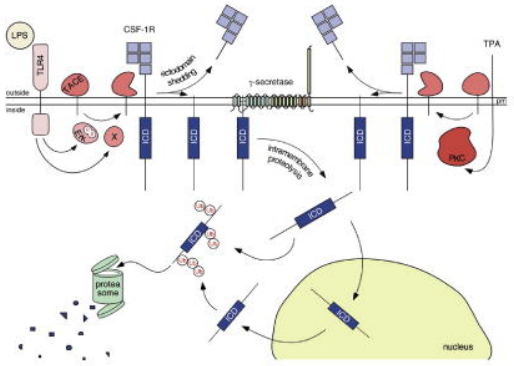
FEBS Letters

The journal for rapid publication
of short reports in molecular biosciences

- Spotlight on… Sandro Sonnino
- A TOM-independent mitochondrial OM insertion pathway
- Toll-like receptors stimulate RIPping



Published by Elsevier on behalf of the Federation of European Biochemical Societies

Submit to:
http://www.ees.elsevier.com/febsletters

ISSN 0014 5793
Volume 582 Number 6 19 March 2008

FEBS Letters 582 (2008) 967–976

Transcriptional changes in response to growth of *Arabidopsis* in high external calcium

Catherine W.M. Chan[a,b,*,1], Dana J. Wohlbach[c,1,2], Matthew J. Rodesch[c,3], Michael R. Sussman[c]

[a] *University of Wisconsin–Whitewater, Department of Biological Sciences, 800 W. Main Street, Upham Hall Room 209, Whitewater, WI 53190, United States*
[b] *University of Wisconsin–Whitewater, Department of Chemistry, 800 W. Main Street, Upham Hall Room 209, Whitewater, WI 53190, United States*
[c] *University of Wisconsin–Madison, Department of Biochemistry, Madison, WI, United States*

Received 5 December 2007; revised 15 February 2008; accepted 18 February 2008

Available online 26 February 2008

Edited by Julian Schroeder

Abstract **Transcriptional responses to growth in high environmental calcium concentrations were characterized and compared between wild-type and mutant *Arabidopsis* plants containing a knockout mutation in the gene encoding a cyclic nucleotide-gated channel (CNGC2). We show that the transcriptional profile of *cngc2* plants grown in normal media resembled that from wild-type plants grown under elevated exogenous calcium conditions. The mutant grown in high-calcium media exhibited transcriptional changes not seen in the wild-type. The pattern of transcription suggests that adaptation to high external calcium overlaps with responses towards various biotic and abiotic stresses.**

Keywords: Cyclic nucleotide; Ion channel; Calcium; Gene expression; Abiotic stress; Biotic stress

1. Introduction

A wealth of physiological, biochemical and genetic data suggest that calcium is an important signaling molecule in plants (for example [1,2]). Given its importance, it is reasonable to hypothesize that plants regulate calcium uptake according to both external and internal concentrations and specific needs. Recent data indicate that the mechanism by which plants adapt to high external calcium concentrations depends on the function of CNGC2 (cyclic nucleotide-gated channel 2, also called DND1 [3]). Growth studies with varying media compositions demonstrate that *cngc2* knockout mutants are specifically sensitive to increases in external calcium [4]. *cngc2* plants grown in Murashige and Skoog (MS) medium supplemented with as little as 10 mM additional calcium are stunted in growth and have significantly reduced seed yield. A calcium concentration of approximately 10 mM is commonly present in many natural plant growth environments (for example [5]) and therefore, the phenotype of *cngc2* mutants is relevant to in situ physiological conditions. CNGC2 can mediate cyclic nucleotide-dependent calcium influx when expressed in a heterologous system [6], and the *cngc2* mutation causes a loss of a cyclic adenosine monophosphate (cAMP)-induced inward calcium current in guard cell protoplasts [7]. Therefore, CNGC2 is likely to be a physiological calcium influx pathway in *Arabidopsis*.

cngc2 mutant plants also show altered pathogen responses. Their constitutively elevated levels of salicylic acid (SA) correlate with broad spectrum disease resistance that is not accompanied by the hypersensitive response (HR) [3]. Current data suggest that CNGC2-mediated calcium influx generates nitric oxide (NO) which, in turn, is required for HR [7]. SA has been shown to be important in systemic acquired resistance, pathogenesis-related (PR) gene expression and other defense responses [8] although the underlying reason(s) for elevated SA in *cngc2* is still not clear.

To better understand the biological response to growth in high external calcium concentrations and the contribution of CNGC2, we compared the global transcriptional profiles of *cngc2* mutant and wild-type plants grown under normal and elevated calcium conditions. We identified significant changes in transcript levels that were associated with: (1) growth of the *cngc2* mutant under normal conditions (*cngc2*/*WS*), yielding visibly normal *cngc2* plants; (2) wild-type responses to elevated calcium (*WS* + Ca^{2+}/*WS*), in which calcium supplementation did not cause any visible growth differences; and (3) growth of the *cngc2* mutant in increased calcium concentration (*cngc2* + Ca^{2+}/*WS* + Ca^{2+}). Mutant plants in this condition were approximately half the weight of the wild-type (refer to [4] for pictures of representative plants). We used a combination of two different approaches to analyze our data. The first utilized statistical analysis to help pinpoint significant changes in each of the three comparisons, and the second relied on hierarchical clustering of large amounts of microarray data to identify similarities in global transcriptional patterns. Our results indicate that the transcriptional response of wild-type plants grown in high external calcium is very similar to that from various biotic and abiotic stresses. Moreover, the transcriptional profile of *cngc2* mutants

*Corresponding author. Fax: +1 262 472 5111.
E-mail address: chanc@uww.edu (C.W.M. Chan).

[1]These authors contributed equally to this paper.
[2]Present address: University of Wisconsin–Madison, Department of Genetics, United States.
[3]Present address: Roche Nimblegen, 1 Science Court, Madison, WI 53711, United States.

Abbreviations: ABC, ATP binding cassette; ACA, Arabidopsis calcium ATPase; AHA, Arabidopsis H^+-ATPase; cAMP, cyclic adenosine monophosphate; CAX, calcium exchanger; CNGC, cyclic nucleotide-gated channel; GLR, glutamate receptor; IRT, iron-regulated transporter; MS, Murashige and Skoog; NO, nitric oxide; PR, pathogenesis-related; qRT-PCR, semi-quantitative reverse transcription polymerase chain reaction; RMA, robust multichip average; SA, aalicylic acid; *WS*, *Wassilewskija*

0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.043

grown under normal conditions closely matches that of the wild-type grown under elevated external calcium. Additional changes in genes responsible for ion homeostasis, hormone-signaling and protein phosphorylation occur when mutants are grown in calcium-supplemented media, implying that CNGC2 affects stress adaptation.

2. Materials and methods

Three week old *Arabidopsis thaliana cngc2-2* and *Wassilewskija* (*WS*) plants were used for our experiments. Standard protocols were used to prepare total RNA for hybridization to Affymetrix ATH1 DNA Microarrays. Each microarray experiment was repeated four times with independent biological samples. Raw hybridization data were pre-processed with robust multichip average (RMA) [9]. Hierarchical clustering was performed using the average linkage method [10], and one-way ANOVA were applied to RMA processed signal intensity values [11]. Semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments were performed with iCycler Real Time PCR System (Bio-Rad, Hercules, California) following standard protocols. This experiment was performed three times, each with an independent biological sample. Detailed description of all materials and methods used are available in Supplementary Materials and Methods section.

