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RESEARCH PAPER

Pathogen and Circadian Controlled 1 (PCC1) regulates polar lipid content, ABA-related responses, and pathogen defence in *Arabidopsis thaliana*

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Abstract

Pathogen and Circadian Controlled 1 (PCC1) was previously characterized as a regulator of defence against pathogens and stress-activated transition to flowering. Plants expressing an RNA interference construct for the *PCC1* gene (iPCC1 plants) showed a pleiotropic phenotype. They were hypersensitive to abscisic acid (ABA) as shown by reduced germination potential and seedling establishment, as well as reduced stomatal aperture and main root length in ABA-supplemented media. In addition, iPCC1 plants displayed alterations in polar lipid contents and their corresponding fatty acids. Importantly, a significant reduction in the content of phosphatidylinositol (PI) was observed in iPCC1 leaves when compared with wild-type plants. A trend in reduced levels of 18:0 and increased levels of 18:2 and particularly 18:3 was also detected in several classes of polar lipids. The enhanced ABA-mediated responses and the reduced content of PI might be responsible for iPCC1 plants displaying a complex pattern of defence against pathogens of different lifestyles. iPCC1 plants were more susceptible to the hemi-biotrophic oomycete pathogen *Phytophthora brassicae* and more resistant to the necrotrophic fungal pathogen *Botrytis cinerea* compared with wild-type plants.

Key words: ABA, *Arabidopsis*, defence, lipids, transcriptome.

Introduction

Plant growth is controlled by endogenous factors as well as through diverse responses to environmental conditions or to biotic interactions. Under photoperiodic growing conditions, the circadian clock machinery controls numerous processes including leaf movement, daily vegetative growth, seed dormancy break, transition to flowering, flower bud opening, stomatal aperture, and sensitivity to light and stress environmental cues (Adams and Carré, 2011). Although light control exerted on those processes shares some common components, significant changes in the respective mechanisms have evolved in each case (Jackson, 2009). For instance, increasing recent evidence suggests that many responses to stress are actually controlled by the circadian clock, including phytopathogenic interactions (Roden and Ingle, 2009; W. Wang *et al.*, 2011), abiotic stress (Sánchez *et al.*, 2011), and wounding (Morker and Roberts, 2011). Moreover, regulation by the circadian clock has also been linked to basal defence against pathogens through salicylic acid (SA)- and phosphate-related responses (G.Y. Wang *et al.*, 2011). *PCC1* was originally identified as a pathogen-responsive gene with a circadian clock-regulated pattern of expression (Sauerbrunn and Schlaich, 2004). PCC1 was further characterized as a potential SA-induced activator of flowering under UV light-stressed conditions (Segarra *et al.*, 2010). Moreover, RNA interference (RNAi) transgenic

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lines with strong down-regulation of *PCC1* gene expression (iPCC1 lines) displayed a late flowering phenotype under non-stressed long-day conditions (Segarra *et al.*, 2010). *PCC1* codes for a small 81 amino acid protein. Its function and mode of action have remained elusive despite its potential involvement in SA-activated defence responses to pathogens and activation of flowering. PCC1 has a cysteine-rich C-terminus (CYSTM) domain proposed to function as a potential site for membrane anchoring and dimerization (Venancio and Aravind, 2010). Here it is reported that the reduced expression of the *PCC1* gene correlates with the appearance of several phenotypes in seeds and leaves, including abscisic acid (ABA)-regulated processes such as seed germination, seedling establishment, and stomatal closure, as well as alterations in the content and composition of polar lipids. Concomitantly, a complex pattern of defence responses to different pathogens was observed in plants with strongly reduced *PCC1* expression, suggesting that PCC1 plays important regulatory roles in controlling metabolic and hormonal responses as well as the execution of defence strategies.

Materials and methods

Plant materials and growing conditions

Transgenic lines expressing RNAi constructs for the *PCC1* gene were previously described (Segarra *et al.*, 2010). Seeds were surface sterilized with 30% bleach and 0.01% Tween-20, washed extensively with miliQ sterile water, and sown in Murashige and Skoog (MS) medium supplemented with 0.8% agar and 1% sucrose. After 3 d of stratification at 4 ºC on MS-containing Petri dishes, seeds were transferred to a growth chamber under white fluorescent light (fluence rate of 70 µmol m^{-2} s^{-1}) with a 16 h light/8 h dark photoperiod and a controlled temperature of 19–23 ºC. Alternatively, stratified seeds were sown on soil and cultivated in environmentally controlled growth chambers under long days (16 h light/8 h dark) or short days (8 h light/16 h dark), as indicated. Plants were grown under a 12 h light/12 h dark photoperiod for pathogen-related experiments.

