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Expression levels of inositol phosphorylceramide synthase modulate plant responses to biotic and abiotic stress in *Arabidopsis thaliana*

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Abstract

This research was undertaken to investigate the global role of the plant inositol phosphorylceramide synthase (IPCS), a non-mammalian enzyme previously shown to be associated with the pathogen response. RNA-Seq analyses demonstrated that over-expression of inositol phosphorylceramide synthase isoforms *At*IPCS1, 2 or 3 in *Arabidopsis thaliana* resulted in the down-regulation of genes involved in plant response to pathogens. In addition, genes associated with the abiotic stress response to salinity, cold and drought were found to be similarly down-regulated. Detailed analyses of transgenic lines over-expressing *At*IPCS1-3 at various levels revealed that the degree of down-regulation is specifically correlated with the level of *IPCS* expression. Singular enrichment analysis of these down-regulated genes showed that *At*IPCS1-3 expression affects biological signaling pathways involved in plant response to biotic and abiotic stress. The up-regulation of genes involved in photosynthesis and lipid localization was also observed in the over-expressing lines.

Introduction

According to UN estimates, growing at a rate of 1.1% per year, the world population is set to reach 9.8 billion by 2050 [1], which would require a 70% increase in food production [2]. A finite amount of arable land, coupled with the detrimental effects of climate change on crop yields, mean that strategies other than intensification will need to be employed to increase production. One that is being adopted, in combination with intensification, is the use of biotechnology to produce genetically modified crops with enhanced yields. The ability to make plants that are more tolerant to biotic and abiotic stress is predicated on identifying molecular targets which modulate plant stress responses.

One such target of interest is the non-mammalian plant enzyme inositol phosphorylceramide synthase (IPCS) which catalyses a key step in sphingolipid biosynthesis. Complex

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sphingolipids can be grouped into two main classes in plants: glycosylceramides and derivatives of inositol phosphorylceramide (IPC) [3]. IPCS is central to synthesis of the latter, catalyzing the transfer of phosphoinositol from phosphatidylinositol to ceramide to form IPC [4]. Ceramide is the base unit of complex sphingolipids and is composed of a long chain base (LCB) and a fatty acid (FA) component [5]. The structural diversity of complex sphingolipids is conferred by the FA and LCB, with variation in carbon chains (C16-26), hydroxylation and desaturation, and the addition of various saccharides/oligosaccharides attached, via a phosphoinositol group in some cases, to the primary hydroxyl of ceramide. These modifications account for the 168 sphingolipid species identified in *Arabidopsis thaliana* [6], and are involved in a plethora of biological pathways, including programmed cell death (PCD) [7], reproduction [8], senescence [9] and cold acclimation [10]. Disruption of the sphingolipid pathway has repeatedly been shown to be inextricably connected to plant defense signaling [11].

First identified in wax bean microsome [12] and later in *A*. *thaliana* [4, 13], IPCS has been shown to play a role as a negative regulator of PCD [13] and is required for reproduction and normal growth [14]. *In planta* three IPCS isoforms exist, and further characterization in *Oryza sativa* showed that the expression of all three *IPCS* isoforms was temporally altered to varying degrees in a tissue and stress specific manner [15]. For example, under cold stress *OsIPCS1* (NP_001044812) and *OsIPCS2* (NP_001055712) were up-regulated in roots and stems, but down-regulated in leaves; in contrast *OsIPCS3* (NP_001055096) was up-regulated in all tissues. Together, these results suggested that *OsIPCS1-3* have key roles in rice growth and abiotic stress responses [15]. With respect to the plant biotic stress response, T-DNA insertion mutants of *AtIPCS2* (AT2G37940) in *A*. *thaliana* showed increased levels of ceramide and phytoceramide, both well-documented inducers of PCD, and displayed necrotic lesions associated with PCD [13]. When exposed to the biotropic pathogen *Golovinomyces cichoracearum* UCSC1, these plants showed a reduction in fungal mass compared to controls [13]. *At*IPCS1 (AT3G54020) and *At*IPCS3 (AT2G29525) have not been characterized so far.

The data from both monocot *O*. *sativa* (rice) and dicot *A*. *thaliana* [13, 15] indicate that manipulation of IPCS activity, chemically or genetically, could be used to modulate biotic and abiotic plant stress responses. To explore this further, in this study, *A*. *thaliana* lines over-expressing each *IPCS* isoform were created and RNA-Seq carried out to monitor conserved changes in the transcriptome.

Materials and methods

Over-expression of *At*IPCS1-3 in *Arabidopsis thaliana*

PCR products of the full-length cDNA of *AtIPCS1-3* [4] were cloned into pENTR/D-TOPO using T4-ligase (ThermoFisher) and into the destination vector pK7WG2 [16] via Gateway LR Clonase (ThermoFisher) to create pK7WG2_AtIPCS1-3.

Primers:

*At*IPCS1-NotI-F:

GCGCGCGGCCGCCACAATGACGCTTTATATTCGCCGCG

*At*IPCS1-AscI-R:

GCGCGGCGCGCCTCATGTGCCATTAGTAGCATTATCAGTGTG

*At*IPCS2-NotI-F:

GCGCGCGGCCGCCACAATGACACTTTATATTCGTCGTGAATCTTCCAAG

*At*IPCS2-AscI-R:

GCGCGGCGCGCCTCACGCGCCATTCATTGTGTTATC

*At*IPCS3-NotI-F:
GCGCGCGGCCGCCACAATGCCGGTTTACGTTGATCGC
*At*IPCS3-AscI-R:
GCGCGGCGCGCCTCAATGATCATCTGCTACATTGTTCTCGTTT

Agrobacterium tumefaciens strain C58C1 was transformed with pK7WG2_AtIPCS1-3, transformants plated on Luria broth (100 μg/μl rifampicin and 100 μg/μl spectinomycin) and incubated for 3 days at 28˚C. Col-0 wild-type *A*. *thaliana* were subsequently transformed using the floral dipping method [17].

Arabidopsis thaliana growth conditions

Col-0 and *AtIPCS1*, *2* and *3* over-expressing plants were grown for 10 days on Murasige and Skoog (MS) agar before transfer to peat plugs. Growth conditions were 20 ˚with a 16-hour day / 8-hour night cycle.

RNA preparation

RNA extraction was carried out on samples flask frozen in nitrogen, using the ReliaPrep™ Tissue Miniprep System (Promega) according to the manufacturer's protocol. Following DNase (ThermoFisher) treatment, the integrity of the RNA was determined by running the samples on a 2100 Bioanalyzer (Agilent) to obtain an RNA Integrity Number (RIN) score.

Quantification of *At*IPCS1-3 in over-expressing *Arabidopsis thaliana* transgenic lines

cDNA samples prepared as above and the Applied Biosystems 7300 Real-Time PCR System and the SYBR Green Jump-Start Taq Ready Mix were used to quantify transcript as previously described [4, 18, 19]. Gene specific primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) for real-time PCR with PEX4 used as a reference gene. Primers: *At*IPCS1_F: TGCGTCCCGTAAACATTACA, *At*IPCS1_R: ACACCGTTCCCATTCAAGAG, A*t*IPCS2_F: TACCAGATCGGACTGCTGTG, *At*IPCS2_F: GTGAACTCCGTTGCTGTCAA, *At*IPCS3_F: CTGGGCCGAATTATCATTGT, *At*IPCS3_R: CCTTCGTGTGCCGTATCTTT

RNA-Seq

Single end libraries for RNA-Seq were generated from DNase treated total RNA using TruSeq Stranded mRNA sample preparation kit according to manufacturer's instructions (Illumina). Briefly, mRNAs were fragmented and purified for use as template for the synthesis of double stranded cDNA. End repair of the double stranded cDNA was carried out and the 3' end adenylated. Sample specific indexing adapters were ligated to the ends of double stranded cDNA samples, amplified by PCR and then purified. Samples were normalized, pooled and then sequenced using a NextSeq 500 instrument (Illumina) to obtain 150 base pair single end reads.

RNA-Seq analyses. The RNA sequence data in Fastq format 11 were filtered and trimmed (sliding window 4:15 and 50 bp minimum) to remove low quality reads using Trimmomatic [20]. Reads were aligned to the *Arabidopsis* genome (*Arabidopsi*s Araport 2017) using STAR [21]. The sequence alignment files were sorted by name14 for HTSeq-count and indexed using SAMtools [22]. Files were converted to BAM files and number of reads mapped onto a gene calculated using HTSeq package [23]. Gene counts were normalized and compared sample by sample using DESeq2 [24] (Bioconductor [25]) in R [26]. Differential expression was determined using with a $\log_2$ fold-change output. GO term enrichment was performed for analyses

of genes up- and down-regulated in both biological replicates using the agriGO analysis tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php) with the default settings [27]. Gene annotations was carried out against *Arabidopsis* gene model (TAIR9) background (https://www.arabidopsis.org/). These data are freely available in GEO (https://www.ncbi.nlm.nih.gov/geo/ GEO Accession GSE129016).

MapMan

Analyses were carried out using MapMan 3.5.1 R2 software [28]. RNA-Seq abundance data from the At2++ transgenic line were uploaded to MapMan and $\log_2$ fold change selected as the experimental data set for analyses. Mapping was carried out using Ath_AGI_TAIR9_Jan2010.

Osmotic stress assay

Seedlings were grown under standard conditions on MS agar for 8 days before floating on the various concentrations of mannitol (without MS) in a sterile culture dish under the same conditions as the plate (16h day photo-period, 20˚C). Photographs were taken after 6 days.

Pathogen stress assay

Control and over-expressing transgenic lines were grown under short day conditions for 5 weeks and leaves excised then incubated on MS agar at 37˚C for 72 hours following the addition of 5μl of *Erwinia amylovora* culture grown to an $OD_{600} = 0.5$.

Genevestigator

Genevestigator [29] was utilised to identify available data showing the upregulation of *At*IPCS1, *At*IPCS2 or *At*IPCS3 in *Arabidopsis thaliana* seedlings exposed to different agents and conditions.

Results

Identification of genes down- or up-regulated on over-expression of *AtIPCS*

A. thaliana plants over-expressing the full-length cDNA of *AtIPCS1*, *AtIPCS2* and *AtIPCS3* respectively, were generated as described in Methods. For each isoform, two transgenic lines (biological replicates) over-expressing the respective *AtIPCS* were selected, one with high levels relative to wild-type Columbia-0 (Col-0) (At1-3++) and one with a lower level of over-expression (At1-3+) (Fig 1A–1C). Importantly, over-expression of one isoform did not affect the expression of the other two *At*IPCS isoforms (S1 Fig). Genome wide transcriptomic data analyses indicated that the number of expressed genes detected was very similar in all transgenic lines and in the Col-0 control (Table 1).

Genes that were identified as differentially expressed in both biological replicates, compared to Col0, were carried forward for further analyses. Function enrichment of the down-regulated genes using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) revealed a significant enrichment of genes (Fisher exact test, two-tailed, p-value < 0.001) under the GO terms *response to stimulus* ($p = 3.50^{E-18}$) and *response to stress* ($p = 1.50^{E-14}$), whilst a significant amount of the up-regulated genes fell under the GO terms *cellular process* ($p = 2.90^{E-07}$) and *cellular metabolic process* ($p = 4.10^{E-08}$) (Fig 2). Those genes identified under GO term *response to stimulus* were the same genes as identified under *response to stress* and *response to abiotic stress*. Subsequently, to add specificity, focus was placed upon the latter two GO terms. *AtIPCS2* over-expression led to the down-regulation of 135 genes, considerably more than

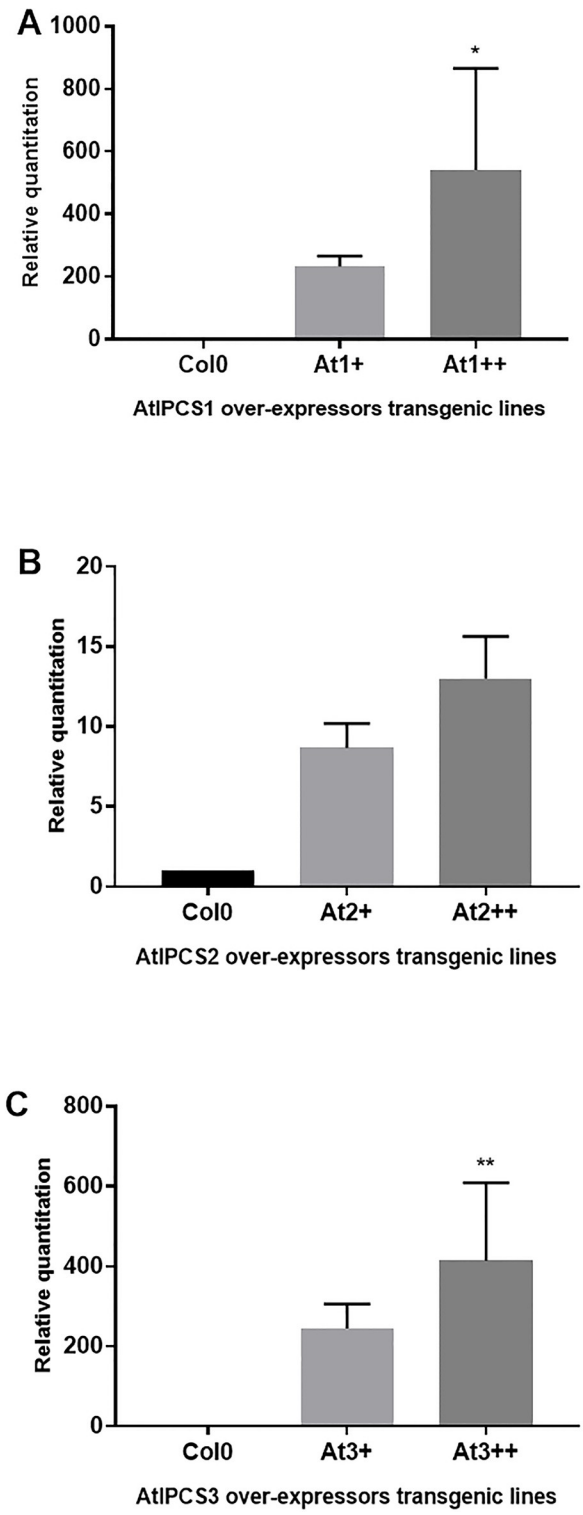

Fig 1. Relative quantitation of mRNA levels of (A) *At*IPCS1 (B) *At*IPCS2 (C) *At*IPCS3 in over-expressor transgenic lines compared to Col0 standardised to equate to a value of 1; qPCR was performed to measure mRNA levels in 10-day old seedlings. Relative quantitation was done after normalization using PEX4 levels; relative quantitation value is the mean of three biological replicates with standard deviation; significance of mRNA levels determined by one-way ANOVA with Turkey's post hoc test ($p < 0.05$).