3. Results

3.1. Transcriptional changes in cngc2 in control media

Although the *cngc2* mutant plants were not visibly different from the wild-type under the control growth condition, changes in the expression of calcium-signaling and homeostasis genes were evident even in the absence of stress. Table 1 shows the genes that were significantly up- and down-regulated in *cngc2*/*WS* (defined here as fold changes of $\geqslant 2$ and $P \leqslant 0.05$ by Student's *t*-test. Genes that had substantial but statistically less reliable differences, i.e. fold changes of $\geqslant 2$ but $P > 0.05$, are listed in Supplementary Table S1). These include a calmodulin-related gene (At5g42380) and a plasma-membrane calcium-ATPase (*ACA13*, At3g22910). Some auxin-responsive genes were also present (At2g33830 and At1g29430/At5g27780), implying altered auxin-signaling in *cngc2*. Such changes in calcium and auxin-signaling may pre-dispose the *cngc2* mutant to calcium-hypersensitivity and stunted growth.

Considering all the genes that showed $\geqslant 2$-fold change in *cngc2*/*WS* (Tables 1 and S1), we found that genes with defense and transport functions are enriched (Fig. 1A). Some of these transcriptional changes may be an indirect effect of the *cngc2* mutation. For example, the enrichment of defense genes is consistent with constitutively elevated levels of SA and pathogen defenses in the mutant (see Section 1), and high concentrations of SA in *cngc2* can explain the observed up-regulation of PR genes (e.g. PR1 and PR5 in Table 1).

3.2. Normal transcriptional changes in response to growth in high external calcium

We next analyzed the wild-type response to growth in elevated levels of calcium. Table 2 lists the genes that were significantly up- and down-regulated in $WS + Ca^{2+}/WS$ (genes with substantial but statistically less reliable changes are listed in Supplementary Table S2). Interestingly, the expression level of *CNGC2* did not change appreciably during calcium-supplemented growth in the wild-type ($WS + Ca^{2+}/WS = 1.18$), suggesting that adaptation to elevated external calcium does not involve transcriptional regulation of CNGC2. We also observed substantial overlap in genes listed in Tables 1 and 2 (highlighted items), suggesting that the transcriptional response of the mutant grown in control media is similar to that of the wild-type in calcium-supplemented media, an interesting point that will be further elaborated in a later section.

Of all the genes in Table 2, only *CAX3* (At3g51860) is clearly related to calcium homeostasis. CAX3, a calcium antiporter in the tonoplast, is most abundant in roots and its expression increases upon overnight exposure to exogenous calcium. *cax3* mutants are also marginally sensitive to increased external calcium although the symptoms are much less severe than *cngc2* [12]. Therefore, current data suggest that up-regulation of *CAX3* is needed for adapting to both short-term and long-term calcium exposure. Unexpectedly, one of the larger changes in $WS + Ca^{2+}/WS$ was in *IRT1* (At4g19690), an iron transporter that is necessary for iron and zinc homeostasis and efficient photosynthesis [13]. This result suggests a previously unknown connection between elevated environmental calcium and a need for increased iron uptake. Interestingly, the transcript levels of *CAX3* and *IRT1* did not change significantly in *cngc2*/*WS*. However, it remains to be determined whether changes in activities of CAX3 and IRT1 (and perhaps other yet-to-be characterized proteins) are responsible for the altered calcium sensitivity in the mutant.

If we consider all of the genes that showed $\geqslant 2$-fold change in $WS + Ca^{2+}/WS$ (Tables 2 and S2), metabolism, defense and transport genes are enriched when compared with their overall representation in the genome (Fig. 1B), suggesting that adjustments in these functions are necessary for adaptive growth in high calcium environments.

3.3. Transcriptional changes in cngc2 in calcium-supplemented media

We then determined the differences between the wild-type and mutant in their responses to growth in high external calcium. Table 3 lists the genes that were significantly up- and down-regulated in $cngc2 + Ca^{2+}/WS + Ca^{2+}$ (those with substantial but statistically less reliable changes are listed in Supplementary Table S3), and we compared them to the changes observed for *cngc2*/*WS* and $WS + Ca^{2+}/WS$ (listed in Tables 1 and 2, respectively). This analysis shows that protein kinases are abundant among the up-regulated genes (29%) and auxin-responsive genes are common among the down-regulated genes (23%) in $cngc2 + Ca^{2+}/WS + Ca^{2+}$. Interestingly, most of these changes were unique to the mutant's response to calcium (see Table 3 – iv), suggesting that the inability of the mutant to adapt to calcified environments may be partly due to aberrant phosphorylation-dependent and auxin-signaling events.

Notably, the mutant up-regulated *IRT1* to a lesser extent than the wild-type (−2.84-fold, see Table 3 – ii). As discussed earlier, *IRT1* was among the largest transcriptional changes observed in $WS + Ca^{2+}/WS$ (see Table 2). The insufficient up-regulation of *IRT1* in the mutant is consistent with our model in which this gene contributes to the stunted growth of the mutant under high external calcium conditions.

CAX1 (At2g38170) exhibited the second largest reduction in expression in the calcium-stressed mutant (Table 3 – iv). This reduction resulted from a combined effect of marginal down-regulation in the mutant under control conditions (*cngc2*/