Generation of pPCC1::GUS transgenic lines

A 1.1 kb fragment of the genomic sequence upstream of the initiation codon of the *PCC1* locus was fused to the β-glucuronidase (GUS) gene using the pMDC162a vector. Primary transformants and further transgenic generation individuals were selected in hygromycin-supplemented MS medium. Three independent homozygous lines were isolated and further used to test *PCC1* expression by GUS staining with X-Gluc in the presence of 5 mM ferricyanide/ferrocyanide redox buffer.

Genome-wide microarray and quantitative real-time PCR (qRT-PCR) analysis

The transcriptomes of iPCC1 lines were compared with that of wild-type seedlings (Col-0 accession) grown *in vitro* under long-day (16 h light/8 h darkness) conditions. Samples were harvested 12 days after sowing (DAS) at 12 h after dawn. Total RNAs from wild-type and iPCC1 plants were extracted with Trizol and purified with an RNeasy kit (QIAGEN). RNAs (three independent biological replicates per genotype) were checked by qRT-PCR for endogenous *PCC1* transcripts and their integrity and purity were further checked by nanocapillary electrophoresis using a Bioanalyzer Agilent 2100. Labelling, hybridization protocols, and statistical analysis are included in a detailed MIAME rules-based description of the microarray experiments in Supplementary Table S1 available at *JXB* online.

To quantify the transcript levels, total RNAs were isolated from wild-type and iPCC1 seedlings and further analysed by qRT-PCR techniques as described previously (Lozano-Juste and León, 2011). Primers used for qPCR were as follows: qMYB28-F, TCCCTGACAAATACTCTTGCT; qMYB28-R, CATTGTGGTTATCTCCTCCGA; qNPR1-F, GATTCGGTTGTGACTGTTTTG; qNPR1-R, TCTCGTTTGTCTTCTTGCTCT; qHRS1-F, TCCGAGGACAAGAACACGAAA; qHRS1-R, TGTCATCGTCTCCTGCTGCAA; qEDS1-F, CGAAGACACAGGGCCGTA; qEDS1-R, AAGCATGATCCGCACTCG; qAOP1-F, TCGAGTTGCCTATTCCGACCAAAC; qAOPI1-2R, TGCTCCGAAAACAGGTGTATCGTCT; qPIF1-F, GTTGCTTTCGAAGGCGGTT; qPIF1-R, GCGCTAGGACTTACCTGCGT; qRGL2-F, GACGGCGCGTAGAGTTCAC; qRGL2-R, TGCATCCCTTGATTAAGCCC; qIRT3-F, GCCCTCACAACCCCGATAG; qIRT3-R, GCTCCGACACTGTGAGAATTGA; qGSTU24-F, CTACTTTGTTATGTTGATCTGTTGTTGC; qGSTU24-R, CATAGACCTCAAAGAAAATAGAACAAAGC; qAt1g44740-F, TCGGCTGGCCAAAAGAAATA; qAt1g44740-R, TCGACCTCTCCTTGGCCTAG; qRPP4-F, GAAGGCACTCAAGGCCTCATTTAC; qRPP4-R, GACAATAATCCCACCATAGCCTTT; qADH1-F, TACCACCGGACAGATTATTCGATGC; qADH1-R, TGGCCGAAGATACGTGGAAACAA; qRAP2.1-F, TCGGGTTTTATTACCGGCGGAGTG; and qRAP2.1-R, TAAGGGAAAAAGCGGCGGAGGT.

In silico analysis of gene ontology and promoter motifs

AgriGO Gene Ontology (GO) tool platforms were used to find significant enrichment of terms for molecular function, biological processes, and cell compartment categories, and the results were confirmed by similar searches in FuncAssociate, ProfCom, and BioMaps. The promoter sequences of the genes differentially expressed in iPCC1 plants were analysed by searching for non-biased over-represented 8-mer motifs using a combination of Bioprospector, DME, MDScan, MEME, and Motif Sampler databases.

