or darkness-treated plants are included in Tables 1 and 2. All genes altered at least twofold in expression by touch or darkness are included in Appendices S3 and S4, respectively. Complete microarray data, including data for genes whose expression is marked absent or marginal on at least one chip, are provided in Appendix S2. We summarize briefly here some prominent examples of genes belonging to the five functional classes demonstrated to be up regulated by the conditions used in our experiments. In addition to the *CAM* and *CML* genes, seven additional putative calcium-binding protein encoding genes are up regulated in expression in touch-stimulated plants, three of these are also up-regulated by darkness. Two additional calcium-binding protein genes are up regulated by darkness. Arabinogalactan protein and pectin esterase encoding genes are among the most highly represented cell wall modification genes in the touch- and touch- and darkness-induced gene sets. In addition, genes in the cellulase synthase, expansin and extensin gene families are also expression regulated, however, expression of most of the cell wall modification genes are induced less than 10-fold by touch (Table 1, Appendix S3). Eighteen genes encoding proteins with leucine-rich repeat motifs, classified as putative

Table 6. Q-PCR primers

Gene	Forward primer	Reverse primer
XTH4	GGCTTATCACACTTACTCAATCCTTTG	TTCGGATTGGTATGTTGTCAACA
XTH6	CGGTCACCGCTTTCTACATGA	CAAGAACTCAAAATCTAGCTCGTCTCT
XTH7	GATTCTGCCGGGACTGTCA	CGTCTCGTACCGAATCGGTATC
XTH8	ATAACGACACCGGATGTGGATT	TTTAGCTTCATACTAAACCATCCGAAT
XTH9	TCGATCCCACCACTGAGTTTC	TACCATGAATACAACACTGCGTTTACT
XTH15	CGGCACCGTCACTGCTTAC	GAAACTCAAAGTCTATCTCGTCATGTG
XTH16	CCGGTAACTCCGCTGGAA	TCTCGTCGTGTGTTGGTCCTT
XTH18	TCAAATGAAACTTGTCCCTGGTAA	AGTTCCCGGAGACTTAAGATAGAATG
XTH22	GAAACTCCGCAGGAACAGTC	TGTCTCCTTTGCCTTGTGTG
XTH23	GCTGGAACTGTCACCGCTTAC	TCAAAGTCAATCTCGTCCCATGT
XTH24	CTCTGCAGGAACAGTCACAACTTT	TCAAAATCAATCTCATCCCAAGTG
XTH27	CGGTTAACGCTCGATGAAAGA	GCACTGAAGAATCCATGCAAGTA
XTH28	AGTATCCTTTGGTCTCTATCTCACATCA	GCCGTACGTTTGACTTCTCTGA
XTH30	GCGTCGTCGTCGCCTTT	TGTCTAACTCGTCGTGTGTTTTCTC
XTH32	GGCTACACTGCTGGAGTCATCA	CATCATGGAACCCTGGATGTG
CML5	GATGATGAAAGGTGGTGGCTTTA	ACACTTCTCTTCTGTCAAATCAAACC
CML9	GAGTGTTCGACAGAGACGGAGA	GCTCCGCTTCTTCTGCTGTTAT
CML10	CAGGCTTATGATGGCCAAGAA	CCAAAGTGCCACCAGTTGTGT
CML12	AAGCCTTCCGCGTATTCGACAAGAA	CACAAACTCAGAGAAACTGATGGTTCC
CML13	TCTAATCTCTAAGAATGTCAAGGTTTGTCT	GTTACCACATTGGGAATCAATGAG
CML16	TGGCTAAATCGGCTGCTGAT	CACAAGTTGCATTGACAGGAGAAG
CML24	GAGTAATGGTGGTGGTGCTTGA	ACGAATCATCACCGTCGACTAA
CML27	CGTCTTCTCTCCTCAACAGCAA	TTATTCTTATTCCCGAAATCCA
CML35	TGTTTGCTTCGATGACTTTTGC	GTCACCATCATCAATCAAACATGA
CML37	TGGAGTTCTCAGCTTTGACGAG	TTGAGGAGGGAAGGAAGATGTC
CML38	TTTTGATCTCAATGCTGATGGA	CAACAACAACAACAACAACAATGG
CML40	GCCATGATGCAATAACATATTCATTAG	GAGAGTACCTTAATTTCAGAGAGTTCATACA
CML42	CAGCTGAAAATGAATCGGATCTC	GAATCCATCACCGTTCTCATCA
CML46	GGGCTTTCCACTGACCAAGA	TCGAAACCTCCTTGGAACTGTAC
CML49	ACCAACAGCAATGTCAGGAAGA	TCAAACCTAGATTGTTGAAGCAAGA
TUB4	CTGTTTCCGTACCCTCAAGC	AGGGAAACGAAGACAGCAAG