Table 1
Significant Changes in *cngc2*/*WS*

Locus identifier	Gene description	Category	*cngc2* / *WS*		*WS*		*cngc2*	
			p-value	Linear fold change	Log_2 average signal	SD (Log_2 Avg.)	Log_2 average signal	SD (Log_2 Avg.)
At3g57260	BGL2 glycosyl hydrolase family 17 protein	1,2	0.0001	**5.36**	7.01	2.23	9.43	1.21
At1g75040	*PR5 pathogenesis-related protein 5*	*2*	*0.0002*	***3.74***	*7.68*	*3.29*	*9.58*	*2.21*
At5g10760	aspartyl protease family protein	7	0.0004	**2.94**	7.01	1.81	8.57	0.91
At2g41730	expressed protein	0	0.0004	**2.78**	6.89	1.55	8.37	0.43
At2g03760	steroid sulfotransferase, putative	1,2	0.0006	**2.77**	7.29	0.92	8.76	0.41
At2g18660	EXPR3 expansin family protein	0	0.0006	**2.77**	5.47	2.32	6.94	2.18
At2g05540	glycine-rich protein	0	0.2920	**2.65**	5.56	0.53	6.96	1.26
At2g14560	expressed protein	0	0.0004	**2.61**	7.85	1.61	9.23	1.37
At5g52760	heavy-metal-associated domain-containing protein	5,6	0.0005	**2.60**	6.31	1.90	7.69	1.37
At1g60740 At1g65970	peroxiredoxin type 2, putative	2	0.0021	**2.56**	5.98	0.51	7.33	1.35
At2g14610	PR1 pathogenesis-related protein 1	2	0.0005	**2.54**	9.06	3.52	10.40	2.14
At5g42380	calmodulin-related protein, putative	6	0.0020	**2.43**	4.29	0.71	5.57	0.92
At1g14870 At1g14880	expressed protein	0	0.0001	**2.35**	9.14	0.63	10.37	1.07
At4g37370	CYP81D8 cytochrome P450, putative	5	0.0004	**2.28**	5.97	0.69	7.16	0.31
At2g33830	dormancy/auxin associated family protein	0	0.0415	**2.24**	9.83	0.86	11.00	1.41
At1g21100	O-methyltransferase, putative	1	0.0083	**2.23**	5.90	0.98	7.05	0.45
At3g26210	CYP71B23 cytochrome P450 71B23	5	0.0004	**2.20**	7.10	1.63	8.24	1.24
At2g46400	WRKY46 WRKY family transcription factor	3	0.0009	**2.18**	7.01	1.56	8.13	0.82
At4g23140 At4g23160	RLK5 receptor-like protein kinase 5	1,4	0.0001	**2.17**	5.37	1.20	6.49	0.88
At4g04490	protein kinase family protein	1,4	0.0009	**2.17**	4.08	0.59	5.20	0.51
At4g14365	zinc finger (C3HC4-type RING finger) family protein	6	0.0017	**2.16**	7.95	1.92	9.06	1.19
At3g56710	SIB1 sigA-binding protein	6	0.0039	**2.15**	7.55	1.40	8.65	0.90
At2g26560	patatin, putative	1	0.0014	**2.14**	7.97	0.93	9.07	0.97
At3g50930	AAA-type ATPase family protein	1	0.0016	**2.13**	6.80	1.30	7.89	0.56
At1g19250	flavin-containing monooxygenase family protein	2,5	0.0046	**2.11**	5.68	1.03	6.76	1.81
At4g39670	expressed protein	0	0.0023	**2.11**	5.83	0.74	6.91	1.32
At4g04500	*protein kinase family protein*	*1,4*	*0.0004*	***2.09***	*5.57*	0.33	*6.63*	0.41
At1g32350	alternative oxidase, putative	5	0.0041	**2.09**	6.17	0.92	7.24	1.00
At2g43570	chitinase, putative	1	0.0003	**2.08**	7.29	2.05	8.34	1.94
At3g46220 At3g46230	expressed protein	0	0.0332	**2.05**	5.10	0.03	6.14	0.08
At2g40750	WRKY54 WRKY family transcription factor	3	0.0005	**2.03**	6.41	1.18	7.42	0.96
At2g30750	CYP71A12 cytochrome P450 71A12	5	0.0002	**2.02**	4.96	1.03	5.97	0.80
At3g22910	calcium-transporting ATPase, plasma membrane-type, putative	5	0.0011	**2.01**	5.70	0.78	6.71	0.74
At2g20870	cell wall protein precursor, putative	0	0.0028	**-2.43**	9.23	0.97	7.95	1.10
At4g20420	tapetum-specific protein-related	0	0.0078	**-2.35**	7.33	0.90	6.09	0.20
At5g13930	CHS chalcone synthase / naringenin-chalcone synthase	1,2	0.0412	**-2.32**	9.96	0.39	8.74	1.31
At1g73600	phosphoethanolamine N-methyltransferase 3, putative	1	0.0035	**-2.27**	7.82	0.65	6.63	0.99
At5g22430	expressed protein	0	0.0013	**-2.27**	7.33	1.12	6.14	1.12
At3g51590	LTP12 lipid transfer protein, putative	5,6	0.0398	**-2.21**	7.15	1.84	6.00	0.92
At5g07550	GRP19 glycine-rich protein	1,6	0.0264	**-2.20**	6.26	1.90	5.13	1.10
At1g29430 At5g27780	auxin-responsive family protein	2	0.0002	**-2.17**	6.71	0.76	5.59	0.87
At1g75940	ATA27 glycosyl hydrolase family 1 protein	1	0.0222	**-2.16**	4.87	1.42	3.76	0.63
At5g16920	expressed protein	0	0.0328	**-2.09**	5.64	1.24	4.57	0.47
At5g08640	FLS flavonol synthase 1	1	0.0301	**-2.08**	7.77	0.71	6.71	0.88
At1g62500	protease inhibitor/seed storage/lipid transfer protein family protein	5,6	0.0239	**-2.08**	7.97	1.04	6.91	0.61
At5g33370	GDSL-motif lipase/hydrolase family protein	1	0.0005	**-2.08**	8.74	0.43	7.68	0.77
At3g08770	LTP6 lipid transfer protein 6	5,6	0.0008	**-2.04**	10.58	0.34	9.55	0.77
At1g67990	caffeoyl-CoA 3-O-methyltransferase, putative	1	0.0335	**-2.00**	5.70	1.54	4.71	0.85

Only genes with fold changes of $\geqslant 2$ (down-regulated genes are indicated by a negative fold-change value) and $P \leqslant 0.05$ are shown. *P*-values reflect the statistical significance of differences in log_2 average signal intensities between *WS* and *cngc2*. 33 genes were significantly up-regulated and 15 were significantly down-regulated. The highlighted entries are common between Tables 1 and 2. Italicized entries are those used in qRT-PCR validation experiments, and the functional categories (based on the MIPS database) are: Unclassified (0), Metabolism (1), Defense (2), Transcription (3), Signal Transduction (4), Transport (5), Binding (6), Protein Fate (7), Energy (8). *CNCG2* (not listed here) actually showed the largest apparent decrease (−10.12, $P = 6.16E{-}08$), as expected for a *CNGC2* null mutant.

WS = −1.84-fold) and a failure in induction when the mutant was grown in calcium-supplemented media (*cngc2* + Ca^{2+}/*cngc2* = −1.14). By contrast, wild-type actually showed a nominal induction in *CAX1* of 1.78-fold when grown in elevated calcium. CAX1 belongs to the same family of tonoplast calcium antiporters as does CAX3, but exhibits predominant