Germination assays

To test ABA sensitivity, seeds were sown in MS medium with 1% (w/v) sucrose, 0.8% (w/v) agar, and increasing concentrations of (±)-*cis*,*trans*-ABA (Sigma) after 3 d of stratification at 4 ºC. Germination was scored as the percentage of seeds with visible radical emergence every 24 h. Seedling establishment was assessed by quantifying seedlings with green expanded cotyledons at 15 DAS. To test the effect of osmotic stress, seeds were sown in medium supplemented with 125 mM NaCl or 250 mM mannitol and quantified as above. Seeds harvested at the same time were used to carry out these assays. When indicated, fresh mature seeds were collected and sown on MS plates without stratification.

Water loss assays and stomatal aperture measurements

To quantify water losses by transpiration, 14-day-old iPCC1 and wild-type seedlings, grown in solid MS medium in Petri dishes, were carefully removed from the medium and gently wiped with absorbent paper to remove water and medium residues. Sets of five seedlings were weighed every 3 min for the first 30 min after removal from the medium and then every 10 min up to 1 h. Water losses were quantified by subtracting the weights at each time from the original fresh weight just after removal from the medium. Values are the mean of three independent replicate experiments ±SE.

Plants grown for 10 d under long-day conditions were incubated in stomatal-opening buffer (30 mM KCl and 10 mM MES-KOH, pH 6.1) in 24-well plates (Iwaki, Japan) for 2.5 h under cool-white light (150 µE m^{-2} s^{-1}) at 22 ºC. To induce stomatal closure, plants were incubated in 50 µM ABA for 2.5 h under light. To quantify stomatal aperture, leaves were stained with propidium iodide, and the same

adaxial regions of the first true leaves of four different plants were captured with a TCS SL confocal laser scanning microscope (Leica). Images were used to measure width and length of the stomata aperture with ImageJ software (National Institutes of Health). Aperture was expressed as the length/width ratio. Values are the mean of 35–50 values for each genotype and condition ±SE.

Lipid analysis

To quantify the lipid content, total lipids from *Arabidopsis* leaves (0.5 g) were extracted with chloroform:methanol (1:2, v:v) as described by Bligh and Dyer (1959), and lipid separation was carried out by thin-layer chromatography according to Hernández *et al.* (2008). Fatty acid methyl esters of total lipids or individual lipid classes were produced by acid-catalysed transmethylation (Garcés and Mancha, 1993) and analysed by gas chromatography using a 7890A (Agilent Technologies, Santa Clara, CA, USA) fitted with a capillary column (30 m length; 0.25 mm i.d.; 0.20 μm film thickness) of fused silica (Supelco, Bellafonte, PA, USA) and a flame ionization detector. Hydrogen was used as carrier gas with a linear flux of 1.34 ml min^{-1} and a split ratio of 1/50. The injector and detector temperature was 220 °C, and the oven temperature 170 °C.

Fungal inoculation of plants

Botrytis cinerea B05.10 spores were isolated from *B. cinerea* mycelia and the density was estimated using a Jenssen cell counter. Droplets of 6 μl of freshly prepared spores (4×10^5 spores $ml^{-1}$of PDB medium) were applied on 5–6 leaves per plant and further incubated under high humidity during 3 d. The diameter of *B. cinerea* lesions was then measured using 5–6 different plants per genotype. This process was repeated independently four times.

Droplets of 30 μl of *Phytophthora brassicae* spores (10^6 spores ml^{-1} of sterile water) were applied on expanded leaves of 3- to 4-week old plants. The susceptibility was assessed on a scale of 0–4 of symptoms 7 d after spore application as previously reported (Schlaeppi *et al.*, 2010).