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Table 1. Genes expressed in Col-0 and *AtIPCS1-3* over-expressing transgenic lines. *A. thaliana* Col-0 parent; At1, 2 or 3 lines over-expressing *AtIPCS1*, *2 or 3* (2 lines of each); Rep—experimental replicates (3); FPKM—Fragments Per Kilobase Million.

Genotype	Rep	Expressed genes (FPKM)	Average	Non-expressed genes (FPKM)	Average
Col-0	1	22740	22749	2524	2515
Col-0	2	22700		2564	
Col-0	3	22807		2457	
At1+	1	22410	22518	2854	2746
At1+	2	22503		2761	
At1+	3	22640		2624	
At1++	1	22914	22805	2350	2459
At1++	2	22823		2441	
At1++	3	22677		2587	
At2+	1	22643	22631	2621	2633
At2+	2	22546		2718	
At2+	3	22705		2559	
At2++	1	22520	22574	2744	2690
At2++	2	22573		2691	
At2++	3	22628		2636	
At3+	1	22657	22595	2607	2669
At3+	2	22593		2671	
At3+	3	22535		2729	
At3++	1	22708	22731	2556	2533
At3++	2	22708		2556	
At3++	3	22777		2487	

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AtIPCS1 (54) and *AtIPCS3* (59) (Fig 3A). Of these, 26 genes were down-regulated in all lines. With respect to up-regulated genes, again most (275) were in response to *AtIPCS2* over-expression, with 70 and 19 in *AtIPCS1* and *AtIPCS3* over-expressers respectively; 15 genes were found to be up-regulated in all lines (Fig 3B).

Analyses of genes identified as responding negatively to *AtIPCS* over-expression

Global analyses of the down-regulated genes in response to *AtIPCS1* over-expression revealed significant enrichment under the GO term GO:0006950, *response to stress* ($p = 1.50^{E-14}$), 42.6% (23/54) when they represent only 6.14% of the *Arabidopsis* transcriptome (S1 Table). Similarly, on *AtIPCS2* over-expression down-regulated genes were enriched under this term, 34.1% (46/135; $p = 2.20^{E-22}$) (S2 Table); and on *AtIPCS3* over-expression 40.7% (24/59; $p = 2.40^{E-14}$). Other biological processes showing a significant enrichment of genes down-regulated in these transgenic lines, included those under GO terms: GO:0042221 (*response to chemical stimulus*); GO:0010033 (*response to organic substances*), GO:0051707 (*response to other organisms*); GO:0009607 (*response to biotic stimulus*); GO:0009628 (*response to abiotic stimulus*); and GO:0006952 (*defense response*) (S1–S3 Tables).

Of particular interest were genes that showed a dose-dependent decrease in expression associated with increased *AtIPCS* expression. Genes (GO:0006950, *response to stress*) whose decrease in expression was negatively correlated ($\log_2$ change ≥ 2) with higher *AtIPCS1* levels (At1++ versus At1+], Fig 1A and Table 2) are: TYROSINE AMINOTRANSFERASE 3 (TAT3; AT2G24850); PLANT DEFENSIN 1.2B (PDF1.2B; AT2G26020); the salicylic acid inducible PATHOGENESIS-RELATED GENE, PR1 (AT2G14610) and PR2 (AT3G57260);

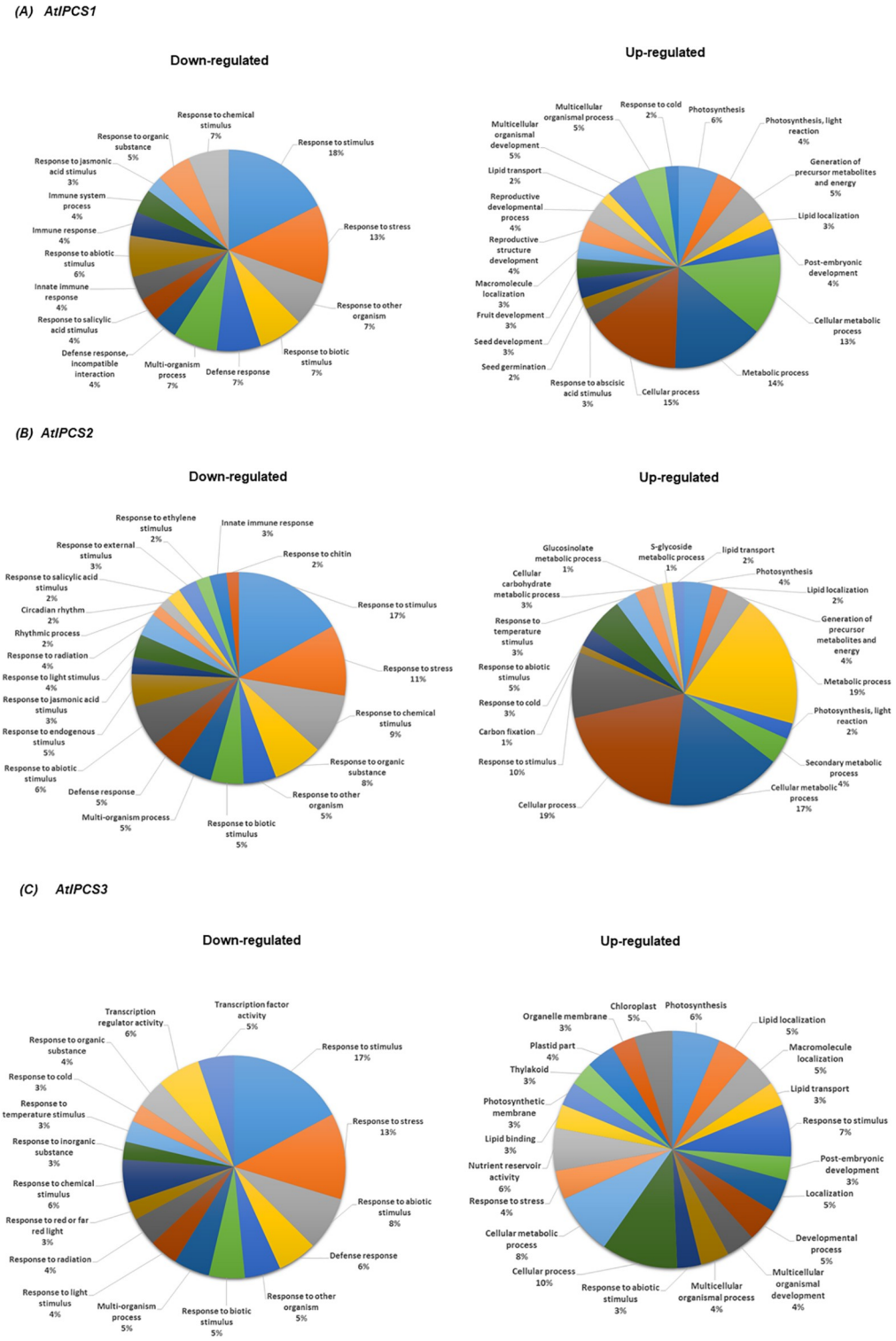

Fig 2. Pie chart of biological processes enriched for genes down- and up-regulated in response to the over-expression of (A) *AtIPCS1*, (B) *AtIPCS2* and (C) *AtIPCS3* isoforms when compared to wild type Col0.

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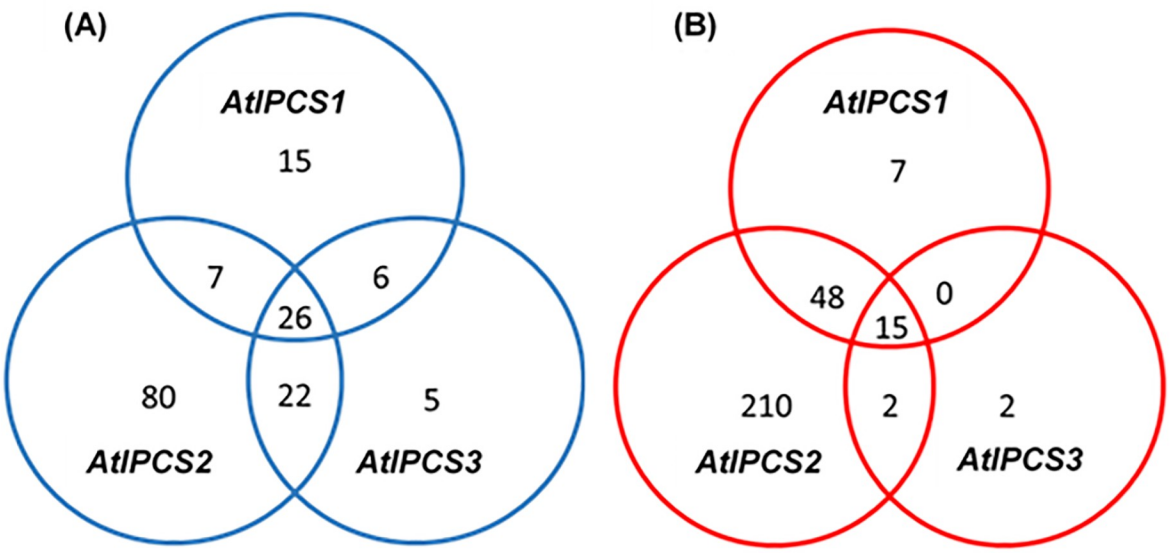

Fig 3. Venn diagrams of number of genes that were down-regulated (A) or up-regulated (B) in response to *AtIPCS1*, *AtIPCS2 and AtIPCS3* overexpression. Log_2 fold change relative to wild type Col-0.

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LIPID TRANSFER PROTEIN (LTP; AT4G12490); and an ETHYLENE- AND JASMONATE-RESPONSIVE PLANT DEFENSIN (AT5G44420), a well characterized component of the defense network against pathogens [30] (Table 2).

Interestingly, the dose response effects noted was largely specific for the *AtIPCS1* isoform (Table 2). However, it is immediately clear that the plant defense system is more sensitive to increased *At*IPCS2 expression (At2+ and At2++), despite the increase being relatively small (up to 10-fold) compared to *At*IPCS1 and 3 (up to 390 and 440-fold respectively) (Fig 1A–1C and Table 2). The overall impact of *AtIPCS2* over-expression may be due to an increases in what represents the most abundant *AtIPCS* transcript (approximately 100-fold *AtIPCS1*) in all tissues of wild type *A*. *thaliana* [4]. Interestingly, there is no major dose response apparent on an increase in the over-expression of *AtIPCS3* (At3++ versus At3+, Table 2), despite an increase in *AtIPCS3* transcript similar to that observed for *AtIPCS1* (Fig 1C). However, transcripts that were down-regulated $\geq$2 log_2 in *AtIPCS3* (either line) and were also suppressed on *AtIPCS1* or *AtIPCS2* over-expression include: TAT3; lipid binding A putative lipid transfer protein (PEARLI 1; AT4G12480); *PDF1.2B; PR1* and *PR2*; LATE UP-REGULATED IN RESPONSE TO *HYALOPERONOSPORA PARASITICA* (LURP1; AT2G14560); GIGANTEA (GI; AT1G22770); ETHYLENE- AND JASMONATE-RESPONSIVE ELEMENT PLANT DEFENSIN. Of the genes down-regulated with a log_2 fold change $\geq$2 in response to *AtIPCS2* over-expression a large number were specific: ELICITOR-ACTIVATED GENE 3–2 (ELI3-2; AT4G37990); RECEPTOR LIKE PROTEIN 23 (RLP23; AT2G32680); PHOSPHOLIPASE A 2A (PLA2A; AT2G26560); CIRCADIAN CLOCK ASSOCIATED 1 (CCA1; AT2G46830); OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF 59 (ORA59; AT1G06160); DARK INDUCIBLE 11 (DIN11; AT3G49620); EXTENSIN 4 (EXT4; AT1G76930); TERPENE SYNTHASE 04 (TPS04; AT1G61120); HISTONE H1-3 (HIS1-3;AT2G18050); SERINE-TYPE ENDOPEPTIDASE INHIBITOR (TI1; AT2G43510); GLUTATHIONE S-TRANSFERASE PHI 2 (GSTF2; AT4G02520). From these analyses, it is clear that *At*IPCS2 expression affects a wider network of genes (including some of those involved in plant defence responses) compared with *At*IPCS1 and *At*IPCS3 transgenic lines, perhaps due to its higher expression pattern in all tissues of *Arabidopsis* [4].

Table 2. Genes showing a negative correlation with *AtIPCS* expression under GO term *GO:0006950* (*response to stress*) compared to Col0. At1-3+ over-expressing *AtIPCS1-3*; At1-3++ higher level expressers of *AtIPCS1-3*. Fold changes are the value of three technical triplicates of a transformed line. Transcripts listed had a p-value <0.001 (Wald test, cut off p-value < 0.05) and highlighted in bold are $\log_2$ fold change ≥ 2.