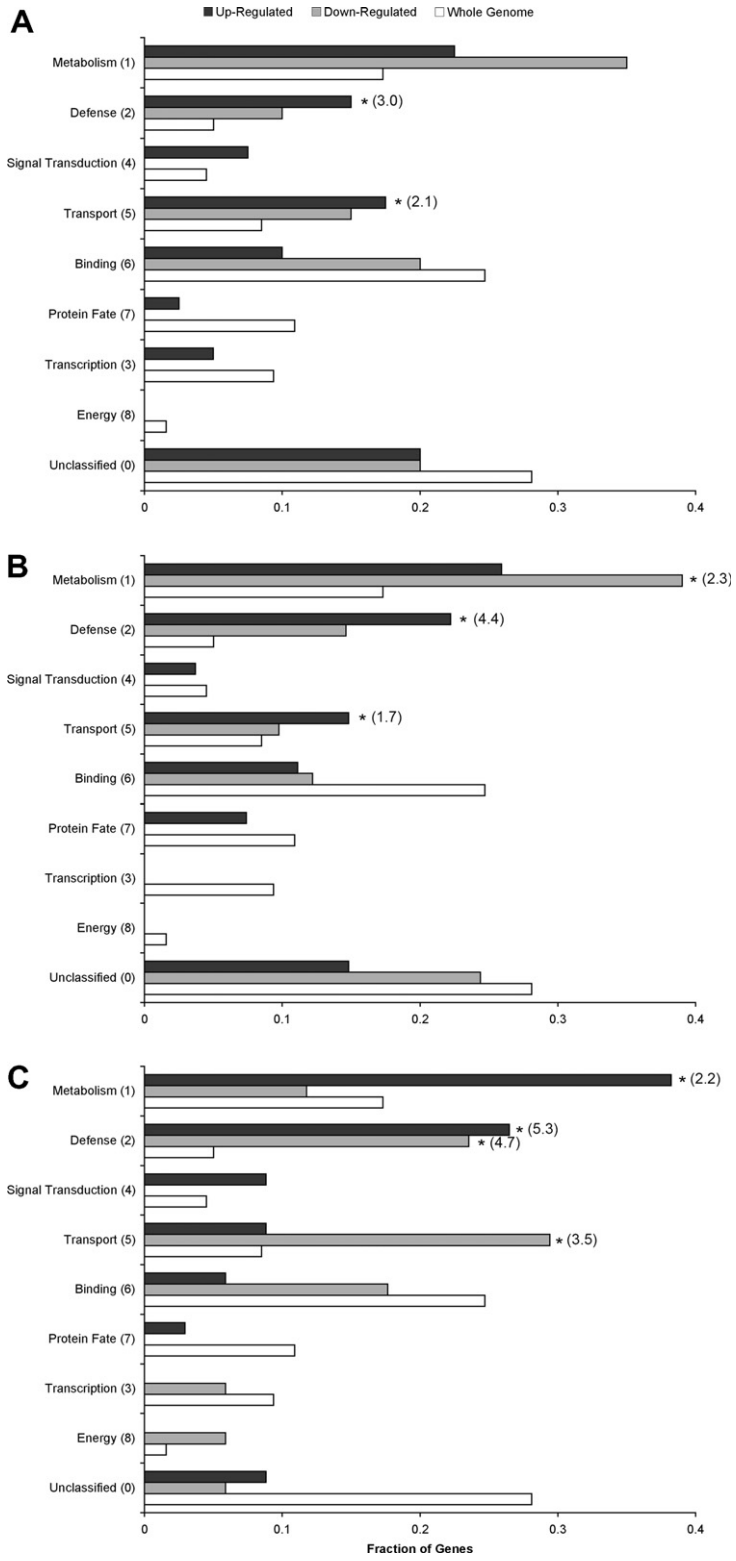


Fig. 1. Functional categories of up- and down-regulated genes and their distribution. The MIPS Functional Category Database (http://mips.gsf.de/projects/funcat) was used to assign broad functional categories to genes that are up- or down-regulated in each of our experimental comparisons, and their distribution is shown here. (A) *cngc2*/*WS*, (B) *WS* + Ca^{2+}/*WS*, (C) *cngc2* + Ca^{2+}/*WS* + Ca^{2+}. * indicates that a particular category is statistically enriched ($p < 0.01$) over the entire genome, and the fold enrichment is indicated beside the corresponding bar in parentheses. Enrichment of a functional category was determined using the hypergeometric function, as implemented by the MIPS FunCatDB website. Note that the functional category assignments are not mutually exclusive.

expression in leaves [12]. Its expression is induced about 12-fold when exposed to 80 mM $CaCl_2$ for 16 h [14], and *cax1*/*cax3* double mutant plants display more severe calcium-sensitivity than either of the single mutants [12]. Insufficient

Table 2
Significant changes in $WS + Ca^{2+}/WS$

Locus identifier	Gene description	Category	$WS + Ca^{2+}$ / WS		WS		$WS + Ca^{2+}$	
			p-value	Linear fold change	Log_2 average signal	SD (Log_2 Avg.)	Log_2 average signal	SD (Log_2 Avg.)
At3g57260	BGL2 glycosyl hydrolase family 17 protein	1,2	0.0002	**7.49**	7.01	2.23	9.91	1.44
At4g19690	IRT1 iron-responsive transporter	5	1.31E-05	**4.74**	5.14	0.57	7.39	1.01
At5g10760	aspartyl protease family protein	7	0.0016	**3.79**	7.01	1.81	8.93	0.93
At2g05540	glycine-rich protein	0	0.0029	**3.26**	5.56	0.53	7.26	1.23
At1g75040	*PR5 pathogenesis-related protein 5*	*2*	*0.0101*	***3.18***	*7.68*	*3.29*	*9.35*	*1.81*
At2g18660	EXPR3 expansin family protein	0	0.0164	**2.60**	5.47	2.32	6.85	1.12
At3g50770	calmodulin-related protein, putative	6	0.0121	**2.59**	5.76	0.71	7.14	1.92
At3g51860	CAX3 cation exchanger, putative	1,5	0.0066	**2.58**	7.52	0.17	8.89	1.58
At5g10380	zinc finger (C3HC4-type RING finger) family protein	6	0.0029	**2.53**	8.26	0.48	9.60	1.29
At5g20230	ATBCB plastocyanin-like domain-containing protein	2,5	0.0219	**2.23**	7.21	1.45	8.37	0.75
At5g24530	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1	0.0080	**2.22**	7.99	0.78	9.14	0.70
At4g25000	AMY1 alpha-amylase / 1,4-alpha-D-glucan glucanohydrolase	1,2	0.0213	**2.19**	5.64	0.17	6.77	1.46
At3g45860	receptor-like protein kinase, putative	1,2	0.0044	**2.18**	6.28	0.71	7.41	0.89
At5g57550	XTH3 xyloglucan:xyloglucosyl transferase	1	0.0418	**2.15**	5.70	0.28	6.80	0.76
At1g26250	unknown protein	0	0.0129	**2.07**	5.81	0.64	6.86	0.85
At2g16660	nodulin family protein	0	0.0010	**2.07**	8.31	0.56	9.36	0.24
At4g15100	SCPL30 serine carboxypeptidase S10 family protein	7	0.0134	**2.04**	4.72	0.13	5.74	1.25
At5g55450	protease inhibitor/seed storage/lipid transfer protein family protein	5,6	0.0098	**2.03**	8.09	1.70	9.11	0.81
At4g33720	pathogenesis-related protein, putative	2	0.0077	**2.03**	5.72	0.84	6.74	1.91
At4g23140 At4g23160	RLK5 receptor-like protein kinase 5	1,4	0.0031	**2.01**	5.37	1.20	6.38	0.93
At1g57750	cytochrome P450, putative	5,6	0.0029	**-4.03**	7.29	1.73	5.27	1.75
At3g51590	LTP12 lipid transfer protein, putative	5,6	0.0254	**-2.71**	7.15	1.84	5.71	1.13
At5g07550	GRP19 glycine-rich protein	1,6	0.0257	**-2.68**	6.26	1.90	4.84	0.94
At5g59320	LTP3 lipid transfer protein 3	2,6	0.0258	**-2.51**	9.54	1.55	8.21	1.13
At1g14960	major latex protein-related / MLP-related	0	0.0024	**-2.43**	8.60	0.71	7.33	0.57
At4g11190	disease resistance-responsive family protein	1,2	0.0078	**-2.38**	8.23	0.80	6.97	0.70
At1g75940	ATA27 glycosyl hydrolase family 1 protein	1	0.0158	**-2.34**	4.87	1.42	3.65	0.66
At3g57010	strictosidine synthase family protein	1	0.0004	**-2.33**	7.03	0.77	5.81	0.38
At5g22430	expressed protein	0	0.0194	**-2.30**	7.33	1.12	6.13	1.41
At5g46890 At5g46900	protease inhibitor/seed storage/lipid transfer protein family protein	5,6	0.0037	**-2.27**	9.13	1.36	7.95	0.97
At1g76680 At1g76690 At1g76700	OPR1 12-oxophytodienoate reductase	1,2	0.0366	**-2.26**	10.26	0.26	9.08	1.28
At4g20420	tapetum-specific protein-related	0	0.0036	**-2.23**	7.33	0.90	6.17	0.35
At1g73600	phosphoethanolamine N-methyltransferase 3, putative	1	0.0102	**-2.23**	7.82	0.65	6.66	0.89
At1g68620	expressed protein	0	0.0244	**-2.22**	6.86	1.31	5.71	0.44
At3g45070 At3g45080	sulfotransferase family protein	1	0.0099	**-2.21**	5.41	0.85	4.26	0.68
At2g20870	cell wall protein precursor, putative	0	0.0362	**-2.21**	9.23	0.97	8.09	1.35
At1g67990	caffeoyl-CoA 3-O-methyltransferase, putative	1	0.0362	**-2.20**	5.70	1.54	4.57	0.83
At1g18870	isochorismate synthase, putative / isochorismate mutase, putative	1	0.0036	**-2.18**	6.82	1.07	5.70	0.47
At5g63660	PDF2.5 plant defensin-fusion protein, putative	2	0.0041	**-2.18**	7.57	0.43	6.45	0.34
At3g15400	ATA20 anther development protein, putative	0	0.0439	**-2.15**	7.37	1.60	6.27	0.99
At5g26260	meprin and TRAF homology domain-containing protein	0	0.0150	**-2.14**	6.81	1.62	5.72	0.28
At3g62680	PRP3 proline-rich family protein	0	0.0211	**-2.14**	8.76	1.08	7.66	0.68
At1g21110 At1g21120	O-methyltransferase, putative	1	0.0095	**-2.13**	4.78	0.38	3.69	0.19
At4g02290	glycosyl hydrolase family 9 protein	1	0.0037	**-2.12**	8.75	0.52	7.66	0.83
At5g13930	CHS chalcone synthase / naringenin-chalcone synthase	1,2	0.0724	**-2.10**	9.96	0.39	8.89	1.44
At3g51720	expressed protein	0	0.0010	**-2.09**	7.30	0.26	6.24	0.72
At1g02520 At1g02530	multidrug resistance P-glycoprotein, putative	1,5	0.0382	**-2.07**	4.90	0.96	3.85	0.18
At5g37990	similar to S-adenosyl-L-methionine:carboxyl methyltransferase	1	0.0161	**-2.05**	8.12	0.68	7.08	0.58
At5g16920	expressed protein	0	0.0353	**-2.04**	5.64	1.24	4.61	0.76
At4g26010	peroxidase, putative	2	0.0402	**-2.03**	7.59	0.99	6.56	0.94
At2g23540	GDSL-motif lipase/hydrolase family protein	1	0.0319	**-2.01**	7.90	0.43	6.89	0.90
At1g75910	EXL4 family II extracellular lipase 4	1	0.0369	**-2.00**	6.58	1.32	5.58	0.43