Results

Enhanced ABA-mediated responses in plants with reduced PCC1 expression

Plants containing very low levels of endogenous *PCC1* transcript due to the expression of a RNAi construct for the *PCCI* gene (iPCC1 plants; Segarra *et al.*, 2010) produce seeds with slightly delayed germination when compared with wild-type plants (Fig. 1A). This phenotype has been characterized in detail with a focus on the sensitivity to ABA, a key hormone in controlling seed dormancy and germination. Experiments were conducted to analyse whether iPCC1 plants displayed altered responses to ABA. Seeds of iPCC1 plants showed reduced germination rates compared with wild-type seeds upon treatment with ABA (Fig. 1A). The hypersensitivity of iPCC1 seeds to ABA seems to be reflected in a delay in radicle emergence and also in a reduced germination potential (Fig. 1A). The hypersensitivity of iPCC1 seeds to ABA in seed germination assays was observed at concentrations as low as 0.2 μM and remained significant up to 1 μM ABA (Fig. 1A). Since seed stratification leads to a decrease in endogenous ABA content, and thus promotes germination (Gubler *et al.*, 2005), the germination rates of fresh wild-type and iPCC1 seeds with no previous stratification treatment were also analysed. Figure 1B shows that the germination rate of non-stratified wild-type seeds was similar to that detected in stratified seeds treated with 0.5 μM ABA (Fig. 1A), thus suggesting that under these conditions endogenous ABA levels keep seeds partially dormant. Importantly, non-stratified iPCC1 seeds germinate at even lower rates than non-stratified wild-type seeds, thus supporting that iPCC1 seeds are more dormant than wild-type seeds. The findings suggest that PCC1 would promote seed germination acting as a negative regulator of the ABA-imposed break to germination.

Seedling establishment was also significantly reduced upon ABA treatment (Fig. 1C). At 0.2 μM ABA, ~55% of the wild-type seedlings but only 20% of the iPCC1 seedlings established (Fig. 1C). Furthermore, iPCC1 seedling establishment was <5% when they were grown on 0.5 μM ABA-containing plates, whereas up to 25% of wild-type seedlings established under these conditions (Fig. 1C). The effect of osmolytes such as mannitol and NaCl on wild-type and iPCC1 seedling establishment was also tested. In the presence of 250 mM mannitol, all three iPCC1 lines established significantly less than wild-type seedlings, but only seedlings of iPCC1 line 25.4 established significantly less than wild-type seedlings in the presence of 125 mM NaCl (Supplementary Fig. S1 at *JXB* online).

In agreement with the enhanced ABA-related responses detected in seeds, stomata of iPCC1 leaves were significantly more closed than those of the wild type under non-stressed conditions (Fig. 2A). iPCC1 stomata were still responsive to ABA. At 50 μM ABA, stomatal closure values in iPCC1 leaves were similar to those observed for ABA-treated wild-type stomata (Fig. 2A). However, at 5 μM ABA, iPCC1 stomata were significantly more closed than those of the wild type (Fig. 2A), thus indicating hypersensitivity to ABA. In connection with the altered stomatal aperture phenotype, it was tested whether iPCC1 plants lost less water by transpiration than wild-type plants. Surprisingly, water loss of iPCC1 seedlings was not significantly different from that of wild-type seedlings under water deficit conditions (Supplementary Fig. S2 at *JXB* online). Despite iPCC1 plants showing ABA-related phenotypes in shoots, the lengths of roots of iPCC1 plants grown on ABA-supplemented media were also analysed and compared with those of wild-type plants. To avoid the effect of ABA on germination, seeds were first sown on ABA-free MS vertical plates and grown for 5 d. Then, seedlings were carefully transferred to different root growth conditions. Root length was scored after 10 d on ABA-supplemented medium. Figure 2B shows that root length on MS medium was similar for wild-type and iPCC1 seedlings. Compared with control MS plates, root length of wild-type plants was reduced ~50% and 70% on 15 μM and 30 μM ABA-supplemented plates, respectively. The reduction of root length of all three different iPCC1 plants was on average 70% and 85% on 15 μM and 30 μM ABA plates, respectively (Fig. 2B). These data confirm that either seeds, shoots, or roots of iPCC1 seedlings were hypersensitive to ABA, thus suggesting that PCC1 must exert a negative regulatory effect on ABA signalling.