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value
AT5G47220	ERF2 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2)			**-2**	1.46E-31	-1.7	4.58E-25	-1.1	7.33E-15				
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-1.8	6.29E-17	**-4**	1.33E-53	**-2.6**	3.03E-28	**-2.3**	4.60E-26	-1.8	6.10E-18	**-2.3**	1.65E-24
AT1G16420	MC8 (METACASPASE 8)			**-2**	1.68E-07	-1.6	3.84E-06	-1.2	0.00047	-1	0.00242862	-1.2	0.00058
AT4G12480	PEARLI 1; LIPID BINDING A PUTATIVE LIPID TRANSFER PROTEIN			**-5**	1.37E-159	-1.9	1.94E-31	**-5.1**	7.83E-172	-1.2	8.99E-13	**-2**	3.20E-32
AT1G78290	SERINE/THREONINE PROTEIN KINASE			**-2**	2.05E-47	-1.8	8.25E-47	**-2**	1.39E-54	-1	2.73E-20	-1.3	1.51E-29
AT4G37990	ELI3-2 (ELICITOR-ACTIVATED GENE 3–2)			**-3**	6.02E-17	**-2.3**	7.52E-12	**-2.3**	2.18E-12	-1.8	3.22E-08	-1.6	6.77E-07
AT3G45290	MLO3 (MILDEW RESISTANCE LOCUS O 3)			-1	7.16E-12	-1.1	5.16E-09	-1.3	7.16E-12				3.50E-08
AT2G26020	PDF1.2B (PLANT DEFENSIN 1.2B)	-1.9	8.15E-18	**-5**	1.44E-66	**-4.9**	1.47E-60	**-4.9**	3.31E-62	**-2.6**	3.65E-30	**-3.4**	3.34E-40
AT2G32680	RLP23 (RECEPTOR LIKE PROTEIN 23)			**-2**	2.69E-17	**-2.2**	1.35E-13	**-2.3**	3.16E-15				2.85E-235
AT2G26560	PLA2A (PHOSPHOLIPASE A 2A)			**-3**	1.57E-78	-1.4	1.57E-26	**-2.6**	1.57E-78				2.27E-14
AT2G14610	PR1 (PATHOGENESIS-RELATED GENE 1)	**-3.4**	1.83E-172	**-7.3**	4.08E-238	**-7.3**	1.05E-206	**-7.9**	4.60E-217	**-4.6**	1.09E-252	**-4.9**	2.85E-235
AT2G46830	CCA1 (CIRCADIAN CLOCK ASSOCIATED 1)			**-2**	9.82E-14	**-2**	3.12E-12	-1.1	1.04E-05				5.91E-13
AT1G06160	ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59)	-1	1.14E-10	-1.4	9.92E-17	**-2.5**	9.89E-37	-1.8	1.67E-24				2.31E-20
AT3G49620	DIN11 (DARK INDUCIBLE 11)			**-4**	1.16E-143	**-3.2**	7.04E-106	**-4.1**	5.37E-147				3.16E-94
AT1G06040	STO (SALT TOLERANCE)	-1.1	2.13E-28	-1.5	1.78E-49	-1.3	2.23E-38	-1.1	8.10E-27	-1	6.04E-26	-1.5	2.41E-48
AT1G48000	MYB112 (MYB DOMAIN PROTEIN 112)			-1	8.79E-05	**-2**	1.97E-10	-1.2	5.54E-05	-1.2	6.45E-05	-1.7	3.23E-08
AT2G14560	LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA)	**-2.7**	5.62E-112	**-3.4**	1.13E-162	**-4.1**	5.67E-173	**-3.3**	1.62E-149	**-3.2**	9.88E-155	-1.7	5.56E-82
AT1G22770	GI (GIGANTEA)			**-2**	2.40E-75	**-2.4**	6.55E-94	**-2.3**	8.70E-91	-1.3	4.07E-33	-1.7	1.27E-94
AT5G10140	FLC (FLOWERING LOCUS C)	-1.2	2.18E-09	-1.5	4.16E-14	-1.4	5.23E-11	-1.3	4.55E-10	-1.1	8.80E-12	-1.7	3.25E-08
AT3G1550	ANAC055 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 55)			**-2**	4.16E-28	-1.3	2.83E-20	-1.5	4.16E-28				

(*Continued*)

Table 2. (Continued)

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value
AT1G76930	EXT4 (EXTENSIN 4)			**-3**	1.47E-142	-1.2	1.21E-28	**-3.1**	3.77E-175				
AT5G44420	AN ETHYLENE- AND JASMONATE-RESPONSIVE PLANT DEFENSIN	**-2.7**	1.49E-54	**-6.1**	3.17E-118	**-6**	3.77E-111	**-6.1**	3.17E-118	**-2.7**	1.34E-55	**-3.6**	7.78E-78
AT2G23680	STRESS-RESPONSIVE PROTEIN			-1	3.11E-14	-1.1	3.58E-14	-1.3	1.09E-18				
AT1G61120	TPS04 (TERPENE SYNTHASE 04)			**-3**	1.57E-22	**-2.6**	2.43E-15	-1.7	5.55E-08	-1.6	4.61E-07	-1.8	6.72E-09
AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	-1.4	3.27E-22	-1.7	2.13E-33	-1.5	1.00E-25	-1.8	5.50E-34				
AT1G52890	ANAC019 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 19)			**-2**	4.84E-09	-1.2	2.90E-05	-1.5	5.04E-07				
AT3G57260	PR2 (PATHOGENESIS-RELATED GENE 2)	-1.9	1.17E-30	**-5.3**	4.64E-102	**-4.8**	2.99E-86	**-4.4**	4.08E-87	**-2**	2.97E-36	**-2.2**	8.40E-38
AT1G18330	EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1)			**-2**	3.87E-53	-1.6	3.85E-29	-1.9	4.62E-40	-1.3	1.98E-23	-1.6	6.68E-30
AT1G78410	VQ MOTIF-CONTAINING PROTEIN			-1	2.59E-05	-1.8	9.42E-08	-1.6	1.24E-06	-1.1	0.00072986	-1.2	0.00016391
AT5G19880	PEROXIDASE					-1.4	0.00010488	-1	0.00418				
AT2G18050	HIS1-3 (HISTONE H1-3)			-1	8.40E-31	**-2.5**	2.90E-96	-1.7	3.90E-55				
AT3G04720	PR4 (PATHOGENESIS-RELATED 4)			-1	8.36E-46	-1.6	7.55E-69	-1.5	1.20E-55				
AT2G43510	TI1; SERINE-TYPE ENDOPEPTIDASE INHIBITOR			**-4**	2.98E-227	**-2.5**	6.34E-132	**-3.9**	2.98E-227	-1.1	2.90E-37	-1.8	9.93E-77
AT3G45860	RECEPTOR-LIKE PROTEIN KINASE	-1.5	6.64E-09	**-2.9**	8.20E-25	**-2.9**	3.03E-22	**-3**	8.20E-25	-1.5	4.12E-09	-1.7	4.81E-10
AT3G51660	MACROPHAGE MIGRATION INHIBITORY FACTOR FAMILY PROTEIN			**-2**	6.63E-15	-1.2	1.40E-16	-1.1	6.63E-15				
AT1G71030	MYBL2 (ARABIDOPSIS MYB-LIKE 2)			**-2**	4.20E-88	-1.2	1.04E-29	-1.7	4.26E-55				
AT1G57630	DISEASE RESISTANCE PROTEIN (TIR CLASS)			**3.1**	1.80E-25	-1.5	8.34E-09	**-2.5**	4.73E-18				
AT5G59780	MYB59 (MYB DOMAIN PROTEIN 59)			**-2**	1.15E-47	-1.2	6.92E-29	-1.7	9.77E-56				
AT4G12490	LIPID TRANSFER PROTEIN (LTP)	-1.3	7.93E-05	**-4.6**	1.76E-45	**-2.8**	8.38E-18	**-4.7**	1.76E-45	-1.9	1.32E-08	**-2.6**	1.46E-15
AT4G23210	PROTEIN KINASE FAMILY PROTEIN ENCODES A CYSTEINE-RICH RECEPTOR-LIKE KINASE (CRK13)			**-3**	1.31E-52	-1.7	8.20E-24	**-3**	1.31E-52				
AT1G09080	ATP BINDING BIP3	-1.1	7.65E-06	**-2.5**	6.79E-21	-1.6	4.53E-10	-1.6	6.04E-11				

(*Continued*)

Table 2. (Continued)

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value
AT4G02520	GSTF2 (GLUTATHIONE S-TRANSFERASE PHI 2)			**-2**		-1.2	2.82E-06	**-2.9**	4.44E-28	-1.4	3.33E-08	-1.6	3.75E-10
AT2G25000	WRKY60					-1.2	7.64E-11	-1.4	9.79E-15				
AT1G08320	TGA9			-1.1	1.21E-22								
AT3G52430	PAD4			-1.0	1.59E-15								
AT1G80840	WRKY40			-1.7	5.02E-23								
AT4G31800	WRKY18			-1.0	1.95E-20								
AT1G01560	MPK11			**-2.0**	5.24E-11			-1.5	4.20E-07				
AT1G73805	SARD1			-1.5	3.18E-29	-1.3	5.55E-20	-1.8	1.56E-35	-1.3	6.19E-23		
AT4G21534	SPHINGOSINE KINASE 2							-1.2	3.12E-21				
AT3G23250	MYB15			-1.9	5.76E-16			**-2.4**	1.09E-20			-1.4	4.96E-10
AT4G25470	DREB1C							-1.3	3.23E-04	-1.1	2.06E-03	-1.1	1.21E-03
AT4G06746	RAP2.9					-1.1	3.27E-13						
AT4G25470	DREB1B			-1.1	0.0014182							-1.0	3.36E-03

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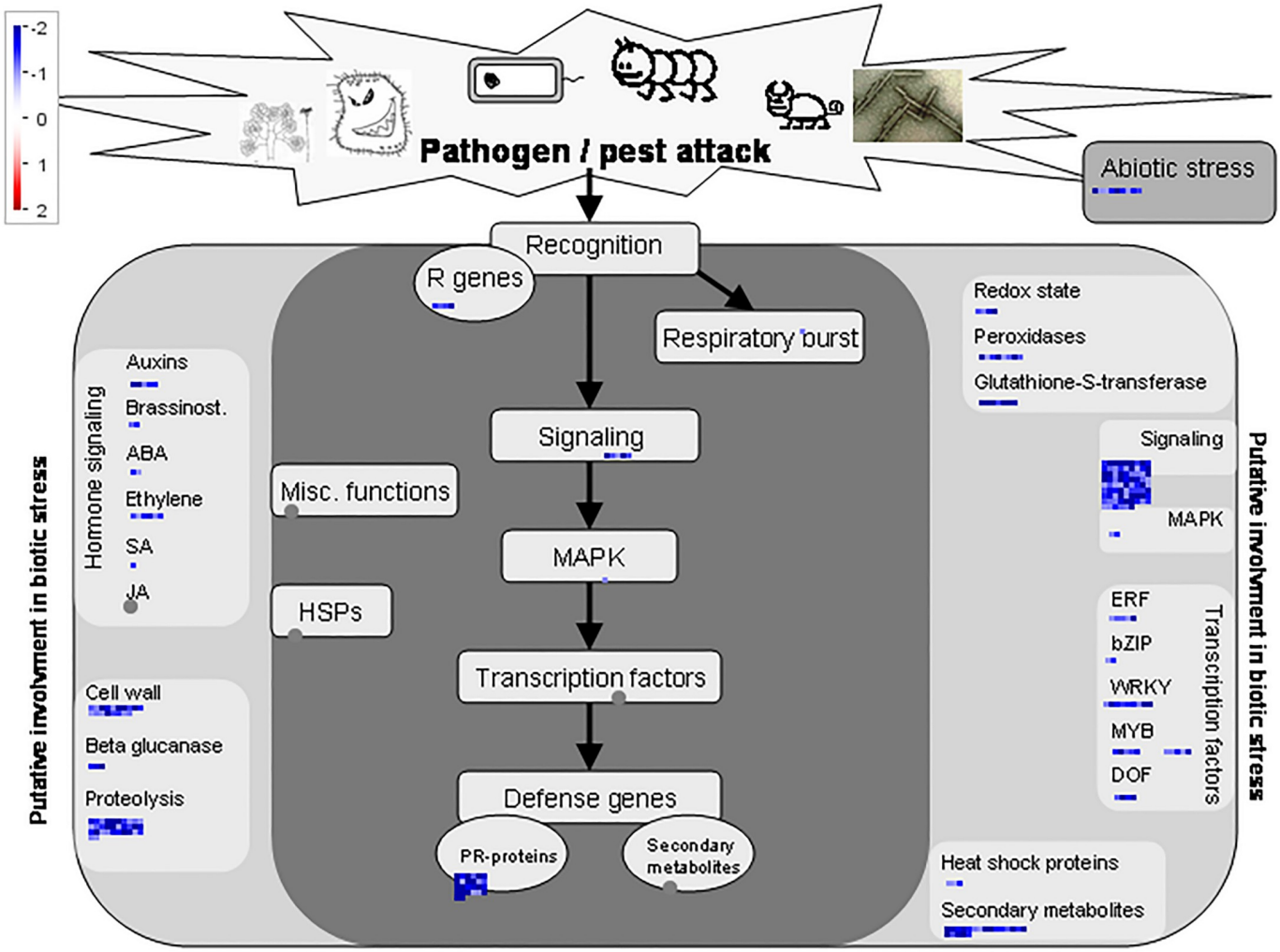

Fig 4. Schematic, generated in MapMan, of genes identified as down-regulated in the At2++ over-expression line and enriched under plant response to biotic stress. Log_2 fold changes in gene expression are indicated by the colour scale. Abbreviations: Resistance (R) genes, salicylic acid (SA), jasmonic acid (JA), ethylene resposnse facotor (ERF), abscisic acid (ABA), DNA binding one zinc finger (DOF), heat-shock protein (HSP) and pathogenesis-related (PR) proteins.

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The effects of over-expression (At2++) were further analysed and visualised using MapMan (https://mapman.gabipd.org). These analyses illustrated multiple negative effects on metabolism (S2 Fig), however the clearest correlation of significantly down-regulated genes (log2-fold change) in At2++ was with the plant response to biotic stress: PR proteins, peroxidases, R genes, B-glucanases, heat shock proteins, transcriptional factors and signalling proteins involved in the response to pathogens (Fig 4). Furthermore, there is a remodelling of plant architecture as seen in the down-regulation of genes involved in cell wall modifications in response to pathogen attack (Fig 4).

The 26 genes down-regulated in response to the over-expression of *AtIPCS1*, *2* and *3* are primarily involved in the plant biotic stress response to pathogens (Table 3), including BDA1 a well characterised transmembrane protein found to be necessary in the regulation and augmentation of the plant response to pathogens [31]; and AED1 [32] an aspartyl protease implicated in *Arabidopsis* systemic acquired resistance. This conserved down-regulation of genes involved in the plant response to pathogens further stresses the functional importance of

Table 3. Genes down-regulated in all three *AtIPCS* over-expression lines compared to Col0. At1-3+ over-expressing *AtIPCS1-3*; At1-3++ higher level expressors of *AtIPCS1-3*. Fold changes are the value of three technical triplicates of a transformed line. Transcripts listed had a p-value <0.001 (Wald test, cut off p-value < 0.05).