Only genes with fold changes of $\geqslant 2$ (down-regulated genes are indicated by a negative fold-change value) and $P \leqslant 0.05$ are shown. *P*-values reflect the statistical significance of differences in log_2 average signal intensities between *WS* and $WS + Ca^{2+}$. 20 genes were significantly up-regulated and 32 were significantly down-regulated. The highlighted entries are common between Tables 1 and 2. Italicized entries are those used in qRT-PCR validation experiments, and the functional categories (based on the MIPS database) are: Unclassified (0), Metabolism (1), Defense (2), Transcription (3), Signal Transduction (4), Transport (5), Binding (6), Protein Fate (7), Energy (8).

Table 3
Significant changes in *cngc2* + Ca^{2+}/*WS* + Ca^{2+}

Locus identifier	Gene description	Category	*cngc2* + Ca^{2+}/ *WS* + Ca^{2+}		*WS* + Ca^{2+}		*cngc2* + Ca^{2+}	
			P-value	Linear fold change	Log_2 average signal	SD (Log_2 Avg.)	Log_2 average signal	SD (Log_2 Avg.)
(i) Common entries between Tables 3 and 1 – genes that were constitutively over- or under-expressed in the mutant and further exacerbated by high Ca^{2+}								
At4g04500	*Protein kinase family protein*	*1*	*0.0348*	***4.65***	*6.54*	0.71	*8.76*	1.04
At1g29430	Auxin-responsive family protein	2	0.0149	**−2.20**	5.87	0.63	4.73	0.55
At5g27780								
(ii) Common entries between Tables 3 and 2 – genes that responded to high Ca^{2+} growth conditions in both the wild-type and mutant								
At4g25000	AMY1 alpha-amylase, putative/ 1,4-alpha-D-glucan glucanohydrolase, putative	1,2	0.0213	**3.71**	6.77	1.46	8.66	0.73
At5g20230	ATBCB plastocyanin-like domain-containing protein	2,5,6	0.0219	**3.56**	8.37	0.75	10.20	0.45
At5g55450	Protease inhibitor/ seed storage/lipid transfer protein family protein	5,6	0.0098	**3.27**	9.11	0.81	10.82	0.58
At3g45860	Receptor-like protein kinase, putative	1,2	0.0044	**3.00**	7.41	0.89	8.99	0.38
At5g24530	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	1	0.0080	**2.71**	9.14	0.70	10.58	0.43
At5g10380	Zinc finger (C3HC4-type RING finger) family protein	6	0.0029	**2.18**	9.60	1.29	10.72	0.25
At1g68620	Expressed protein	0	0.0244	**2.10**	5.71	0.44	6.78	0.60
At5g59320	LTP3 lipid transfer protein 3	5,6	0.0258	**−3.31**	8.21	1.13	6.48	0.79
At4g19690	IRT1 iron-responsive transporter	5	1.31E−05	**−2.84**	7.39	1.01	5.88	1.00
At4g33720	Pathogenesis-related protein, putative	2	0.0077	**−2.40**	6.74	1.91	5.48	1.20
(iii) Common entries between Tables 1, 2, and 3 – combined effect of (i) and (ii)								
At1g75040	*PR5 pathogenesis-related protein 5*	*2*	*0.0101*	***7.94***	*9.35*	1.81	*12.33*	0.36
At3g57260	BGL2 glycosyl hydrolase family 17 protein	1,2	0.0002	**5.05**	9.91	1.44	12.25	0.41
At2g18660	EXPR3 expansin family protein	0	0.0164	**4.52**	6.85	1.12	9.03	0.75
At5g10760	Aspartyl protease family protein	7	0.0016	**3.58**	8.93	0.93	10.77	0.69
At4g23140	RLK5 receptor-like protein kinase 5	1	0.0031	**2.68**	6.38	0.93	7.80	0.58
At5g22430	Expressed protein	0	0.0194	**−2.63**	6.13	1.41	4.73	0.44
(iv) Genes that are unique to Table 3 – novel induction or repression in the mutant when exposed to high Ca^{2+}								
At4g10500	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	1	0.0432	**5.85**	6.60	1.24	9.15	0.78
At4g11890	Protein kinase family protein	1	0.0427	**4.88**	5.75	0.88	8.04	0.58
At4g23150	Protein kinase family protein	1	0.0250	**4.32**	4.82	0.50	6.93	0.96
At2g32680	Disease resistance family protein	1,2,4	0.0065	**3.14**	7.08	1.46	8.73	0.62

Table 3 (*continued*)