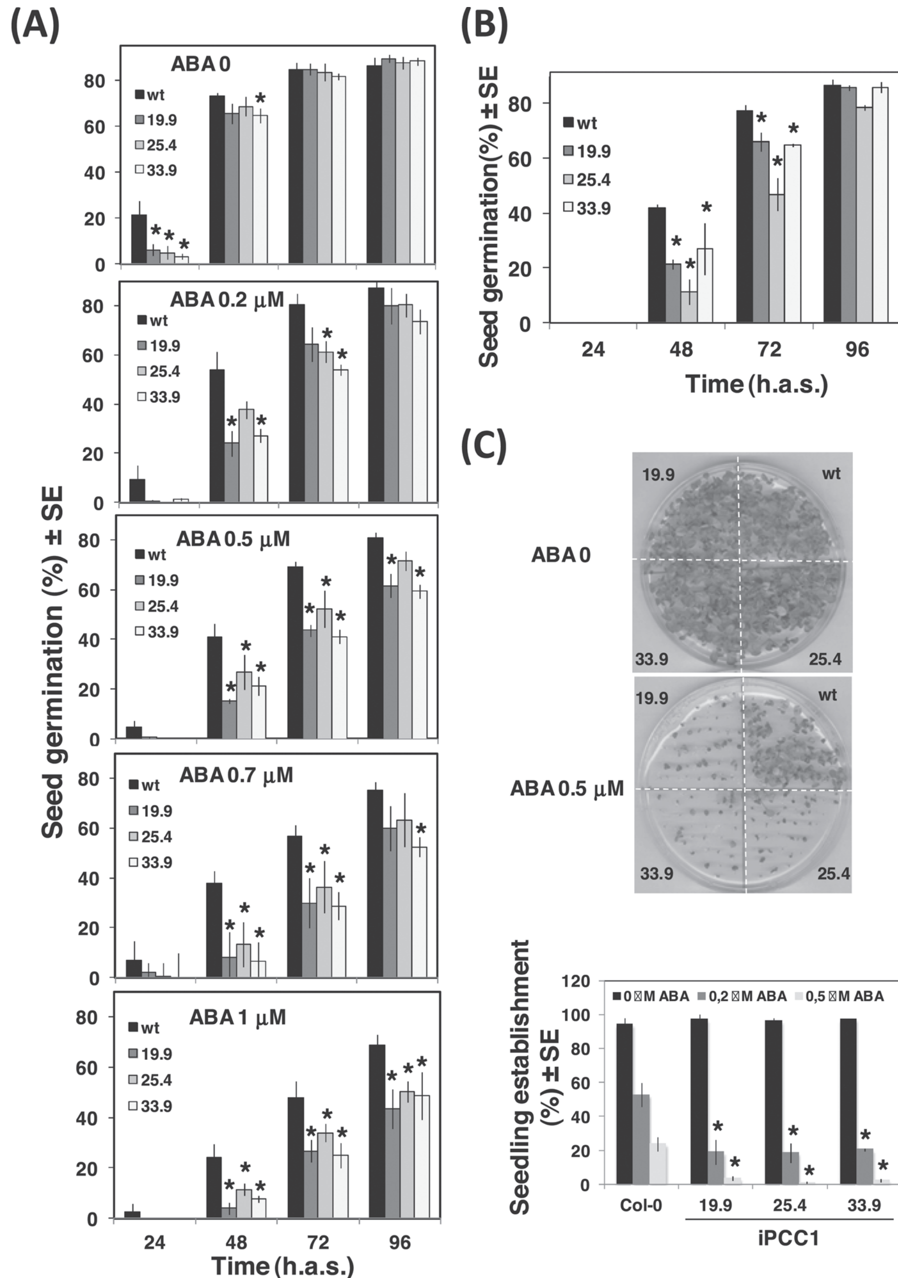

Fig. 1. Wild-type and iPCC1 seed germination and seedling establishment. (A) Germination rates of wild-type (wt) and iPCC1 (lines 19.9, 25.4, and 33.9) seeds at the indicated ABA concentrations and times in hours after sowing (h.a.s.). Values represent the mean of the main seed germination (%) of four independent replicates (populations of 50 seeds each) per genotype and condition ±SD. (B) Germination of non-stratified seeds was calculated from the percentage of germination of fresh seeds that were sown without stratification treatment. (C) Seedling establishment was calculated as the percentage of seedlings with open green cotyledons by 10 DAS at the indicated ABA concentrations. *Mean significantly different in iPCC1 seeds when compared with a similar condition for wild-type seeds with a *P*-value <0.05 by Student's *t*-test.