Gene ID	Gene annotation	At1+		At1 ++		At2+		At2++		At3+		At3++	
		log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value
AT2G26020	PDF1.2B (PLANT DEFENSIN 1.2B)	-1.9	8.15E-18	-5	1.44E-66	-4.9	1.47E-60	-4.9	3.31E-62	-2.6	3.65E-30	-3.4	3.34E-40
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-1.8	6.29E-17	-4	1.33E-53	-2.6	3.03E-28	-2.3	4.60E-26	-1.8	6.10E-18	-2.3	1.65E-24
AT2G14610	PR1 (PATHOGENESIS-RELATED GENE 1)	-3.4	1.83E-172	-7.3	4.08E-238	-7.3	1.05E-206	-7.9	4.60E-217	-4.6	1.09E-252	-4.9	2.85E-235
AT1G06040	STO (SALT TOLERANCE)	-1.1	2.13E-28	-1.5	1.78E-49	-1.3	2.23E-38	-1.1	8.10E-27	-1	6.04E-26	-1.5	2.41E-48
AT2G14560	LURP1 (LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA)	-2.7	5.62E-112	-3.4	1.13E-162	-4.1	5.67E-173	-3.3	1.62E-149	-3.2	9.88E-155	-2.2	5.56E-82
AT5G10140	FLOWERING LOCUS C	-1.2	2.18E-09	-1.5	4.16E-14	-1.4	5.23E-11	-1.3	4.55E-10	-1.1	8.80E-12	-1.4	3.25E-08
AT5G44420	AN ETHYLENE- AND JASMONATE-RESPONSIVE PLANT DEFENSIN	-2.7	1.49E-54	-6.1	2.23E-122	-6	3.77E-111	-6.1	3.17E-118	-2.7	1.34E-55	-3.6	7.78E-78
AT3G57260	PR2 (PATHOGENESIS-RELATED GENE 2)	-1.9	1.17E-30	-5.3	4.64E-102	-4.8	2.99E-86	-4.4	4.08E-87	-2	2.97E-36	-2.2	8.40E-38
AT3G45860	RECEPTOR-LIKE PROTEIN KINASE	-1.5	6.64E-09	-2.9	3.18E-24	-2.9	3.03E-22	-3	8.20E-25	-1.5	4.12E-09	-1.7	4.81E-10
AT4G12490	LIPID TRANSFER PROTEIN (LTP)	-1.3	7.93E-05	-4.6	4.17E-43	-2.8	8.38E-18	-4.7	1.76E-45	-1.9	1.32E-08	-2.6	1.46E-15
AT1G13470	UNCHARACTERIZED	-2.1	2.12E-11	-3.7	3.73E-29	-3.3	2.67E-23	-2.8	2.70E-18	-2.1	6.88E-12	-2.4	3.04E-14
AT1G33960	AVRRPT2-INDUCED GENE 1	-1.5	6.39E-23	-5.4	1.23E-127	-5.7	9.38E-116	-5.2	1.80E-119	-2.2	7.45E-48	-3.3	6.79E-81
AT3G47480	CALCIUM-BINDING EF-HAND FAMILY PROTEIN	-1.3	3.75E-11	-3.0	3.27E-39	-2.8	4.26E-33	-3.3	6.57E-42	-1.6	4.36E-16	-2.2	5.55E-24
AT4G03450	ANKYRIN REPEAT FAMILY PROTEIN	-2.0	3.19E-10	-3.4	9.96E-24	-3.1	1.96E-20	-3.2	1.03E-21	-2.3	8.75E-13	-2.1	1.90E-10
AT4G23150	CYSTEIN-RICH RLK	-1.7	5.61E-07	-3.2	1.05E-20	-2.9	5.61E-18	-3.1	5.53E-20	-2.1	1.08E-09	-2.5	2.97E-13
AT5G52760	COPPER TRANSPORT PROTEIN FAMILY	-1.2	4.82E-05	-2.6	2.28E-17	-2.4	3.65E-14	-2.7	1.53E-17	-1.4	5.87E-07	-2.2	2.08E-12
AT5G54610	BDA1	-2.9	1.16E-25	-4.2	8.48E-45	-4.0	6.73E-39	-3.7	2.34E-36	-2.9	1.54E-26	-2.2	4.99E-16
AT5G10760	AED1	-1.7	2.88E-39	-4.6	1.85E-126	-4.2	6.95E-105	-4.1	1.41E-112	-2.1	1.09E-55	-1.9	6.10E-45
AT5G60900	RLK1	-1.1	9.02E-06	-2.9	1.86E-24	-2.3	1.51E-16	-2.8	2.43E-22	-1.3	1.25E-07	-1.5	2.05E-09
AT2G18660	EGC2	-1.6	2.23E-11	-3.5	8.48E-35	-3.9	7.62E-38	-3.5	1.52E-33	-2.3	8.03E-20	-2.6	6.45E-22
AT2G26400	ARD1	-2.0	2.90E-12	-2.2	6.94E-15	-2.9	2.32E-20	-2.3	1.82E-15	-2.4	2.33E-16	-3.1	9.05E-23
AT2G29120	GLR2.7	-1.2	6.10E-09	-2.3	6.40E-25	-1.8	1.01E-15	-2.0	9.39E-20	-1.0	1.54E-07	-1.4	3.64E-11
AT3G28510	AAA-ATPase	-2.1	2.93E-23	-1.4	2.97E-13	-2.9	1.42E-32	-1.8	1.70E-17	-1.5	2.91E-14	-2.2	6.87E-23
AT1G52400	BGLU18	-1.4	1.35E-49	-1.9	3.86E-91	-1.8	1.92E-85	-1.0	2.44E-28	-1.1	2.69E-34	-1.0	5.51E-28

(*Continued*)

Table 3. (Continued)

Gene ID	Gene annotation	At1+		At1 ++		At2+		At2++		At3+		At3++	
		log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value	log_2 fold change	p-value
AT1G32960	SBT3.3	-1.4	5.56E-06	-3.4	1.61E-25	-3.5	3.54E-26	-3.2	2.01E-22	-2.0	6.04E-11	-2.0	5.74E-10
AT3G17609	HYH	-1.7	1.92E-14	-1.5	3.92E-12	-1.6	8.78E-12	-1.5	4.75E-11	-1.5	1.75E-12	-1.7	1.87E-13

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*At*IPCS as a non-discriminate and far-reaching negative regulator of the response to biotic stress.

Genes involved in the plant response to abiotic stress are also highlighted as down-regulated in Fig 4, although they did not map to specific pathways. Notably, the sphingolipid biosynthetic pathway itself has been linked to the abiotic stress response. Only one gene in this pathway, sphingosine kinase (AT4G21534), had significantly altered expression (down-regulated) in At2++ (see data deposited in GEO, Accession GSE129016). The protein product of this gene phosphorylates sphingosine (to S1P) and phytosphingosine (to phytoS1P) in plants [33], and increased levels of S1P and abscisic acid dependent stomatal closure have been reported in response to drought [34]. Knock-down of sphingosine kinase expression significantly decreased sensitivity to abscisic acid induced stomatal closure compared to Col0 [35], indicating that At2++ with reduced sphingosine kinase may be more sensitive to drought. One gene showed a negative correlation under GO term *GO:009819* (*drought recovery*), a serine/theronine kinase (AT1G78290), a member of the SNF1-related protein kinase (SnRK2) family whose activity is activated by osmotic stress and dehydration [36]. Similarly, DREB genes have been implicated in the plant response to abiotic stress, including osmotic stresses such as high salinity and drought [37], and DREB1B and DREB1C were down-regulated in all the higher level over-expressors (At1-3++; Table 2). Together, these data place *At*IPCS at the heart of the abiotic, as well as biotic, stress response.

Overall, these data show a complex picture of the modulation of the plant stress response on over-expression of *AtIPCS* isoforms. Some changes were relatively specific for an isoform, some showed dose response effects that correlated with *At*IPCS expression levels, and all are genes modulated in response to biotic and abiotic stress.

Analyses of genes identified as responding positively to *AtIPCS* over-expression

Analyses of genes whose expression was positively correlated with *AtIPCS1* over-expression (At1+ and At1++) revealed a significant enrichment (Fisher exact test, two tailed, $p < 0.001$) for those under GO term GO:0015979, *photosynthesis* ($p = 9.10^{E-27}$) 25.7% (18/70) when they represent 0.43% of the *Arabidopsis* transcriptome (S4 Table). Similarly, for *AtIPC*2 over-expressor transgenic lines (At2+ and At2++) there was a significant enrichment ($p = 1.70^{E-31}$) with 13.3% (30/226) of the genes associated under this GO term (S5 Table). *AtIPC*3 over-expressor lines (At3+ and At3++) also had a significant enrichment under *photosynthesis* ($p = 6.00^{E-18}$)—34.5% (10/29). Other GO terms with significant enrichment for up-regulated genes in response to *AtIPCS1*, *2* or *3* over-expression include: GO:0010876 (*lipid localization*), GO:0006091 (*generation of precursor metabolites and energy*) and GO:0033036 (macromolecular localization) (S4–S6 Tables).

Utilising the differential over-expression in the analysed lines, the influence of *AtIPCS1-3* expression levels on gene up-regulation was analysed. A dose dependent increase in the expression of up-regulated genes under GO term GO:0015979 (*photosynthesis*) was associated with the increase in *AtIPCS1* and *2* transcript found in the transgenic lines (At1+ and At1++, and At2+ and At2++; Table 4). Those showing $\geq$2 $\log_2$ fold change dose response to both *AtIPCS1* and *AtIPCS2* are: YCF4, a PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY (ATCG00520); a SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX, NDHI (ATCG01090); NADH DEHYDROGENASE ND1 NDHA (ATCG01100); PSAC SUBUNIT OF PHOTOSYSTEM I (ATCG01060); 49KDA PLASTID NAD(P)H DEHYDROGENASE SUBUNIT H PROTEIN (ATCG01110); NDHC, NADH DEHYDROGENASE D3 SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX (ATCG00440); YCF3, a PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY (ATCG00360). All isoforms clearly influence the expression of the genes under this GO term and are therefore likely to influence photosynthesis itself. Over-expression of *AtIPCS2* has the broadest and largest effect, correlating again with its status as the most abundant, and perhaps most important, *AtIPCS* isoform (approximately 100-fold *AtIPCS1*) in all tissues of wild type *A*. *thaliana* [4].

The effects of over-expression (At2++) were further analysed and visualised using MapMan (https://mapman.gabipd.org). This illustrated multiple positive transcriptional effects on genes associated with metabolism (S3 Fig), a greater effect than those negatively affected (S2 Fig). Amongst those particularly influenced were genes associated with the metabolism of light reactions and flavonoids. The effects clustered under light reactions correlated with the enrichment under GO term GO:0015979, *photosynthesis* discussed above. Further analyses using MapMan showed that genes up-regulated in At2++ had high enrichment under photorespiration, the Calvin cycle and light reactions (Fig 5). This indicated that there was an associated increase in energy production and, perhaps, the rate of growth.

The MapMan analyses (S3 Fig) also indicated an upregulation of flavonoid metabolism. Flavonoids are antioxidant molecules usually produced as a result of ROS accumulation in response to abiotic and biotic stress [38]. These data indicated that over-expression of *At*IPCS2 may play a protective role in plant defense, not only as a negative regulator of plant pathogen defense genes, but also as a positive regulator of metabolites that have antioxidant properties.

After *photosynthesis*, the next best supported GO term for up-regulated genes across all *AtIPCS* isoform over-expressers was GO:0010876 (*lipid localization*; $p = 8.20^{E-16}$, 1.80^{E-26} and 6.00^{E-18} for *AtIPCS1*, *2* and 3 respectively (S4–S6 Tables). The degree of enrichment across these genes (8/70, 17/275, and 7/19 for *AtIPCS1*, *2* and 3 respectively) was greater than that observed with GO:0015979 (*photosynthesis*); in addition, higher transcript $\log_2$ fold changes correlated with the rise in *AtIPCS* isoform expression levels (Table 5). Increased *AtIPCS1* expression (At1+ to At1++; 200–390 fold wild type Col-0 (Fig 1A) saw $\geq$2 $\log_2$ increase in expression of the following genes under GO:0010876: OLEOSIN 1, 2 and 4 (OLEO1, 2 and 4; AT4G25140, AT5G40420 and AT3G27660); 2S SEED STORAGE PROTEIN 1–4 (AT4G27140, AT4G27150, AT4G27160 and AT4G27170); and LIPID TRANSFER PROTEIN (LPT; AT5G54740). All of these genes were also up-regulated in response to *AtIPCS2* over-expression, including all 4 SEED STORAGE PROTEIN genes (AT4G27140, AT4G27150, AT4G27160 and AT4G27170). However, only isoforms 1, 3 and 4 increased $\geq$2 $\log_2$ in expression on further increase in *AtIPCS2* expression (At2+ to At2++; 7–9 fold wild type Col-0; Fig 1B; Table 5). In addition, LIPID TRANSFER PROTEIN 4 (LTP4; AT5G59310); LIPID BINDING PROTEIN PREDICTED TO ENCODE A PR (PATHOGENESIS-RELATED) protein (LTP6; AT3G08770); and LIPID TRANSFER PROTEIN (LPT;

Table 4. Genes showing a positive correlation with *AtIPCS* expression under GO term GO:0015979 (*photosynthesis*). At1-3+ over-expressing *AtIPCS1-3*; At1-3++ higher level expressers of *AtIPCS1-3*. Fold changes are the value of three technical triplicates of a transformed line. Transcripts listed had a p-value <0.001 (Wald test, cut off p-value < 0.05) and highlighted in bold log_2 fold change ≥ 2.