Locus identifier	Gene description	Category	*cngc2* + Ca^{2+}/ *WS* + Ca^{2+}		*WS* + Ca^{2+}		*cngc2* + Ca^{2+}	
			P-value	Linear fold change	Log_2 average signal	SD (Log_2 Avg.)	Log_2 average signal	SD (Log_2 Avg.)
At4g23310	Receptor-like protein kinase, putative	1,2,4	0.0374	**2.98**	4.25	0.48	5.83	1.01
At5g46050	Proton-dependent oligopeptide transport (POT) family protein	5	0.0422	**2.60**	6.78	0.42	8.15	0.48
At5g64000	SAL2 3′(2′),5′-bisphosphate nucleotidase, putative/inositol polyphosphate 1-phosphatase, putative	1,4	0.0423	**2.25**	6.14	1.67	7.31	0.60
At1g56150	Auxin-responsive family protein	2	0.0190	**2.19**	7.09	0.71	8.23	0.70
At1g51890	Leucine-rich repeat protein kinase, putative	1	0.0485	**2.12**	6.20	0.58	7.29	0.73
At1g72060	Expressed protein	0	0.0310	**2.02**	7.07	0.48	8.09	0.32
At5g50200	WR3 expressed protein	2,5	0.0039	**2.01**	7.66	0.94	8.66	0.69
At5g59310	LTP4 lipid transfer protein 4	5,6	0.0371	**−3.84**	6.96	1.41	5.02	0.58
At2g38170	*CAX1 calcium exchanger*	*2,5*	*0.0070*	***-3.72***	*11.19*	0.40	*9.30*	0.45
Atcg00350	PSAA protein comprising the reaction center for photosystem I	8	0.0401	**−2.45**	9.09	0.63	7.79	0.58
At5g50915	Basic helix–loop–helix (bHLH) family protein	3,6	0.0279	**−2.28**	7.01	0.39	5.82	0.17
At1g29510	Auxin-responsive protein, putative	2	0.0075	**−2.25**	5.96	0.51	4.78	0.15
At1g29500	Auxin-responsive protein, putative	2	0.0500	**−2.06**	5.39	0.37	4.35	0.18
At4g08870	Arginase, putative	1	0.0410	**−2.03**	6.90	1.16	5.88	0.55
At1g52190	Proton-dependent oligopeptide transport family protein	5	0.0120	**−2.00**	8.71	0.98	7.70	0.97

Only genes with fold changes of $\geqslant 2$ (down-regulated genes are indicated by a negative fold-change value) and $P \leqslant 0.05$ are shown. *P*-values reflect the statistical significance of differences in log_2 average signal intensities between *WS* + Ca^{2+} and *cngc2* + Ca^{2+}. 24 genes were significantly up-regulated and 13 genes were significantly down-regulated. Italicized entries are those used in qRT-PCR validation experiments, and the functional categories (based on the MIPS database) are: Unclassified (0), Metabolism (1), Defense (2), Transcription (3), Signal Transduction (4), Transport (5), Binding (6), Protein Fate (7), Energy (8).

levels of CAX1 may affect calcium homeostasis in *cngc2* and contribute to its stunted growth under long-term calcium exposure. Interestingly, *CAX3* induction, which is likely part of the normal response to growth in high external calcium, was not significantly different in the mutant.

If we consider all of the genes with $\geqslant$ 2-fold change in *cngc2* + Ca^{2+}/*WS* + Ca^{2+} (Tables 3 and S3), defense, transport, and metabolism genes are over-represented (Fig. 1C). Changes in these functions in the mutant may have contributed to its stunted growth.

3.4. Comparison of transcriptional profiles

We compared the global transcriptional pattern exhibited by our experimental conditions with others listed in a public Affymetrix microarray database. Hierarchical clustering analysis revealed that the *cngc2*/*WS* comparison co-clustered with *WS* + Ca^{2+}/*WS* and 12 other experimental conditions (Cluster A in Fig. 2), and *cngc2* + Ca^{2+}/*WS* + Ca^{2+} co-clustered with *cngc2* + Ca^{2+}/*cngc2* and 11 other conditions (Cluster B in Fig. 2). This result indicates that the transcriptional profile of *cngc2* mutants grown in control media was similar to that obtained from the wild-type grown in elevated levels of calcium, as if the mutants were pre-disposed to initiating stress responses. The other experimental conditions in Cluster A include a variety of stresses, including pathogen infections, mechanical and temperature-induced wounding and DNA damage. This suggests an overlap in the adaptive response to high external calcium with the responses to these biotic and abiotic stimuli. The co-regulated conditions in Cluster B are also associated with various stresses but are slightly different from the ones in Cluster A, suggesting identifiable differences in the transcriptional response between mutant and wild-type plants towards

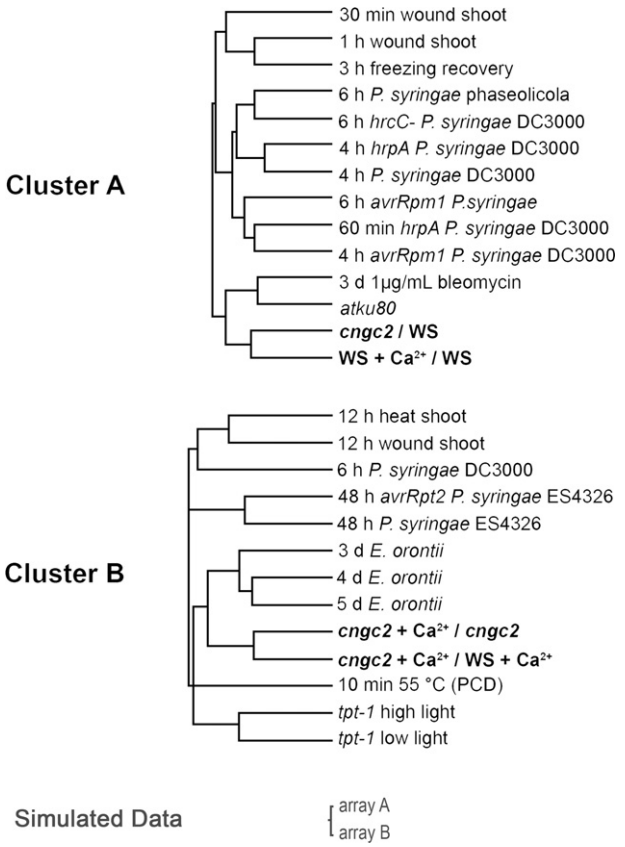


Fig. 2. Results of hierarchical clustering. 1704 Affymetrix 23 K ATH1 microarray data sets (representing 444 different experimental conditions) were compiled and hierarchically clustered in two dimensions. The resulting tree organizes data so that gene expression profiles adjacent to each other are more similar than those farther away, and tree branch lengths reflect degree of similarity. For tree branch length 'scale' reference, we show a pair of 'simulated experiments' whose expression profiles we have created to be nearly identical. Cluster A contains *cngc2*/*WS* and $WS + Ca^{2+}/WS$, and Cluster B contains $cngc2 + Ca^{2+}/cngc2$ and $cngc2 + Ca^{2+}/WS + Ca^{2+}$. The correlation coefficients of these clusters are significantly different from a set of ten different randomly sampled groups of the same size (Cluster A: $r = 0.140$, $p < 0.0001$; Cluster B: $r = 0.065$, $p < 0.025$; Random clusters: $r_{AVG} = 0.052$). Additional details on the specific experimental conditions contained within each cluster are available from Supplementary Tables S4A and S4B.

adapting to external calcium. This is supported by the observation that the genes listed in Table 3 (mutants' response to growth in high calcium) only partially overlap with entries in Table 2 (wild-type response to growth in high calcium).