iPCC1 plants display altered defence against pathogens

The *PCC1* gene was originally identified and characterized as a pathogen-responsive gene that was up-regulated upon infection with *P. syringae* DC3000 carrying the *avrRpt2* gene, and its overexpression in *Arabidopsis* confers resistance to the oomycete *Peronospora parasitica* (Sauerbrunn and Schlaich, 2004). On the other hand, as mentioned above, iPCC1 plants displayed enhanced ABA-mediated responses (Figs 1, 2) and ABA has recently been characterized as an important regulator of defence against a wide array of pathogens (Cao *et al.*, 2011). The resistance phenotype of iPCC1 plants against pathogens with different lifestyles has also been analysed here. As expected by the enhanced resistance of *PCC1*-overexpressing plants to the oomycete *P. parasitica*

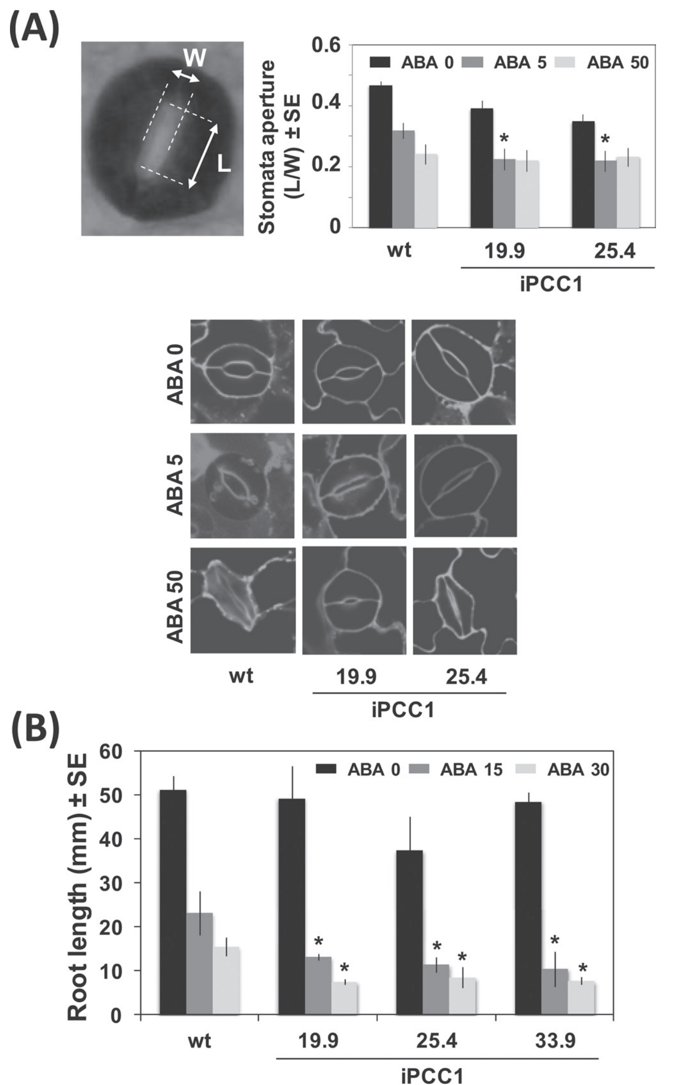

Fig. 2. Effect of ABA on wild-type and iPCC1 stomatal aperture and root growth. (A) The stomatal aperture, expressed as the ratio of length to width as indicated in the image in the left panel, was calculated for the wild type and two iPCC1 lines either in the absence of exogenously added ABA or in plants treated with 5 μM or 50 μM ABA as indicated. The central panel is a representative image of propidium iodide-stained stomata for every genotype and condition indicated. Values are the mean ±SE of between 35 and 50 stomata for each condition and genotype. (B) Root length of wild-type and iPCC1 seedlings on ABA-supplemented medium. Seedlings were grown in vertical square Petri dishes containing Murashige and Skoog (MS) medium supplemented or not with the indicated concentrations of ABA. Values represent the mean of the main root length (mm) of eight seedlings per genotype and condition ±SD. *Represents significantly different values from wild-type and iPCC1 roots with a *P*-value <0.05 by Student's *t*-test..