Primers are listed in 5′ to 3′ orientation.

disease resistance genes, are touch inducible, 12 of which are also darkness inducible. Two of these genes are more than 10-fold up regulated by touch and darkness. Genes identified as encoding leucine-rich repeat transmembrane kinases are among the most abundant touch-induced putative kinase genes with 14 members increased in expression by touch. Touch-induced expression is a characteristic of multiple genes encoding mitogen-activated protein kinases, calcium-dependent protein kinases (CDPKs), serine/threonine kinase, CBL-interacting protein kinases and wall-associated kinases. Many of these genes also have darkness-inducible expression. *CDPK18* and *CDPK32*, which are both touch and darkness inducible in expression, are among the most strongly up-regulated kinase-encoding genes. *CDPK18* is 13-fold up-regulated by touch and 7.5-fold up-regulated by darkness; *CDPK32* is 11-fold up regulated by touch and seven-fold up-regulated by darkness. Touch- and darkness-inducible transcription factor encoding genes include 10 members of the WRKY family, 15 others with zinc finger motifs, eight putative no apical meristem (NAM)-related proteins, seven with myb domains, two heat shock factor relatives, three with potential roles in regulating ethylene induced genes, three with bHLH domains and four with AP2 domains. Experiments to elucidate the physiological relevance of these regulatory changes will be the next important step to revealing the cellular, metabolic and physiological changes that occur in plants in response to mechanical perturbation.

Discussion

Touch sensitivity of plants

Although touch-inducible genes have been previously described, a systematic screen for genes altered in expression by touch had not been completed. Using Affymetrix chip hybridization, we report that touch-responsive gene expression is remarkably widespread. Expression of over 2.5% of the genome is up regulated at least twofold in plants subjected to a simple touch stimulation (Appendix S3). This finding has broad implications. First, it reveals the potential importance of mechano-sensitivity of plants. The original isolation of the five original Arabidopsis *TCH* genes was accomplished through differential cDNA screening (Braam & Davis, 1990), a laborious technique that tends to result in preferential