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value
ATCG00520	ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY	1.3	0.000136	**2**	6.06E-09	**2.4**	3.56E-12	**3.6**	7.23E-26			**2**	1.23E-08
ATCG01090	ENCODES SUBUNIT OF THE CHLOROPLAST NAD (P)H DEHYDROGENASE COMPLEX NDHI	1.5	1.14E-05	**2.5**	1.60E-13	**2.7**	1.01E-15	**4**	4.89E-33			**2.2**	6.40E-11
ATCG00540	ENCODES CYTOCHROME F APOPROTEIN	1.1	0.00043	1.9	3.70E-09	**2.2**	1.72E-12	**3.4**	7.67E-27			1.7	3.65E-08
ATCG01010	CHLOROPLAST ENCODED NADH DEHYDROGENASE UNIT. NDHF					**2.2**	2.06E-10	**3.4**	7.33E-22			1.8	2.59E-07
ATCG00280	CHLOROPLAST GENE ENCODING A CP43 SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER	1.5	2.31E-06	1.6	1.08E-06	1.9	2.22E-09	**3**	1.05E-20			1.7	7.88E-08
ATCG00680	ENCODES FOR CP47, SUBUNIT OF THE PHOTOSYSTEM II REACTION CENTER	1	0.000857	1.2	0.000146	1.5	8.93E-07	**2.5**	2.18E-15			1.3	2.90E-05
AT4G28660	PSB28 (PHOTOSYSTEM II REACTION CENTER PSB28 PROTEIN)					1.6	6.17E-59	1.2	8.53E-37	0.5			
ATCG01100	NADH DEHYDROGENASE ND1 NDHA	1.6	2.92E-06	**2.6**	2.62E-15	**2.9**	1.82E-18	**4**	3.49E-33			**2.4**	9.53E-13
AT3G04790	RIBOSE 5-PHOSPHATE ISOMERASE-RELATED					1.8	1.32E-101	1.3	6.95E-58	0.8		1.3	1.00E-52
ATCG00270	PSII D2 PROTEIN PSBD	1.8	5.79E-08	1.9	4.30E-09	**2.4**	7.28E-13	**3.4**	1.13E-25			**2.1**	3.43E-10
ATCG01060	ENCODES THE PSAC SUBUNIT OF PHOTOSYSTEM I	1.3	5.19E-05	**2.2**	3.40E-12	**2.6**	8.05E-17	**3.6**	2.28E-30			**2.1**	1.50E-11
AT3G55800	SBPASE (SEDOHEPTULOSE-BISPHOSPHATASE);					1.2	1.64E-100	1	4.49E-67	0.3			
AT4G09650	ATPD (ATP SYNTHASE DELTA-SUBUNIT GENE);					1.5	7.39E-119	1.2	3.91E-76	0.3		1.1	3.83E-57
ATCG00730	A CHLOROPLAST GENE ENCODING SUBUNIT IV OF THE CYTOCHROME B6	1.3	2.93E-05	1.6	1.02E-06	1.9	4.38E-09	**2.8**	6.43E-19			1.6	3.63E-07
ATCG01080	NADH DEHYDROGENASE ND6 NDHG					1.9	9.92E-08	**3.1**	5.52E-19			1.5	1.37E-05
ATCG00710	ENCODES A 8 KD PHOSPHOPROTEIN	1	0.000866	1.2	1.80E-06	1.7	5.64E-08	**2.8**	1.16E-17			1.5	6.42E-06
AT2G01590	CRR3 (CHLORORESPIRATORY REDUCTION 3)					1.2	1.06E-40	1	1.79E-31				
ATCG00580	PSII CYTOCHROME B559	1.1	0.0003654	1.3	8.33E-05	1.8	8.75E-09	**2.4**	2.18E-13			1.3	2.94E-05
ATCG00720	ENCODES THE CYTOCHROME B(6) SUBUNIT OF THE CYTOCHROME B6F COMPLEX	1.1	0.0005436	1.4	5.73E-06	1.7	7.29E-08	**2.9**	4.62E-20			1.4	5.58E-06
AT3G01440	PSB-LIKE PROTEIN 2 (PQL2)					1.8	7.06E-107	1.5	2.18E-13	0.5	4.69E-10	1.3	2.89E-50
AT1G14150	PSB-LIKE PROTEIN 1 (PQL1)					1.5	3.82E-75	1.2	1.54E-44	0.5	7.91E-09	1	4.06E-35

(*Continued*)

Table 4. (Continued)

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value
ATCG01110	ENCODES THE 49KDA PLASTID NAD(P)H DEHYDROGENASE SUBUNIT H PROTEIN	**2.8**	3.73E-19	**4**	4.72E-37	**4.2**	1.35E-40	**4.7**	5.23E-52	1	0.001626	**3.6**	5.95E-30
AT1G19150	LHCA6; CHLOROPHYLL BINDING PSI TYPE II CHLOROPHYLL A/B-BINDING PROTEIN	-		1.2		**2**	2.50E-114	1.6	5.98E-73	0.7	1.71E-15	1.5	1.95E-66
AT1G60950	FED A; 2 IRON, 2 SULFUR CLUSTER BINDING					1.6	1.03E-63	1.5	1.68E-54			1.2	1.99E-33
ATCG00300	ENCODES PSBZ, WHICH IS A SUBUNIT OF PHOTOSYSTEM II	1.1	0.0022523	1.6	6.17E-06	1.7	8.98E-07	**3.2**	1.04E-19			1.6	2.72E-06
ATCG00440	ENCODES NADH DEHYDROGENASE D3 SUBUNIT OF THE CHLOROPLAST NAD(P)H DEHYDROGENASE COMPLEX NDHC	1.3	0.0001679	**2**	3.59E-09	**2.7**	6.65E-16	**3.4**	4.14E-24			1.9	1.08E-08
ATCG00340	ENCODES THE D1 SUBUNIT OF PHOTOSYSTEM I AND II REACTION CENTERS. PSAB	1.2	0.0001204	1.6	1.29E-06	1.8	1.35E-08	**2.9**	4.71E-19			1.6	1.30E-06
ATCG00360	ENCODES A PROTEIN REQUIRED FOR PHOTOSYSTEM I ASSEMBLY AND STABILITY	1.5	6.99E-06	**2.3**	5.20E-12	**2.7**	1.25E-15	**3.8**	1.03E-29			**2.1**	4.94E-10
AT3G16250	NDF4 (NDH-DEPENDENT CYCLIC ELECTRON FLOW 1);					1.4	1.02E-79	1.1	5.91E-52	0.4	1.70E-07		
AT3G62410	PROTEIN BINDING CP12-2 ENCODES A SMALL PEPTIDE FOUND IN THE CHLOROPLAST STROMA					1.8	5.62E-79	1.4	5.57E-49	0.3	0.006318	1.5	2.39E-51

https://doi.org/10.1371/journal.pone.0217087.t004

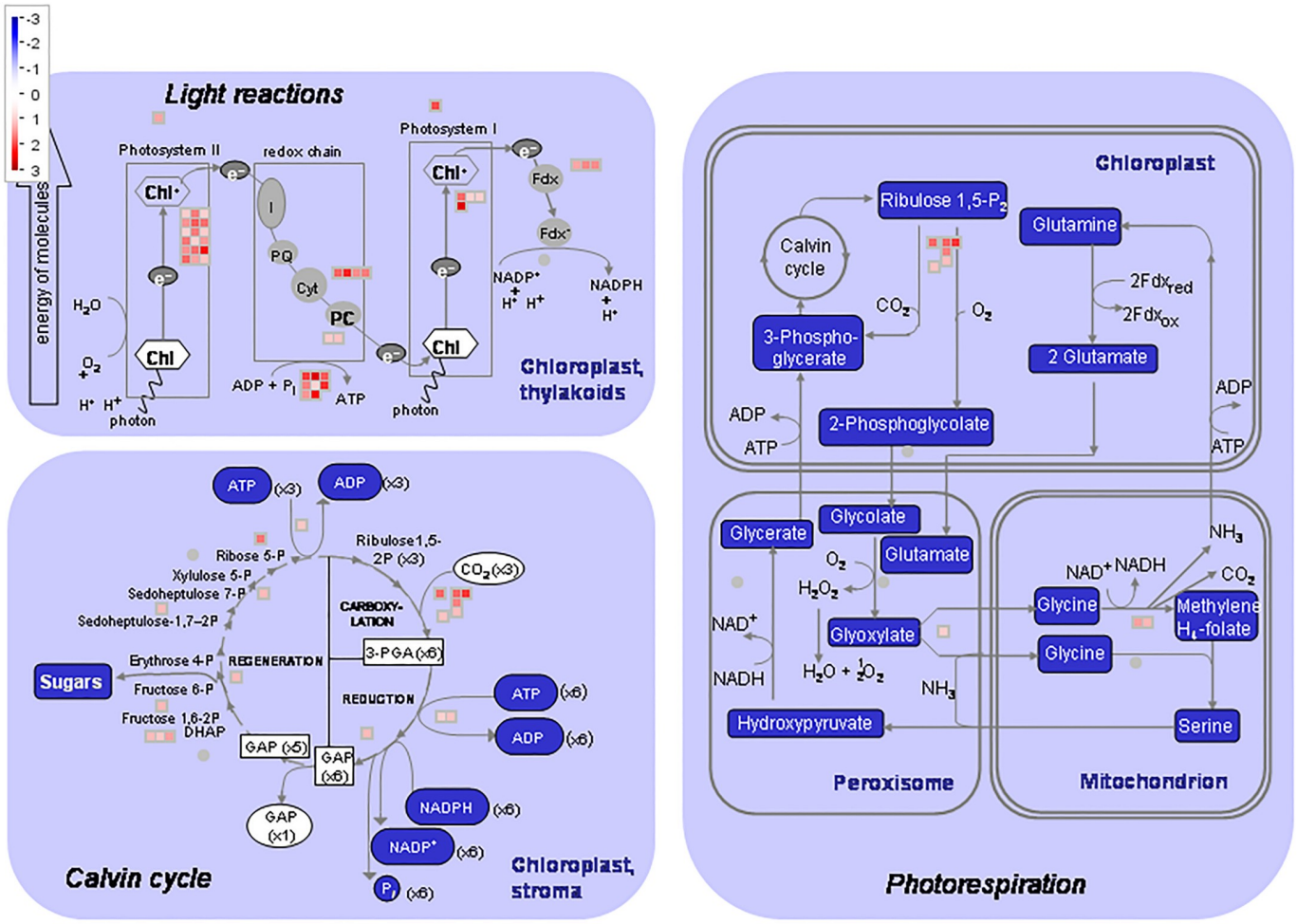

Fig 5. MapMan schematic of showing genes identified as upregulated in the At2++ over-expression line enriched under the plant light reaction, Calvin cycle and photorespiration. Log_2 fold changes in gene expression are indicated by the colour scale.

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AT5G55410 and AT2G37870) are all up-regulated ≥2 log_2 on over-expression of this isoform. None of these are increased ≥2 log_2 in the higher *AtIPCS2* expressors, however LPT4 is decreased. As above, *AtIPCS2* over-expression had the broadest effect on the selected genes (GO:0010876), again presumably due to its high levels in all tissues of wildtype *A. thaliana* [4]. All 4 SEED STORAGE PROTEIN genes, LTP (AT5G54740) and OLEO1 and 4, are up-regulated in response to *AtIPCS3* over-expression. Furthermore, all are further up-regulated (≥2 log_2) on increased expression (At3+ to At3++; 220–440 fold wild type Col-0; Fig 1C; Table 5). Although isoform specific effects, particularly with respect to *AtIPCS2*, were observed, over-expression of each lead to an up-regulation in expression of genes associated with GO term GO:0010876 (*lipid localization*). Analyses of the genes up-regulated in all over-expression lines demonstrated that they mainly encode seed storage proteins (Table 6), including CRUCIFERIN 2 and 3 genes (*CRU2* and *3*). *CRU2* and *3* are expressed during the later stages of embryogenesis [39], with CRU3 having a role in protein and oil storage [40]. Together, these data indicated that the increased metabolic activity, perhaps induced by *At*IPCS over-expression (S3 Fig), may result in an increase in protein and lipid storage during seed development.

Table 5. Genes showing a positive correlation with *AtIPCS* expression under GO term GO:0010876 (*lipid localization*). At1-3 over-expressing *AtIPCS1-3*; At1-3 + higher level expressers of *AtIPCS1-3*. Fold changes are the value of three technical triplicates of a transformed line. Transcripts listed had a p-value <0.001 (Wald test, cut off p-value < 0.05) and highlighted in bold $\log_2$ fold change ≥ 2.

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value	$\log_2$fold	p-value
AT5G59310	LTP4 (LIPID TRANSFER PROTEIN 4)					**3.7**	2.07E-28	1	0.00358				
AT5G48490	DIR1 LIPID TRANSFER PROTEIN					1.2	4.21E-16	1.3	4.78E-18				
AT3G08770	LTP6; LIPID BINDING PREDICTED TO ENCODE A PR (PATHOGENESIS-RELATED) PROTEIN					**2**	3.96E-65	1.3	4.44E-27				
AT5G40420	OLEO2 (OLEOSIN 2)	1.4	1.45E-07	**4.6**	3.63E-70	**2.5**	9.95E-21	**2.8**	5.74E-25				
AT4G25140	OLEO1 (OLEOSIN 1)	**3.5**	4.38E-45	**7.1**	2.82E-192	**4.6**	2.85E-80	**5**	1.20E-91	**2.5**	1.01E-21	**6.1**	3.11E-141
AT4G27140	2S SEED STORAGE PROTEIN 1	**4.2**	5.35E-65	**8**	1.03E-238	**4.3**	1.28E-68	**6.3**	1.95E-148	**3**	4.86E-32	**7.2**	6.10E-193
AT4G27150	2S SEED STORAGE PROTEIN 2	**3.8**	2.16E-40	**7.3**	2.16E-147	**4.7**	1.09E-61	**5.4**	1.89E-81	**2.3**	8.24E-16	**6.4**	7.03E-115
AT4G27160	2S SEED STORAGE PROTEIN 3	**5**	1.84E-81	**8.5**	2.51E-239	**4.2**	4.18E-58	**6.3**	1.28E-129	**3.6**	4.03E-43	**7.3**	2.59E-176
AT5G64080	LPT (LIPID TRANSFER PROTEIN)					1.6	1.28E-30	1.2	3.85E-18				
AT5G05960	LPT (LIPID TRANSFER PROTEIN)					1.7	4.62E-51	1.2	6.31E-26				
AT3G18280	LPT (LIPID TRANSFER PROTEIN)					1.3	7.33E-28	1.1	3.46E-19				
AT5G54740	LPT (LIPID TRANSFER PROTEIN)	**5.1**	3.42E-97	**8.2**	3.86E-259	**5.8**	1.57E-127	**6.3**	5.98E-153	**3.9**	4.20E-57	**7.7**	9.99E-230
AT5G55410	LPT (LIPID TRANSFER PROTEIN)					**2.2**	4.37E-12	**2**	1.50E-09				
AT4G27170	2S SEED STORAGE PROTEIN 4	**2.7**	1.03E-19	**5.7**	5.84E-88	1.8	6.48E-09	**3.9**	2.71E-39	**1.8**	6.80E-09	**4.9**	9.93E-64
AT3G27660	OLEO4 (OLEOSIN 4)	**2.1**	1.35E-15	**5.2**	7.26E-100	**2.8**	6.81E-27	**2.6**	5.28E-23	1.1	8.65E-05	**4**	2.28E-58
AT2G37870	LPT (LIPID TRANSFER PROTEIN)					**2.9**	2.88E-27	1.5	4.73E-08				
AT3G01570	OLE05 (OLEOSIN 5)					1.5	8.26E-13	1.3	1.92E-08				

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Phenotype of *Arabidopsis* over-expressing *AtIPCS* isoforms

To examine the effects of these global alterations in gene expression on plant development, the phenotypes of *A. thaliana* over-expressing each of the *AtIPCS* isoforms (both levels) were analysed. All lines showed early flowering (4 days earlier than wild type Col-0) associated with the formation of bolts (Fig 6). This correlated with a slight (<2-fold $\log_2$) up-regulation of the florigen FT (see data deposited in GEO, Accession GSE129016), a well characterized systematic signal for plant transition from the vegetative to the reproductive (flowering) phase [41]. However, the mechanism underlying this phenotype remains unclear, and perhaps reflects the metabolic changes indicated in the analyses above.