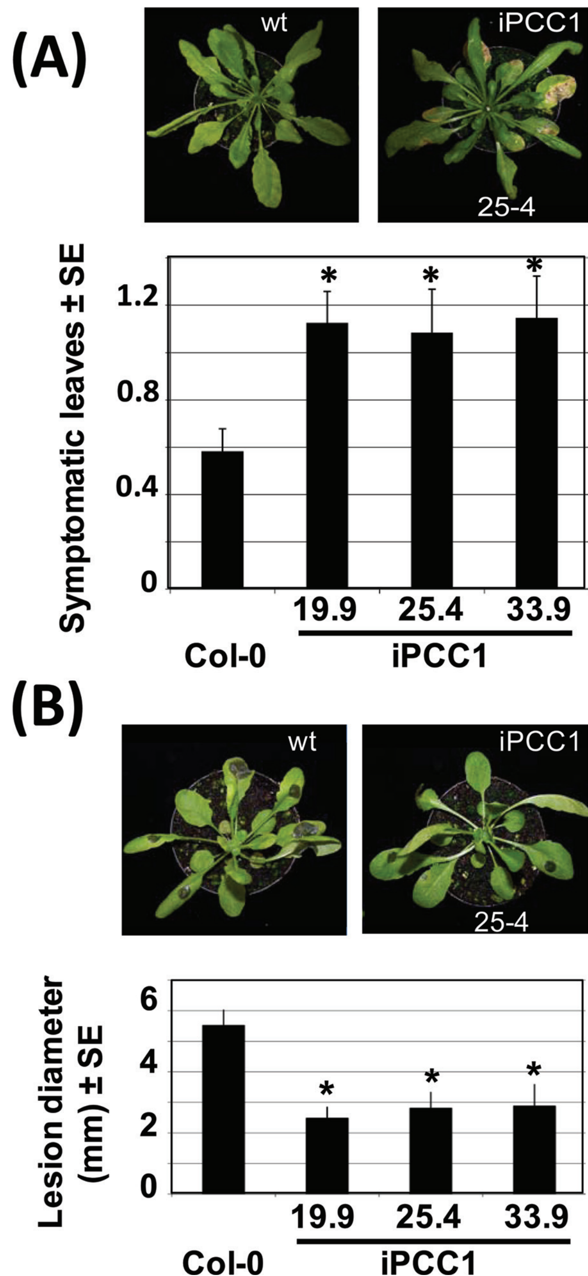

Fig. 3. Performance of wild-type and iPCC1 plants against *Phytophthora brassicae* and *Botrytis cinerea*. (A and B) The relative symptoms on a scale of 0–4 from the least to the most symptomatic leaves, and the lesion diameter of plants inoculated with *P. brassicae* and *B. cinerea*, respectively. Images on top of the graphs corresponding to symptoms for the wild type and iPCC1 25.4 line are also representative for the other iPCC1 lines. Values are the mean ±SE of four replicates and * means that iPCC1 values were all significantly different from wild-type values with a *P*-value <0.05 by Student's *t*-test.

(Sauerbrunn and Schlaich, 2004), iPCC1 plants were significantly more susceptible than wild-type plants to another oomycete pathogen *P. brassicae* (Fig. 3A). In turn, iPCC1 plants were surprisingly more resistant than wild-type plants to the necrotrophic fungus *B. cinerea*, with a reduction of ~50% in the necrotic lesion diameter (Fig. 3B).

Altered lipid content in iPCC1 plants

Alterations to physiological processes as diverse as seed germination and defence responses to pathogens in iPCC1 plants might be indicative of PCC1 affecting either common signalling events or a central metabolic process. Regarding