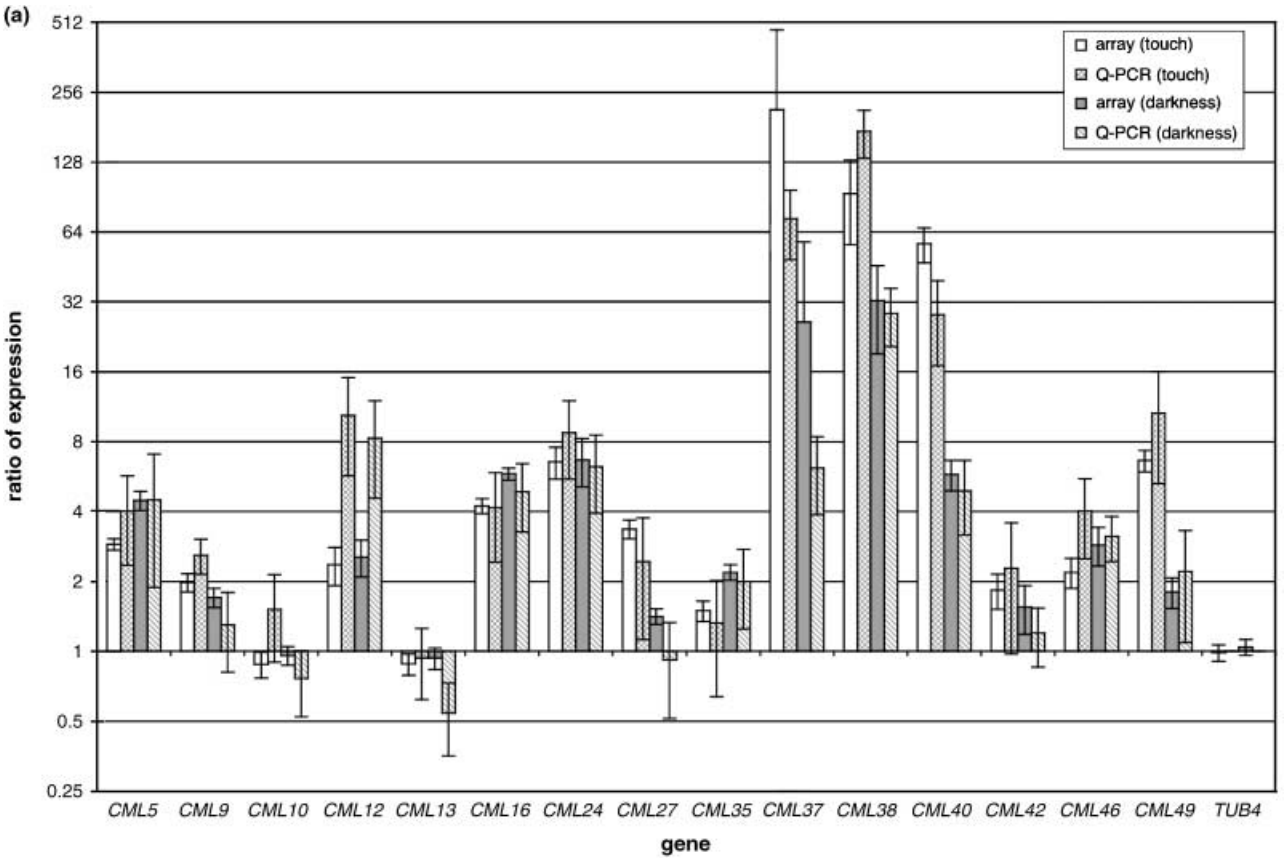

Fig. 3 Expression induction and repression of *CMLs* and *XTHs* in touched and darkness-treated *Arabidopsis thaliana* plants as assessed by microarray and Q-PCR. Expression of each gene is shown with four bars which, from left to right, denote: expression ratios of touched vs control plants as calculated from microarray (solid white), touched vs control plants as calculated from Q-PCR (gray checkerboard), darkness-treated vs control plants as calculated from microarray (solid gray), and darkness-treated vs control plants as calculated from Q-PCR (gray stripes). (a) *CMLs*; (b) *XTHs*. Error bars represent standard deviations. Three biological replicates resulted in nine pair-wise comparisons for each analysis.

identification of highly expressed genes. The differential screen was not conducted to saturation and identified only a very small subset of the touch-inducible genes in the Arabidopsis genome. Indeed, even some very highly touch-inducible genes identified by the microarray analyses and verified by Q-PCR, such as *CML37* and *CML38* that are induced over 60-fold by touch (Table 4 and Fig. 3), had not been previously identified.

These results are also evidence that researchers should be aware that common laboratory manipulation can have potentially large effects on gene expression and thus can confound genome-wide transcript profiling, in addition to other types of analyses. These expression changes can occur very quickly and can be of high magnitude. Over 60 genes were found to have at least 10-fold expression increases within 30 min of mechanical perturbation (Table 1, Appendix S3).

Touch-inducible genes

Based on the sequence identities of previously identified touch-inducible genes, it is not surprising to find that genes encoding calcium-binding proteins and cell wall modifying proteins make up a significant proportion of the total touch-regulated gene set (Table 1, Appendix S3) because of previously determined identities of the original *TCH* genes (Braam & Davis, 1990). It is less expected, however, that the third most represented functional class of genes in the touch-regulated set is the class with potential roles in disease resistance (Table 1, Appendix S3). It will be interesting to further investigate the potential role of mechanical perturbation responses in disease resistance in plants.

Genes encoding transcription factors and kinases are among those highly regulated at the transcriptional level by many stimuli. We find that 66 of the 634 transcription factor and 72 of the 613 kinase genes with detectable expression on all nine chips have increased transcript levels in touched plants (Table 1, Appendix S3).

Relationship between Touch and Darkness

Regulation of *TCH* expression is unusual in that these genes are not only responsive to mechanical perturbation but also