Table 6. Genes up-regulated in all three *AtIPCS* over-expression lines compared to Col0. At1-3+ over-expressing *AtIPCS1-3*; At1-3++ higher level expressers of *AtIPCS1-3*. Fold changes are the value of three technical triplicates of a transformed line. Transcripts listed had a p-value <0.001 (Wald test, cut off p-value < 0.05).

GeneID	Gene annotation	At1+		At1++		At2+		At2++		At3+		At3++	
		log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value	log_2fold	p-value
AT1G03880	CRUCIFERIN 2	2.4	1.77E-17	6.9	2.47E-149	2.8	1.65E-22	4.4	2.05E-59	1.9	1.19E-10	5.5	6.22E-96
AT1G14940	POLYKETIDE CYCLASE	1.3	0.00013457	3.9	3.80E-37	2.9	1.09E-19	1.2	0.000415868	1.2	0.000363067	3.9	5.89E-37
AT1G68250	UNCHARACTERIZED	3.3	1.29E-35	5.5	4.67E-100	3.3	8.42E-35	4.7	2.00E-72	1.6	1.42E-08	4.7	1.07E-72
AT1G75830	PDF1.1	2.7	3.86E-42	6.1	4.61E-234	4.3	6.31E-116	4.1	6.34E-102	1.6	6.48E-15	5.3	1.15E-174
AT4G27150	2S SEED STORAGE PROTEIN 2	3.8	2.16E-40	7.3	2.16E-147	4.7	1.09E-61	5.4	1.89E-81	2.3	8.24E-16	6.4	7.03E-115
AT2G27380	EPR1	2.9	3.08E-31	6.0	6.18E-135	5.1	6.03E-100	2.9	4.27E-33	1.5	4.85E-09	5.6	8.36E-120
AT3G27660	OLEO4 (OLEOSIN 4)	2.1	1.35E-15	5.2	7.26E-100	2.8	6.81E-27	2.6	5.28E-23	1.1	8.65E-05	4.0	2.28E-58
AT4G25140	OLEO1 (OLEOSIN 1)	3.5	4.38E-45	7.1	2.82E-192	4.6	2.85E-80	5.0	1.20E-91	2.5	1.01E-21	6.1	3.11E-141
AT4G28520	CRU3	5.8	9.91E-131	9.6	0	6.0	5.56E-142	7.3	9.09E-207	4.2	6.51E-67	8.3	2.55E-271
AT5G44120	CRA1	5.6	2.15E-133	9.0	0	6.8	2.63E-201	6.1	5.74E-158	3.6	1.13E-55	7.9	9.89E-267
AT3G22640	PAP85	3.2	2.74E-52	5.5	5.80E-166	3.2	9.47E-51	2.6	1.55E-32	1.5	6.57E-11	4.1	4.59E-89
AT4G27160	2S SEED STORAGE PROTEIN 3	5.0	1.84E-81	8.5	2.51E-239	4.2	4.18E-58	6.3	1.28E-129	3.6	4.03E-43	7.3	2.59E-176
AT4G27140	2S SEED STORAGE PROTEIN 1	4.2	5.35E-65	8.0	1.03E-238	4.3	1.28E-68	6.3	1.95E-148	3.0	4.86E-32	7.2	6.10E-193
AT4G27170	2S SEED STORAGE PROTEIN 4	2.7	1.03E-19	5.7	5.84E-88	1.8	6.48E-09	3.9	2.71E-39	1.8	6.80E-09	4.9	9.93E-64
AT5G54740	LPT (LIPID TRANSFER PROTEIN)	5.1	3.42E-97	8.2	3.86E-259	5.8	1.57E-127	6.3	5.98E-153	3.9	4.20E-57	7.7	9.99E-230

https://doi.org/10.1371/journal.pone.0217087.t006

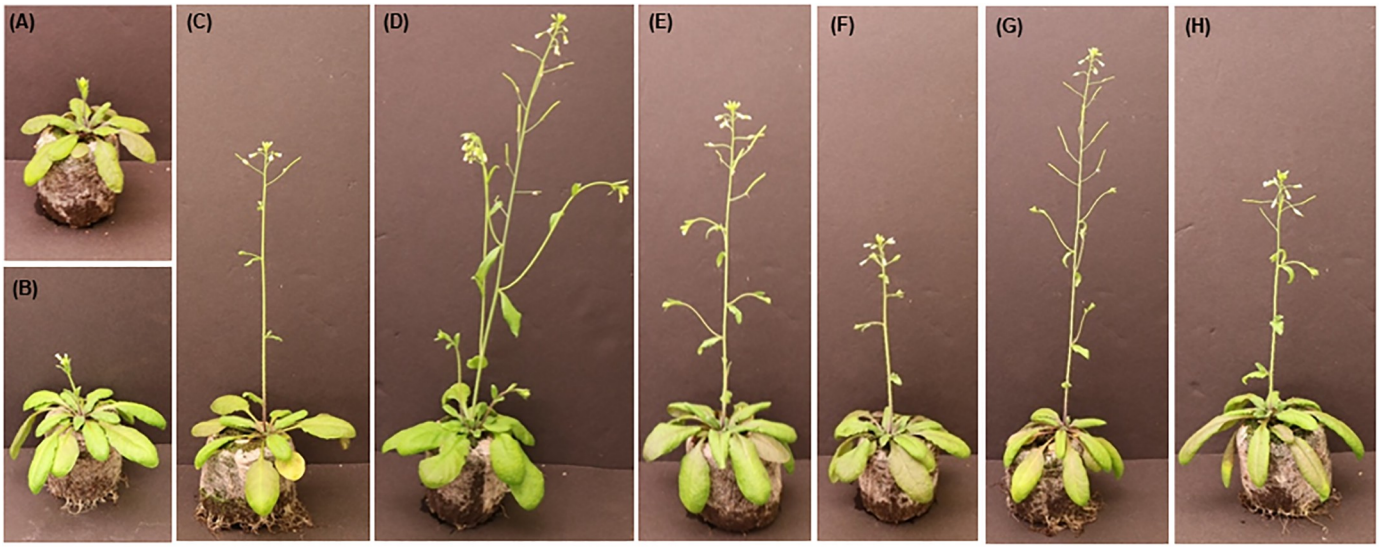

Fig 6. At 44 days wild type Col-0 had flowered (A and B). The *AtIPCS* over-expressing lines had also flowered at this time point, however all had bolted: (C) At1+; (D) At1++; (E) At2+; (F) At2++; (G) At3+; (H) At3++.

https://doi.org/10.1371/journal.pone.0217087.g006

Reflecting the broad negative regulatory effect *AtIPCS* over-expression has on biotic and abiotic stress responses in *Arabidposis* (transcriptomic data—Table 2 and Fig 4), the phenotypes of *Arabidopsis* At1++, At2++ and At3++ were observed under osmotic (abiotic) and pathogen (biotic) stress. Firstly, the over-expressing lines were analysed for tolerance to osmotic stress using the non-ionic osmolyte, mannitol (S4 Fig). In agreement with the down-regulation of genes involved in the abiotic stress response (Table 2; Fig 4), At1++, At2++ and At3++ over-expressing lines were all more susceptible to osmotic stress at high concentrations of mannitol (500mM).

Subsequently, the phenotype of the pathogen response was assessed. Previously, a specific role for *At*IPSC2 in plant resistance to biotrophic pathogens (powdery mildew, *G. cichoracearum*) was proposed. A homozygote *At*IPSC2 T-insert mutant showed a reduction in fungal mass compared with a *G. cichoracearum* infected control, whereas resistance to the hemibiotrophic pathogen *Pseudomonas syringae* was unaffected [13]. The *At*IPSC2 T-insert mutant resistance to *G. cichoracearum* was associated with increased *PR1* [13], whereas as we demonstrated that *AtIPCS* over-expression reduced *PR1* (and *PR2*) expression. This places *AtIPCS* at the centre of the biotic stress response. Therefore, to examine the potential role of *At*IPCS in the necrotophic pathogen response, *Arabidopsis* At1++, At2++ and At3++ were challenged with *Erwinia amylovora*. Interestingly, based on the spread of the pathogen across the surface of the leaves, At2++ and At3++, but not At1++, were less susceptible than the Col0 controls (S5 Fig). At first glance these results appear counter intuitive, the over-expression lines showing down-regulation of the biotic response transcriptome but phenotypically showing a rise in pathogen resistance. Therefore, these data were considered in light of the expression data available in Genevestigator for *At*IPCS1-3 (Fig 7). Response to various stimuli is observed for *AtIPCS2* with a fold increase of up to 600 compared to the highest 100 fold increase observed for *AtIPCS1* during developmental leaf senescence [42]. The highest increase in *AtIPCS2* transcript levels was found to be in response to plants treated with ozone to activate apoplastic reactive oxygen species (ROS) signalling, correlating with the increase in transcript associated with antioxidant flavonoid metabolism seen in At2++ (S3 Fig). *AtIPCS1* also showed an increase in the transcript, although 40-fold lower than *AtIPCS2* [43]. Notably, *AtIPCS1* and *2*

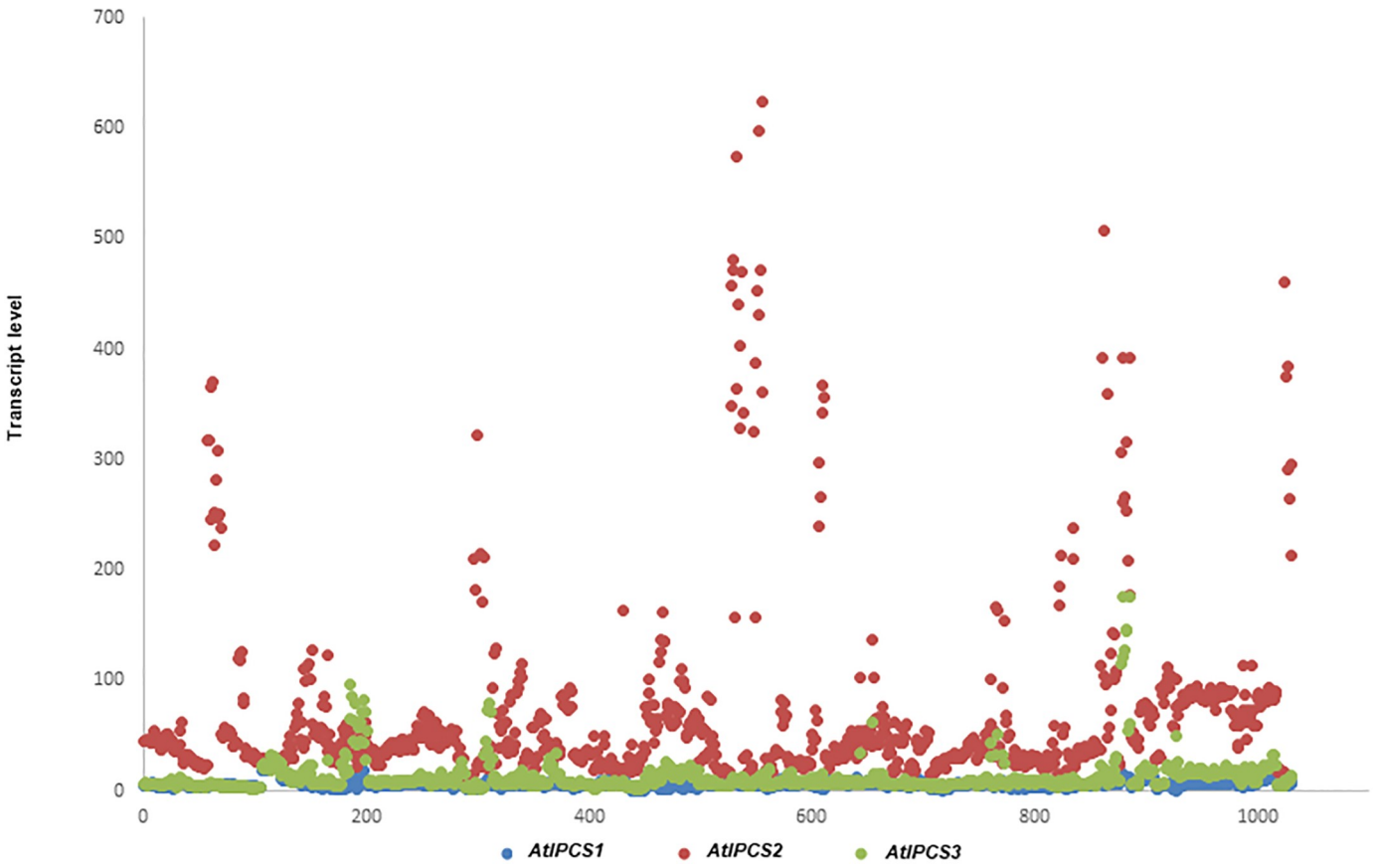

Fig 7. Predicted *AtIPCS1-3* expression in response to pathogens, pathogen effectors and chemical stimuli. x-axis: experiment number; y-axis: transcript level. Produced from RNA-Seq data using Genevestigator.

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transcript increases were also observed in ozone tolerant plants [44]. Other elicitors of *AtIPCS2* transcript increase include the fungi *Botryris cinereal* [45] and *Blumeria graminis* [46], the bacterium *P. syringae* [47], and bacterial flagellin protein [48]. Therefore, increased expression of *AtIPCS2* (and perhaps *AtIPCS*1 and 3) is part of the response to necrotropic, biotrophic and hemibiotropic pathogens. All this in a background of the suppression of the biotic stress response at the transcriptomic level.