3.5. Validation of microarray data

To validate our result obtained from microarray data, we selected six genes for further testing with qRT-PCR. These genes have varied average expression values and show larger than 2-fold change in at least one experimental comparison according to our microarray data. The normalized average fold change for each of the six genes obtained from qRT-PCR and microarray data, respectively, was compared (Supplementary Fig. 1). Overall, the same trends in changes in gene expression were observed regardless of experimental techniques used – when a microarray result indicated an increase in expression for a particular comparison, so did qRT-PCR. However, the absolute extent of change varies depending on the method of analysis. This may not be unexpected since microarray hybridizations and qRT-PCR experiments utilize very different methodologies to quantify mRNA levels.

4. Discussion

4.1. Growth in calcified environments

To the best of our knowledge, this is the first report that characterizes and compares transcriptional responses of wild-type plants and a known calcium-sensitive mutant grown under elevated environmental calcium. The only other report we are aware of that investigates the transcriptome of plants in response to calcium exposure is by Kaplan et al. [15]. In that work, cytosolic calcium transients were induced in *Arabidopsis* by the application of calmodulin antagonists, and transcriptional activities of ~6900 genes were monitored after one hour of treatment. The authors deduced that perhaps more than 3% of the genome is responding to the calcium transient. In contrast, our experiments investigated the long-term effect of growth in elevated calcium. The proportion of genes that changed in our study was lower (e.g. 52 out of a total of 22 810 genes, or approximately 0.23%, showed $\geqslant$2-fold change in $WS + Ca^{2+}/WS$), and there was little overlap in the list of genes compiled from the two different studies. This implies that the biological response towards short- and long-term calcium exposure may be fundamentally different.

In another report, Maathuis and colleagues investigated the expression of 1096 *Arabidopsis* root transporters under conditions of calcium starvation for up to 96 h [16], an experimental condition that is opposite to ours. The transcriptional level of various transporters changed when *Arabidopsis* were subjected to calcium deprivation or high sodium stress (which can induce calcium starvation) and in our study, we also observed that transcription of transport genes was disproportionately affected by our experimental conditions relative to their representation in the genome (see Fig. 1). However, there is little correlation between the genes identified in Maathuis et al. and this work (for example, we did not observe any significant changes in the transcript levels of the *Arabidopsis* H^+-ATPase (AHA) family of proton-ATPases, V-type ATPase, and Aquaporins), except for four areas of interesting overlap. Firstly, *ACA13*, a plasma membrane calcium-ATPase that was up-regulated in the *cngc2* mutant under control conditions, was also induced by high sodium treatment. This result is consistent with the hypothesis that the mutant has a constitutive defect in sensing or maintaining proper calcium homeostasis. Secondly, some peptide transporters were observed to be down-regulated in calcium-starved plants. We found that one peptide transporter (At5g46050) had an increase in transcript level in the mutant when grown in calcium-supplemented media and another peptide transporter (At1g52190) had a decrease in the same condition. Consistent with the suggestion made by Maathuis et al., this result indicates that protein metabolism is sensitive to perturbations in calcium-signaling and homeostasis. Thirdly, the transcription of various ABC (ATP binding cassette) transporters was affected by high sodium treatment and calcium deprivation. We also observed down-regulation of such a transporter when wild-type plants were grown in calcium-supplemented media and therefore, our result is consistent with the suggestion made by Maathuis and colleagues that this family of proteins has functions outside of herbicide and heavy metal detoxification.

Lastly, the transcript level of *CAX3* decreased in high sodium treatment and increased in the wild-type in elevated external calcium. Therefore, results from both reports indicate that transcriptional regulation of *CAX3* is an important component in the regulation of calcium/ion homeostasis.

A common stress transcriptome for abiotic, biotic and chemical stresses has recently been proposed [17]. However, there is virtually no overlap between the proposed common stress response genes and the changes we observed in the mutant. This implies that the transcriptional response we observed is specific to the experimental condition imposed.

We have not investigated the precise reasons that similar transcriptional responses are apparently evoked in response to prolonged calcium exposure and various other stimuli. One possibility is that high external calcium promotes calcium influx, which may be mimicking selected biotic (e.g. *Pseudomonas* infection) and abiotic (e.g. wounding) stresses and hence, creates the overlap in responses. A study by Klüsener et al. [18] strongly suggests that abscisic acid (an abiotic-stress hormone) and elicitors of plant defense can both activate the same type of plasma membrane calcium channel in guard cells. Furthermore, Mateo and colleagues [19] recently found a tight coupling between the concentration of SA, H_2O_2, and glutathione. High SA content not only affects pathogen response but also photosynthetic performance and redox homeostasis, two parameters traditionally associated with abiotic stresses. The previously suggested link between SA and redox status is supported by our observation that a putative peroxiredoxin (At1g60740/At1g65970, see Table 1), a protein with anti-oxidant function, is up-regulated in the *cngc2* mutant. Therefore, we suggest that signaling pathways and related physiological responses for a myriad of biotic and abiotic stimuli are more intimately linked than previously thought.

To see if the transcriptional responses identified in our work are unique to plants, we compared our results to a report by Feske and colleagues, who investigated the transcriptional effect induced by a defect in calcium influx in human T lymphocytes [20]. The authors concluded that this defect caused some genes to be up-regulated and others to be down-regulated, and a large proportion of this gene expression effect was mediated through the function of the calcium-dependent phosphatase calcineurin. In our work, we also observed both up- and down-regulation of gene expression but the genes we identified as having significant changes do not overlap with those mentioned in the work by Feske et al. We also did not observe any significant changes in transcript levels in calcineurin or any phosphatase genes although the expression of a number of protein kinases was clearly affected. This comparison suggests that the transcriptional effect of calcium influx is probably mediated through different signaling pathways in human and plant systems but phosphorylation and dephosphorylation cycles most likely play important roles in the control of gene expression in both.

4.2. CNGC2 and stress adaptation

An array of genetic, physiological and electrophysiological data clearly support a role for CNGC2 in pathogen responses and growth in calcified environments [3,4,7]. The data presented here suggest that CNCG2 may be needed for adapting to selected abiotic stresses as well since the transcriptional profile of *cngc2* plants co-clustered with those under various abiotic stimuli (see Fig. 2). We believe that this co-clustering result is quite specific as the microarray dataset we used contains a large number of wild-types, mutants, and transgenic lines grown under many different conditions and yet, our experimental comparisons only co-clustered with specific abiotic stress conditions. For example, neither of the mutant comparisons co-clustered with profiles from plants with altered levels of or sensitivity towards various chemicals (including SA) and plant hormones, or with plants under numerous other abiotic stresses. Presently, we have no direct physiological data to support a role for CNGC2 in adapting to abiotic stresses other than high external calcium, but this is a testable hypothesis for future experiments.