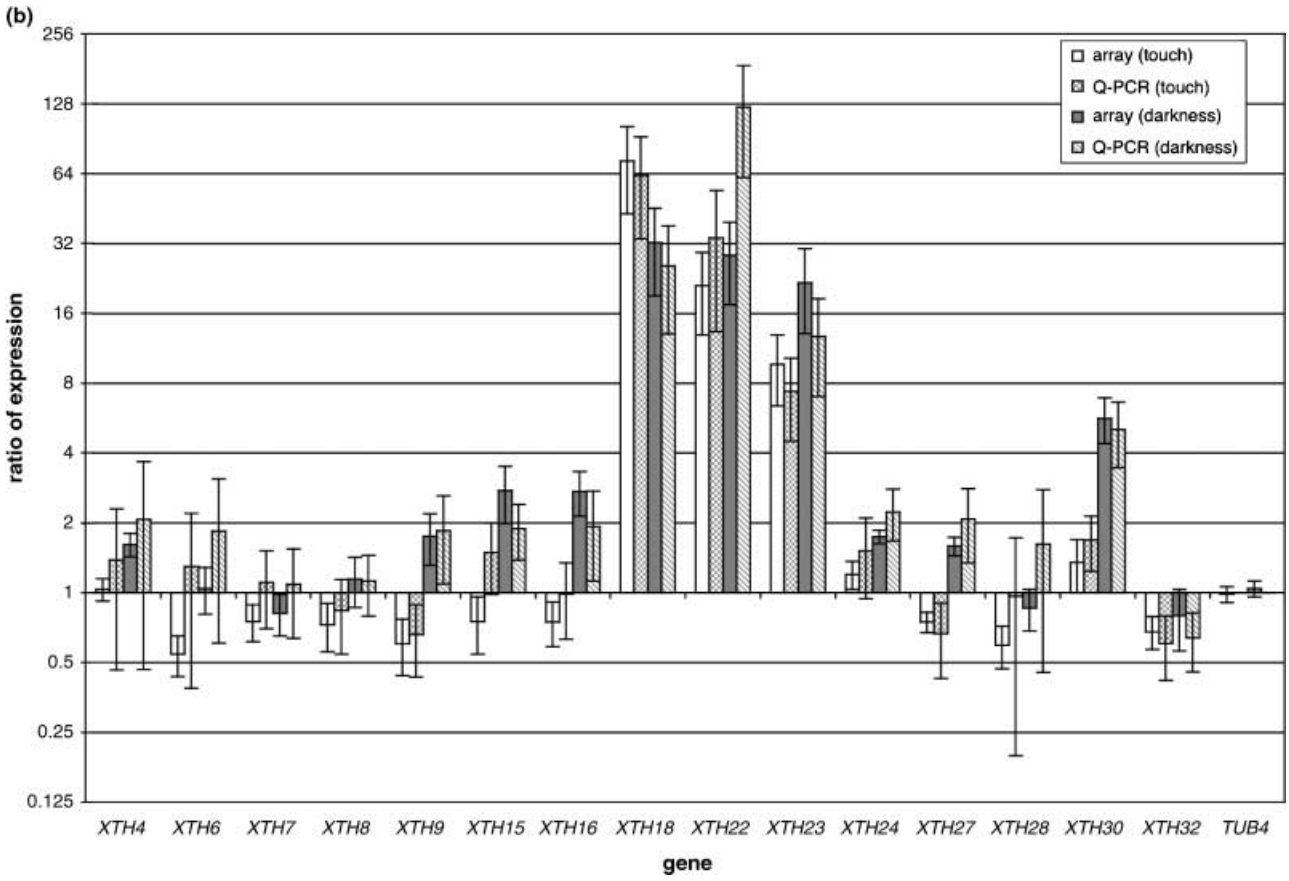

Fig. 3 continued

to diverse stimuli such as darkness, cold, heat, and some hormones (Braam & Davis, 1990; Braam, 1992; Sistrunk *et al.*, 1994; Antosiewicz *et al.*, 1995; Xu *et al.*, 1995; Polisensky & Braam, 1996). This latter group of stimuli might be predicted to be unrelated in properties, and thus in perception mechanisms, to touch. Alternatively, it may be that all the stimuli capable of inducing *TCH* expression may cause mechanical perturbations and thus lead to *TCH* expression up regulation indirectly (Braam, 2000). To begin to differentiate between these possibilities, we sought to determine whether other touch-inducible genes behave like the *TCH* genes in also being up-regulated in response to darkness. Remarkably, there is a strong correlation between touch and darkness inducibility. 52.6% of genes at least twofold up regulated by touch are also up-regulated by darkness (Table 1, Appendix S3); 67.2% of genes at least twofold up-regulated by darkness are also up-regulated by touch (Table 1, Appendix S4). Furthermore, all but four of the 60 genes that have greater than 10-fold increased expression in touch-stimulated plants are also darkness inducible (Table 2, Appendix S3). Of the 68 most strongly darkness up-regulated genes, only three are not touch inducible (Table 2, Appendix S4). These results strongly suggest a relationship between either the perception of these two seemingly unrelated stimuli or the signal transduction pathways that they activate. One possibility is that the darkness stimulus results in mechanical perturbations. This may occur through turgor changes, possibly as a consequence of darkness-inducible closure of stomata or through an indirect effect on humidity.

Future experiments are aimed toward determining whether coregulated genes share sequence motifs that may function as touch- and/or darkness-inducible regulatory elements. In addition, it will be interesting to determine if the genes found to be both touch and darkness responsive are also up-regulated in response to the other stimuli that induce *TCH* gene expression, including heat and cold shock.

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Supplementary Material

The following material is available as Supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1238/NPH1238sm.htm

Appendix S1 Source code to the software utility that averages intensity values from the individual cells on the chip to their neighbors

Appendix S2 Full list of genes inducible by both touch and darkness

Appendix S3 Full list of genes with increased and decreased expression levels in response to touch

Appendix S4 Full list of genes with increased and decreased expression levels in response to darkness

Appendix S5 Categorizations of the full 22 810-gene complements

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