Discussion

Each of the 3 *IPCS* isoforms are differentially expressed in the tissues of *A. thaliana* [4], and in *Oryza sativa* (rice) *IPCS* expression in response to specific abiotic stimulus is tissue specific [15]. To further probe the downstream effects of IPCS, in this study we analysed the transcriptomic response to the over-expression of *AtIPCS1*, *2* and *3*.

Multiple genes responded both positively and negatively, and specifically, in response to elevated *AtIPCS1*, *2* and *3*. Analyses of genes up-regulated in response to *AtIPCS* over-expression showed most enrichment under GO term GO:0010876 (*lipid localization*), with levels showing strong correlation with increased expression of *AtIPCS1* and *3*, indicating a regulatory (rheostat) function (Table 5). Specifically, 2S SEED STORAGE PROTEIN 1, 2, 3 and 4 genes, LIPID TRANSFER PROTEIN gene (*LPT*; AT5G54740) and OLEOSIN 1, 2 and 4 genes (*OLEO1*, *2* and *4*) are up-regulated in response in *AtIPCS1*, and further up-regulated with

increased expression. In addition, all apart from *OLEO2* responded similarly to *AtIPCS3* (Table 5), and all were up-regulated in response to *AtIPCS2* over-expression. Several other *LPT* genes also positively responded to *AtIPCS2* over-expression. *OLE* genes encode oleosins that prevent the abnormal fusion of oil bodies in seeds during imbibition and thereby protect the seeds from undergoing mechanical stress that would result in mortality [49]. Also upregulated were the 2S SEED STORAGE PROTEIN genes which act as nitrogen and sulphur reserves for seeds during germination [50]. Both were particularly influenced by the expression levels of *AtIPCS1* and 3 (Table 5) suggesting these isoforms have a role in seed development. A small number of genes were enriched under GO term GO:000979 (*post-embryonic development*) and up-regulated in response to *AtIPCS1* and *3* (S4 and S6 Tables). An shown in S7 Table, the effects seen with *AtIPCS1* and 3 were again magnified on increased expression. Notably, in addition to those genes already discussed, up-regulated CRUCIFERIN 2 and 3 genes (*CRU2* and *3*) are seed storage proteins expressed during the later stages of embryogenesis [39] with CRU3 having a role in protein and oil storage [40]. From these data, it appears that *AtIPCS* is involved in regulating protein and lipid storage in seeds. This potential role is further supported by the observation that 1-CYSTEIN PEROXIREDOXIN 1 (*AtPER1*) and EXTENSIN PROLINE-RICH 1 (*AtEPR1*) are also up-regulated in response to *AtIPCS*. Both are expressed in the embryo and in developing seeds, providing protection from ROS during seed desiccation [51, 52]. EMBRYOGENIC CELL PROTEIN 31 gene (*AtECP31*) and CALEOSIN PROTEIN gene 1 (*AtCOL1*), both expressed in the later stages of seed development, are important for seed viability and desiccation tolerance [53, 54]. Therefore, *At*IPCS may be important in the protection, viability and therefore the germination of seeds. The mechanisms behind this are not known, however the function of these enzymes in regulating ceramide and phytoceramide levels point towards multiple roles in the signal transduction networks underlying development [5].

The next most enriched genes up-regulated in response to *AtIPCS* over-expression were under GO term GO:0015979 (*photosynthesis*). The importance of this in relation to IPCS functionality is unclear. However, the most compelling change in transcript levels was seen under the influence of *AtIPCS2* over-expression; whilst *AtIPCS3* appeared to function as a rheostat (Table 4) with increased expression inducing the up-regulation of all 6 genes influenced ($\geq$2 $\log_2$). Notably this isoform is least expressed in rosette and cauline leaves of *A*. *thaliana* [4] perhaps indicating a role of up-regulation in photosynthetic regulation. However, the mechanism behind this possible function is unclear.

To further visualise the possible effects of *At*IPCS over-expression, and given the scale and scope of its influence on the transcriptome, the *AtIPCS2* higher level over-expressor (At2++) data was analysed using MapMan (S3 Fig). The expression of many genes associated with metabolism were positively influenced, particularly those associated light reactions and antioxidative flavonoids. Further analyses showed up-regulated genes to be enriched under photorespiration, the Calvin cycle and light reactions (Fig 5), perhaps positively influencing energy production and the rate of growth. Interestingly, all *At*IPCS over-expressing lines showed bolts (Fig 6) perhaps indicating accelerated growth, however this requires further analysis.

Flavonoid metabolism was also up-regulated in At2++ *Arabidopsis* at the transcriptome level, and these antioxidant molecules are synthesized to protect plant tissues from ROS produced in response to abiotic and biotic stress [38]. Therefore, over-expression of *At*IPCS2 may also have a protective role in plant defense, a stress response. Interestingly, and perhaps correlating with this, on over-expression of each of the isoforms down-regulated genes were significantly enriched under GO term GO:0006950 (*response to stress*) (S1–S3 Tables). These included Pathogenesis-Related (*PR*) genes, *PR1* and 2 were particularly effected and transcript levels were reduced $\geq$2 $\log_2$ (Table 2), this effect was magnified on increased expression of

AtIPCS1 suggesting that this isoform may have a regulatory role in the stress response. (Table 2). Systematic Acquired Resistance (SAR) is characterised by the increased expression of the *PR* genes which are induced in response to elevated endogenous growth hormones such as salicylic acid (SA) and ethylene (ET), the levels of which increase in response to infection [55]. *A. thaliana* manipulated to express elevated levels of *PR1*, *2* and *5* are resistant to the oomycete obligate biotroph *Hyaloperonospora parasitica* and the bacterium biotroph *Pseudomonas syringae pv. Maculicola* [56]. Furthermore, *PR* genes are also induced by environmental stress such as cold and light [57]; *PR1*, *PR2* and *PR5* were induced in cold treated and drought stressed *A. thaliana* [58, 59].

Plant Defensin genes, *PDF1.2B* and an ET- AND JA-RESPONSIVE PLANT DEFENSIN, are similarly repressed in response to over-expression of *AtIPCS1*, *2* and *3*, and again the effect was magnified on increased expression of (rheostatic) *AtIPCS1* (Table 2). Like the *PR* genes, *PDF* genes are markers of SAR induced by endogenous growth hormones in response to biotic and abiotic stress [55]. Wound associated TYROSINE AMINO TRANSFERASE 3 (TAT3) was also induced in *A. thaliana* in response to an endogenous growth hormone (JA) [60], and was down-regulated in response to all isoforms but with a magnified effect on increased expression of *AtIPCS1* (Table 2). LATE UP-REGULATED IN RESPONSE TO *HYALOPERONOSPORA PARASITICA* (*LURP1)* was down-regulated in response to over-expression of all isoforms and, as the name suggests, has been shown to be needed for basal resistance to the oomycete *Hyaloperonospora parasitic* [61]. More specifically PHOSPHOLIPASE 2A (*PLA2A*) expression is negatively regulated only by over-expression of *AtIPCS2*. An orthologue of this enzyme is induced in mosaic virus infected tobacco leaves independently of the growth hormone JA [62] (Table 2).

Similarly, in relation to abiotic stress response, MYB112, which is induced in salt stressed plants [63], responded specifically and negatively to *AtIPC2* over-expression. CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) and GIGANTEA (*GI*), which are part of the photoperiodic control of flowering [64, 65], are specifically negatively regulated by over-expression of *AtIPCS2* and *3*, and *AtIPCS2* respectively (Table 2). Notably, in support of these effects, the *AtIPCS* over-expressing lines displayed an early flowering phenotype.

These data indicating the role of *AtIPCS* over-expression in the suppression of biotic and, to a lesser extent, abiotic stress responses, are supported by the MapMan analyses (Fig 4). At a phenotypic level, the transcriptomic findings correlate with a decreased tolerance for osmotic (abiotic) stress, albeit only at high concentrations of the non-ionic osmolyte, mannitol (S5 Fig). However, the relative resistance of the *At*IPCS2 and 3 over-expressor lines (At2++ and At3++) to challenge with the necrotroph *Erwinia amylovora* (S4 Fig) is difficult to reconcile with the transcriptomic data showing down-regulation of the pathogen response. However, Genevestigator analyses (Fig 7) indicated that increased expression of *AtIPCS2* (and perhaps *AtIPCS*1 and 3) is part of the response to necrotropic, biotrophic and hemibiotropic pathogens. The mode of this in biotic stress response is unclear, however Genevestigator showed that the *AtIPCS2* transcript level positively correlated with ROS signalling, as could the indicated increase in antioxidant flavonoid metabolism noted in this work (S3 Fig). Clearly this warrants further investigation, however it notable that the response to *E. amylovora*, and other pathogens, in *Arabidopsis* includes ROS [66].

Together these data suggest some specificity in the influence of each *AtIPCS* isoform and that *AtIPCS1* expression may act as a rheostat of SAR and the response to biotic and abiotic stress. Furthermore, the observation that *AtIPCS* expression negatively influences both growth hormone dependent (e.g. *PR*) and independent responses (*PL2A*) indicated its role in a wide variety of defence networks perhaps reflecting the role of phytoceramide as an indiscriminate pro-apoptotic signal [13].

Conclusion

Transcriptomic analyses of *A*. *thaliana* indicated that *AtIPCS1-3* over-expression positively correlated with the expression of genes encoding storage proteins essential for normal seed development (S7 Table). As such, the enzyme may be crucial for seed survival, maturation and germination. Furthermore, these data also indicated that *At*IPCS acts as a negative regulator of the plant defense response to pathogens and abiotic stress (Tables 2–4), a process associated with PCD. Importantly, these findings were also corroborated by data available from Genevestigator (Fig 7) and phenotypic observations (S4 and S5 Figs).

The negative association of biotic and abiotic stress responses to *At*IPCS expression indicates the potential to engineer tolerance in crop plants.

Supporting information

S1 Table. GO enrichment of genes down-regulated in response to the constitutive over-expression of *AtIPCS*.
(TIF)

S2 Table. GO enrichment of genes down-regulated in response to the constitutive over-expression of *AtIPCS2*.
(TIF)

S3 Table. GO enrichment of genes down-regulated in response to the constitutive over-expression of *AtIPCS3*.
(TIF)

S4 Table. GO enrichment of genes up-regulated in response to the constitutive over-expression of *AtIPCS1*.
(TIF)

S5 Table. GO enrichment of genes up-regulated in response to the constitutive over-expression of *AtIPCS2*.
(TIF)

S6 Table. GO enrichment of genes up-regulated in response to the constitutive over-expression of *AtIPCS3*.
(TIF)

S7 Table. Genes showing a positive correlation with *AtIPCS* expression under GO term GO: 0009791 (*post-embryonic development*). At1-3+ over-expressing *AtIPCS1-3*; At1-3++ higher level expressers of *AtIPCS1-3*. Log_2 change ≥ 1 shown, ≥ 2 in bold.
(TIF)

S1 Fig. Relative quantitation of mRNA levels of (A) *At*IPCS1 (B) *At*IPCS2 (C) *At*IPCS3 in over-expressor transgenic lines compared to Col0 standardised to equate to a value of 1; qPCR was performed to measure mRNA levels in 10-day old seedlings. Relative quantitation was done after normalization using PEX4 levels; relative quantitation value is the mean of three biological replicates with standard error.
(TIF)

S2 Fig. Metabolisim overview generated in MapMan for genes down-regulated in At2++ transgenic line. Log_2 fold changes in gene expression are indicated by the colour scale, with the distribution of genes in different pathways and expression levels shown. Abbreviations: carbohydrates (CHO), tricarboxylic acid (TCA) cycle, oxidative pentose phosphate (OPP)

pathway, sulphur containing glucosinates synthesis (S-misc), nitrogen containing glucosinate synthesis (N-misc).
(TIF)

S3 Fig. Metabolisim overview generated in MapMan for genes up-regulated in At2++ transgenic line. Log_2 fold changes in gene expression are indicated by the colour scale, with the distribution of genes in different pathways and expression levels shown. Abbreviations: carbohydrates (CHO), tricarboxylic acid (TCA) cycle, oxidative pentose phosphat pathway, sulphur containing glucosinates synthesis (S-misc), nitrogen containing glucosinate synthesis (N-misc).
(TIF)

S4 Fig. 8 day *Arabidopsis thaliana* seedlings treated with the non-ionic osmolyte mannitol for a further 6 days. Col0 and overexpressing lines At1++, At2++ and At3++. Mannitol concentrations in mM. At the highest concentration (500mM) chlorosis in the over-expressing lines, but not Col0, was apparent.
(TIF)

S5 Fig. *Arabidopsis thaliana* leaves challenged with *Erwinia amylovora* 3 dpi. (A) Col0 (B) At1++ (C) At2++ (D) At3++ (E) Plot of ratio of area infected by *Erwinia amylovora* to uninfected area for Col0 and over-expression lines. *At*IPCS2 and 3 over-expressors are less susceptible to the pathogen compared to Col0 and *At*IPCS1 over-expressor, based on the spread of the pathogen across the surface of the leaf. The pathogen has spread to occupy a large area of Col0 and *At*IPCS1 over-expressor leaves, compared to a less aggressive spread seen on the leaves of *At*IPCS2 and 3 over-expressors. In addition, a distinctive yellow colour is observed at the outer boundaries of the area occupied by the pathogen, indicating a measured response that favours plant survival. These observations may be linked to the role of *At*IPCS as a negative regulator of the plant response to biotic stress.
(TIF)