One may expect that *cngc2*, with a likely defect in calcium uptake, should have a calcium-tolerant phenotype. This is clearly opposite to the observed result. One possible explanation is that CNGC2 may not be involved in the uptake of calcium from the growth media or its transport from roots to leaves but instead, may be primarily responsible for mediating signal transduction and ion homeostasis events that are associated with high external calcium. The predominant expression of CNGC2 in aerial tissues [21] and the types of genes that are affected in the mutant (e.g. ion transporters, kinases and hormone-responsive genes from our study) are consistent with this interpretation. Moreover, there is precedence by which a defect in a potential calcium channel causes calcium-sensitivity. Kang and Turano [22] found that *Arabidopsis* seedlings with antisense *GLR1.1*, a glutamate receptor which can function as a calcium channel, are more sensitive to external calcium. However, there is no overlap in the genes that are transcriptionally regulated in the antisense *GLR1.1* line and *cngc2*. *GLR1.1* is proposed to be a regulator of carbon/nitrogen metabolism, abscisic acid biosynthesis, and water balance [22,23]. Interestingly, *Arabidopsis* over-expressing a radish GLR (*RsGluR*) showed increased glutamate-induced calcium influx and delays in fungal infection [24] but again, we do not observe any correlation in the transcriptionally regulated genes in this transgenic line and *cngc2*. Our current hypothesis is that CNGC2 and various GLRs are each linked to unique sets of signaling, transcriptional and physiological pathways.

We cannot exclude the possibility that *cngc2* is also defective in potassium uptake since CNGC2 can mediate potassium influx in several heterologous systems [6,25]. However, we have focused our discussion on the potential role of CNGC2 in calcium influx and subsequent physiological changes because all data to date strongly imply a connection between calcium flux, CNGC2 function and biological responses to various stimuli. In addition, our data do not indicate that the mutant is potassium-deficient (no change in the sensitivity of *cngc2* plants to external potassium [4], minimal overlap between our data and those from potassium-starved *Arabidopsis* [26], no change in the transcription of potassium uptake genes observed, and no sign of potassium deficiency through direct measurement (C.W.M. Chan, unpublished work).

Current data do not provide information on the precise mechanism by which CNGC2 is activated upon various environmental stresses. However, it is presumed that certain stimuli can increase the concentration of cyclic nucleotides, which then leads to channel activation. In our study, wild-type plants adapting to calcium stress did not show significant

changes in transcript levels in any of the components of the cyclic nucleotide signaling system. Therefore, activation of CNGC2 most likely occurs at the post-transcriptional rather than the transcriptional level, perhaps by regulating relevant protein activities and/or localization.

5. Conclusion

Our result is consistent with other reports that suggest an overlap in the responses towards selected biotic and abiotic stresses. CNGC2 is likely involved in some of these adaptive responses since without its input, plants are pre-disposed to initiate various stress responses even under normal growth conditions. Our work lays the foundation for future work that investigates the molecular mechanisms by which this channel affects physiological processes.

Acknowledgements: The authors thank Ms. Heather Burch and Mr. Lee Breunig for their expert technical assistance, Professor Brian S. Yandell (Departments of Statistics and Horticulture, UW–Madison) for his help and advice in the earliest stages of our microarray data analysis, and the UW Gene Expression Center for carrying out the microarray hybridizations and scannings. C.W.M. Chan was supported by a USDA Grant (Award Number 2002-01387) from 2002 to 2004, and a UW Faculty Diversity Research Award in 2007. D.J. Wohlbach was supported by an NIH Graduate Student Genetics Training Grant.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.043.

References

[1] Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. Plant Cell 14 Suppl., S401–S417.
[2] Yang, T. and Poovaiah, B.W. (2003) Calcium/calmodulin-mediated signal network in plants. Trends Plant Sci. 8, 505–512.
[3] Clough, S.J., Fengler, K.A., Yu, I.C., Lippok, B., Smith Jr., R.K. and Bent, A.F. (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. USA 97, 9323–9328.
[4] Chan, C.W.M., Schorrak, L.M., Smith Jr., R.K., Bent, A.F. and Sussman, M.R. (2003) A cyclic nucleotide-gated ion channel, CNGC2, is crucial for plant development and adaptation to calcium stress. Plant Physiol. 132, 728–731.
[5] Kelling, K. and Schulte, E. (1998) A2523 Soil and Applied Calcium, Cooperative Extension Publications, University of Wisconsin-Extension, pp. 1–2.
[6] Leng, Q., Mercier, R.W., Yao, W. and Berkowitz, G.A. (1999) Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol. 121, 753–761.
[7] Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., von Bodman, S. and Berkowitz, G.A. (2007) Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell 19, 1081–1095.
[8] Mou, Z., Fan, W. and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113, 935–944.
[9] Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B. and Speed, T.P. (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31, e15.
[10] Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95, 14863–14868.
[11] Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V. and Quackenbush, J. (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374–378.
[12] Cheng, N.H., Pittman, J.K., Shigaki, T., Lachmansingh, J., LeClere, S., Lahner, B., Salt, D.E. and Hirschi, K.D. (2005) Functional association of *Arabidopsis* CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol. 138, 2048–2060.
[13] Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F. and Leister, D. (2002) The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. Plant J. 31, 589–599.
[14] Hirschi, K.D. (1999) Expression of *Arabidopsis* CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell 11, 2113–2122.
[15] Kaplan, B., Davydov, O., Knight, H., Galon, Y., Knight, M.R., Fluhr, R. and Fromm, H. (2006) Rapid transcriptome changes induced by cytosolic Ca^{2+} transients reveal ABRE-related sequences as Ca^{2+}-responsive cis elements in *Arabidopsis*. Plant Cell 18, 2733–2748.
[16] Maathuis, F.J., Filatov, V., Herzyk, P., Krijger, G.C., Axelsen, K.B., Chen, S., Green, B.J., Li, Y., Madagan, K.L., Sanchez-Fernandez, R., Forde, B.G., Palmgren, M.G., Rea, P.A., Williams, L.E., Sanders, D. and Amtmann, A. (2003) Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. Plant J. 35, 675–692.
[17] Ma, S. and Bohnert, H.J. (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. Genome Biol. 8, R49.
[18] Klusener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V. and Schroeder, J.I. (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. Plant Physiol. 130, 2152–2163.
[19] Mateo, A., Funck, D., Muhlenbock, P., Kular, B., Mullineaux, P.M. and Karpinski, S. (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J. Exp. Bot. 57, 1795–1807.
[20] Feske, S., Giltnane, J., Dolmetsch, R., Staudt, L.M. and Rao, A. (2001) Gene regulation mediated by calcium signals in T lymphocytes. Nat. Immunol. 2, 316–324.
[21] Kohler, C., Merkle, T., Roby, D. and Neuhaus, G. (2001) Developmentally regulated expression of a cyclic nucleotide-gated ion channel from *Arabidopsis* indicates its involvement in programmed cell death. Planta 213, 327–332.
[22] Kang, J. and Turano, F.J. (2003) The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 100, 6872–6877.
[23] Kang, J., Mehta, S. and Turano, F.J. (2004) The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss. Plant Cell Physiol. 45, 1380–1389.
[24] Kang, S., Kim, H.B., Lee, H., Choi, J.Y., Heu, S., Oh, C.J., Kwon, S.I. and An, C.S. (2006) Overexpression in *Arabidopsis* of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca^{2+} influx and delays fungal infection. Mol. Cells 21, 418–427.
[25] Leng, Q., Mercier, R.W., Hua, B.G., Fromm, H. and Berkowitz, G.A. (2002) Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol. 128, 400–410.
[26] Armengaud, P., Breitling, R. and Amtmann, A. (2004) The potassium-dependent transcriptome of *Arabidopsis* reveals a prominent role of jasmonic acid in nutrient signaling. Plant Physiol. 136, 2556–2576.
[27] Zhao, S. and Fernald, R.D. (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comp. Biol. 12, 1047–1064.