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References

1. Agricultural Development Economics Division FaAo, United Nations. How to feed the world 2050—High level expert forum. 2009.
2. United Nations DoEaSA, Population Division. World Population Prospects: The 2017 Revision, Key Findings and Advance Tables ESA/P/WP/248. 2017.
3. Lynch DV, Dunn TM. An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. New phytologist. 2004; 161(3):677–702.
4. Mina J, Okada Y, Wansadhipathi-Kannangara N, Pratt S, Shams-Eldin H, Schwarz R, et al. Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase. Plant molecular biology. 2010; 73(4–5):399–407. https://doi.org/10.1007/s11103-010-9626-3 PMID: 20309609
5. Young SA, Mina JG, Denny PW, Smith TK. Sphingolipid and ceramide homeostasis: potential therapeutic targets. Biochem Res Int. 2012; 2012:248135.
6. Markham JE, Jaworski JG. Rapid measurement of sphingolipids from Arabidopsis thaliana by reversed-phase high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 2007; 21(7):1304–14. https://doi.org/10.1002/rcm.2962
7. Liang H, Yao N, Song JT, Luo S, Lu H, Greenberg JT. Ceramides modulate programmed cell death in plants. Genes & development. 2003; 17(21):2636–41.
8. Dietrich CR, Han G, Chen M, Berg RH, Dunn TM, Cahoon EB. Loss-of-function mutations and inducible RNAi suppression of Arabidopsis LCB2 genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. The Plant Journal. 2008; 54(2):284–98. https://doi.org/10.1111/j.1365-313X.2008.03420.x PMID: 18208516
9. Coupe SA, Watson LM, Ryan DJ, Pinkney TT, Eason JR. Molecular analysis of programmed cell death during senescence in Arabidopsis thaliana and Brassica oleracea: cloning broccoli LSD1, Bax inhibitor and serine palmitoyltransferase homologues. Journal of Experimental Botany. 2004; 55(394):59–68. https://doi.org/10.1093/jxb/erh018 PMID: 14645391
10. Dutilleul C, Benhassaine-Kesri G, Demandre C, Rézé N, Launay A, Pelletier S, et al. Phytosphingosine-phosphate is a signal for AtMPK6 activation and Arabidopsis response to chilling. New Phytologist. 2012; 194(1):181–91. https://doi.org/10.1111/j.1469-8137.2011.04017.x PMID: 22236066
11. Berkey R, Bendigeri D, Xiao S. Sphingolipids and plant defense/disease: the "death" connection and beyond. Front Plant Sci. 2012; 3(68):68.
12. Bromley PE, Li YO, Murphy SM, Sumner CM, Lynch DV. Complex sphingolipid synthesis in plants: characterization of inositolphosphorylceramide synthase activity in bean microsomes. Archives of Biochemistry and Biophysics. 2003; 417(2):219–26. PMID: 12941304
13. Wang W, Yang X, Tangchaiburana S, Ndeh R, Markham JE, Tsegaye Y, et al. An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in Arabidopsis. The Plant Cell. 2008; 20(11):3163–79. https://doi.org/10.1105/tpc.108.060053 PMID: 19001565
14. Tartaglio V, Rennie EA, Cahoon R, Wang G, Baidoo E, Mortimer JC, et al. Glycosylation of inositol phosphorylceramide sphingolipids is required for normal growth and reproduction in Arabidopsis. The Plant Journal. 2017; 89(2):278–90. https://doi.org/10.1111/tpj.13382 PMID: 27643972
15. Liao P, Huang J, Tong P, Nie W, Yan X, Feng Y, et al. Characterization and expression analysis of inositolphosphorylceramide synthase family genes in rice (Oryza sativa L.). Genes & Genomics. 2017; 39 (5):485–92.
16. Karimi M, Inzé D, Depicker A. GATEWAY™ vectors for Agrobacterium-mediated plant transformation. Trends in plant science. 2002; 7(5):193–5.
17. Clough SJ, Bent AF. Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana. The plant journal. 1998; 16(6):735–43. PMID: 10069079
18. Ali HZ, Harding CR, Denny PW. Endocytosis and Sphingolipid Scavenging in Leishmania mexicana Amastigotes. Biochem Res Int. 2012; 2012:691363.
19. Alqaisi AQI, Mbekeani AJ, Llorens MB, Elhammer AP, Denny PW. The antifungal Aureobasidin A and an analogue are active against the protozoan parasite Toxoplasma gondii but do not inhibit sphingolipid biosynthesis. Parasitology. 2018; 145(2):148–55. https://doi.org/10.1017/S0031182017000506 PMID: 28486997

20. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30(15):2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

21. Dobin A, Gingeras TR. Mapping RNA-seq Reads with STAR. Current protocols in bioinformatics. 2015: 11.4. 1-.4. 9.

22. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009; 25(16):2078–9. https://doi.org/10.1093/bioinformatics/btp352 PMID: 19505943

23. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015; 31(2):166–9. https://doi.org/10.1093/bioinformatics/btu638

24. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology. 2014; 15(12):550. https://doi.org/10.1186/s13059-014-0550-8 PMID: 25516281

25. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. Nature methods. 2015; 12(2):115–21. https://doi.org/10.1038/nmeth.3252 PMID: 25633503

26. Ihaka R, Gentleman R. R: a language for data analysis and graphics. Journal of computational and graphical statistics. 1996; 5(3):299–314.

27. Du Z, Zhou X, Ling Y, Zhang Z, Su Z. agriGO: a GO analysis toolkit for the agricultural community. Nucleic acids research. 2010; 38(suppl_2):W64–W70.

28. Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. The Plant Journal. 2004; 37(6):914–39. PMID: 14996223

29. Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, et al. Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Advances in bioinformatics. 2008; 2008.

30. Penninckx I, Eggermont K, Terras F, Thomma B, De Samblanx GW, Buchala A, et al. Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. The Plant Cell. 1996; 8(12):2309–23. https://doi.org/10.1105/tpc.8.12.2309 PMID: 8989885

31. Yang Y, Zhang Y, Ding P, Johnson K, Li X, Zhang Y. The ankyrin-repeat transmembrane protein BDA1 functions downstream of the receptor-like protein SNC2 to regulate plant immunity. Plant physiology. 2012; 159(4):1857–65. https://doi.org/10.1104/pp.112.197152 PMID: 22740615

32. Breitenbach HH, Wenig M, Wittek F, Jorda L, Maldonado-Alconada AM, Sarioglu H, et al. Contrasting roles of apoplastic aspartyl protease AED1 and legume lectin-like protein LLP1 in Arabidopsis systemic acquired resistance. Plant Physiology. 2014:pp. 114.239665.

33. Coursol S, Fan L-M, Le Stunff H, Spiegel S, Gilroy S, Assmann SM. Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature. 2003; 423(6940):651–4. https://doi.org/10.1038/nature01643 PMID: 12789341

34. Egan JF, Maxwell BD, Mortensen DA, Ryan MR, Smith RG. 2, 4-dichlorophenoxyacetic acid (2, 4-D)–resistant crops and the potential for evolution of 2, 4-D–resistant weeds. Proceedings of the National Academy of Sciences. 2011; 108(11):E37–E.

35. Worrall D, Liang YK, Alvarez S, Holroyd GH, Spiegel S, Panagopulos M, et al. Involvement of sphingosine kinase in plant cell signalling. The Plant Journal. 2008; 56(1):64–72. https://doi.org/10.1111/j.1365-313X.2008.03579.x PMID: 18557834

36. Mizoguchi M, Umezawa T, Nakashima K, Kidokoro S, Takasaki H, Fujita Y, et al. Two closely related subclass II SnRK2 protein kinases cooperatively regulate drought-inducible gene expression. Plant Cell Physiol. 2010; 51(5):842–7. https://doi.org/10.1093/pcp/pcq041 PMID: 20375108

37. Lata C, Prasad M. Role of DREBs in regulation of abiotic stress responses in plants. Journal of experimental botany. 2011; 62(14):4731–48. https://doi.org/10.1093/jxb/err210 PMID: 21737415

38. Winkel-Shirley B. Biosynthesis of flavonoids and effects of stress. Current opinion in plant biology. 2002; 5(3):218–23. PMID: 11960739

39. Pang PP, Pruitt RE, Meyerowitz EM. Molecular cloning, genomic organization, expression and evolution of 12S seed storage protein genes of Arabidopsis thaliana. Plant Molecular Biology. 1988; 11 (6):805–20. https://doi.org/10.1007/BF00019521 PMID: 24272631

40. Lin Y, Pajak A, Marsolais F, McCourt P, Riggs CD. Characterization of a cruciferin deficient mutant of Arabidopsis and its utility for overexpression of foreign proteins in plants. PLoS One. 2013; 8(5): e64980. https://doi.org/10.1371/journal.pone.0064980 PMID: 23724110

41. Araki T. Transition from vegetative to reproductive phase. Curr Opin Plant Biol. 2001; 4(1):63–8. PMID: 11163170

42. Brusslan JA, Bonora G, Rus-Canterbury AM, Tariq F, Jaroszewicz A, Pellegrini M. A genome-wide chronological study of gene expression and two histone modifications, H3K4me3 and H3K9ac, during developmental leaf senescence. Plant physiology. 2015; 168(4):1246–61. https://doi.org/10.1104/pp.114.252999 PMID: 25802367

43. Xu E, Vaahtera L, Brosché M. Roles of defense hormones in the regulation of ozone-induced changes in gene expression and cell death. Molecular plant. 2015; 8(12):1776–94. https://doi.org/10.1016/j.molp.2015.08.008 PMID: 26348016

44. Xu E, Vaahtera L, Horak H, Hincha DK, Heyer AG, Brosche M. Quantitative trait loci mapping and transcriptome analysis reveal candidate genes regulating the response to ozone in Arabidopsis thaliana. Plant, cell & environment. 2015; 38(7):1418–33.

45. Liu S, Kracher B, Ziegler J, Birkenbihl RP, Somssich IE. Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards Botrytis cinerea 2100. Elife. 2015; 4:e07295. https://doi.org/10.7554/eLife.07295 PMID: 26076231

46. Maekawa T, Kracher B, Vernaldi S, van Themaat EVL, Schulze-Lefert P. Conservation of NLR-triggered immunity across plant lineages. Proceedings of the National Academy of Sciences. 2012; 109 (49):20119–23.

47. Howard BE, Hu Q, Babaoglu AC, Chandra M, Borghi M, Tan X, et al. High-throughput RNA sequencing of pseudomonas-infected Arabidopsis reveals hidden transcriptome complexity and novel splice variants. PLoS One. 2013; 8(10):e74183. https://doi.org/10.1371/journal.pone.0074183 PMID: 24098335

48. Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, De Lorenzo G, et al. Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. Molecular plant. 2008; 1 (3):423–45. https://doi.org/10.1093/mp/ssn019 PMID: 19825551

49. Shimada TL, Shimada T, Takahashi H, Fukao Y, Hara-Nishimura I. A novel role for oleosins in freezing tolerance of oilseeds in Arabidopsis thaliana. The Plant Journal. 2008; 55(5):798–809. https://doi.org/10.1111/j.1365-313X.2008.03553.x PMID: 18485063

50. Krebbers E, Herdies L, De Clercq A, Seurinck J, Leemans J, Van Damme J, et al. Determination of the processing sites of an Arabidopsis 2S albumin and characterization of the complete gene family. Plant Physiology. 1988; 87(4):859–66. https://doi.org/10.1104/pp.87.4.859 PMID: 16666238

51. Dietz K-J. Plant peroxiredoxins. Annual review of plant biology. 2003; 54(1):93–107.

52. Stacy RA, Nordeng TW, Culiáñez-Macià FA, Aalen RB. The dormancy-related peroxiredoxin anti-oxidant, PER1, is localized to the nucleus of barley embryo and aleurone cells. The Plant Journal. 1999; 19 (1):1–8. PMID: 10417721

53. Poxleitner M, Rogers SW, Lacey Samuels A, Browse J, Rogers JC. A role for caleosin in degradation of oil-body storage lipid during seed germination. The Plant Journal. 2006; 47(6):917–33. https://doi.org/10.1111/j.1365-313X.2006.02845.x PMID: 16961733

54. Yang H, Saitou T, Komeda Y, Harada H, Kamada H. Late embryogenesis abundant protein in Arabidopsis thaliana homologous to carrot ECP31. Physiologia Plantarum. 1996; 98(3):661–6.

55. Durrant WE, Dong X. Systemic acquired resistance. Annu Rev Phytopathol. 2004; 42:185–209. https://doi.org/10.1146/annurev.phyto.42.040803.140421 PMID: 15283665

56. Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. The Plant Cell Online. 1994; 6(12):1845–57.

57. Seo PJ, Lee AK, Xiang F, Park CM. Molecular and functional profiling of Arabidopsis pathogenesis-related genes: insights into their roles in salt response of seed germination. Plant Cell Physiol. 2008; 49 (3):334–44. https://doi.org/10.1093/pcp/pcn011 PMID: 18203731

58. Liu W-X, Zhang F-C, Zhang W-Z, Song L-F, Wu W-H, Chen Y-F. Arabidopsis Di19 functions as a transcription factor and modulates PR1, PR2, and PR5 expression in response to drought stress. Molecular plant. 2013; 6(5):1487–502. https://doi.org/10.1093/mp/sst031 PMID: 23404561

59. Seo PJ, Kim MJ, Park JY, Kim SY, Jeon J, Lee YH, et al. Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. The Plant Journal. 2010; 61(4):661–71. https://doi.org/10.1111/j.1365-313X.2009.04091.x PMID: 19947982

60. Titarenko E, Rojo E, Leon J, Sanchez-Serrano JJ. Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in Arabidopsis thaliana. Plant Physiol. 1997; 115 (2):817–26. https://doi.org/10.1104/pp.115.2.817 PMID: 9342878

61. Knoth C, Eulgem T. The oomycete response gene LURP1 is required for defense against Hyaloperonospora parasitica in Arabidopsis thaliana. The Plant Journal. 2008; 55(1):53–64. https://doi.org/10.1111/j.1365-313X.2008.03486.x PMID: 18346188

62. Dhondt S, Geoffroy P, Stelmach BA, Legrand M, Heitz T. Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. The Plant Journal. 2000; 23(4):431–40. PMID: 10972869

63. Lotkowska ME, Tohge T, Fernie AR, Xue G-P, Balazadeh S, Mueller-Roeber B. The Arabidopsis transcription factor MYB112 promotes anthocyanin formation during salinity and under high light stress. Plant physiology. 2015:pp. 00605.2015.

64. Levy YY, Dean C. Control of flowering time. Current opinion in plant biology. 1998; 1(1):49–54. PMID: 10066558

65. Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, et al. Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. The Plant Cell. 2005; 17(8):2255–70. https://doi.org/10.1105/tpc.105.033464 PMID: 16006578

66. Vrancken K, Holtappels M, Schoofs H, Deckers T, Valcke R. Pathogenicity and infection strategies of the fire blight pathogen Erwinia amylovora in Rosaceae: state of the art. Microbiology. 2013; 159(Pt 5):823–32. https://doi.org/10.1099/mic.0.064881-0 PMID: 23493063