this, lipid involvement in seed germination (Theodoulou and Eastmond, 2012) and plant–pathogen interactions (Christensen and Kolomiets, 2011) has gained increasing interest. A lipidomic profile analysis focused on fatty acids and polar lipids for iPCC1 and wild-type leaves was performed. Quantification of the total content of polar lipids and their fatty acids by thin-layer chromatography followed by gas chromatography showed that iPCC1 leaves contained ~20% less fatty acids than wild-type leaves (Fig. 4A). This reduction affected the total content of saturated and desaturated 16C and 18C fatty acids similarly (Fig. 4A), but when the percentage composition was calculated a significant decrease was only observed in 18:0 (Fig. 4A). On the other hand, quantification of the total content of polar lipids showed no significant difference in iPCC1 leaves when compared with wild-type leaves (Fig. 4B). However, the analyses of the levels of different classes of polar lipids showed increases in the abundant chloroplastic monogalactosyl and digaloctosyl diacylgycerol (MGDG and DGDG) and phosphatidylglycerol (PG) in iPCC1 plants (Fig. 4B). In turn, the levels of polar lipids widely involved in signalling, such as phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanoalmine (PE) were significantly decreased in iPCC1 leaves compared with those of the wild type (Fig. 4B). Of importance, iPCC1 leaves contained only ~30% of the PI content detected in wild-type leaves (Fig. 4B). This dramatic reduction in a lipid class extensively characterized as important signalling components in a wide array of plant processes (Munnik and Nielsen, 2011) may help to explain some of the above-mentioned phenotypes as well as some others as yet unexplored. The reduced levels of 18:0 in iPCC1 leaves might suggest that the reduced expression of the *PCC1* gene could correlate with enhanced desaturation of fatty acids. Table 1 shows a more detailed quantification of fatty acids in each polar lipid class. The most significant differences between iPCC1 and wild-type plants were observed in 16:0, 18:0, 18:1, 18:2, and 18:3 fatty acids of PI and PS (Table 1). A 55% decrease in the 18:0 content of iPCC1 PI and the concomitant increase of >2-fold in the unsaturated fatty acids 18:1, 18:2, and 18:3 of PI was observed (Table 1). A similar trend was also observed for the fatty acid composition of PS (Table 1), thus supporting the hypothesis that reduced *PCC1* expression correlates well with enhanced desaturation of fatty acids in these classes of polar lipids.

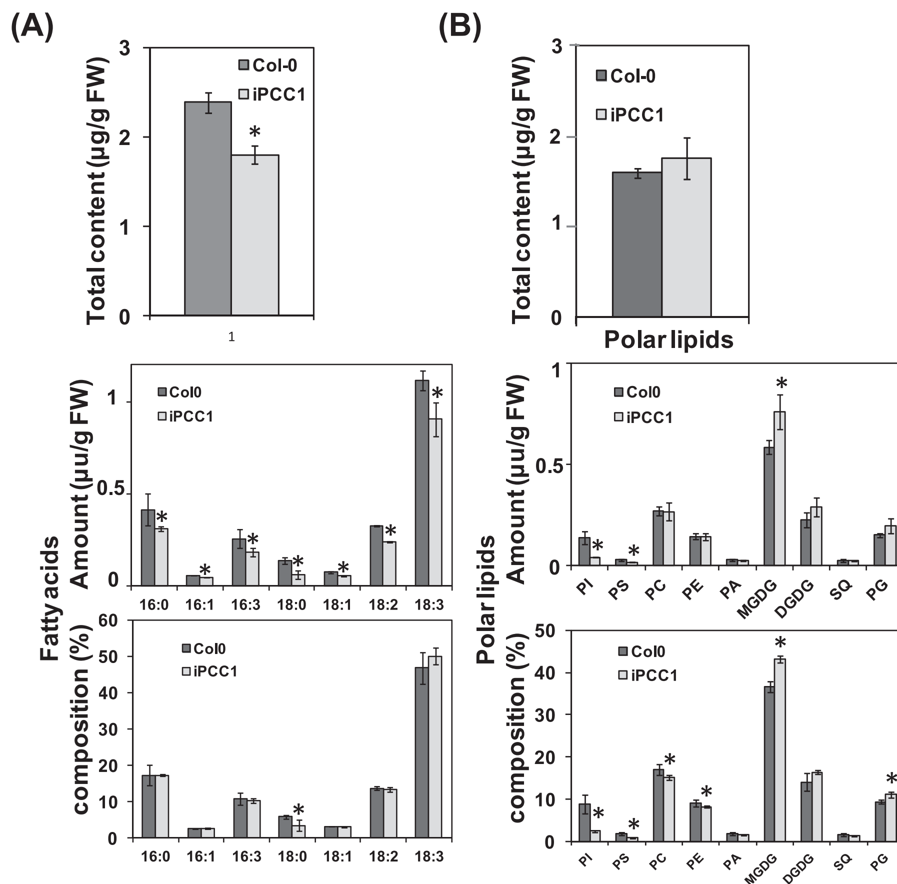

Fig. 4. Comparative polar lipid profile of iPCC1 and wild-type plants. The total amounts and percentage composition of each class of (A) fatty acids and (B) polar lipid are shown from the top to bottom panels as indicated. Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyl and digaloctosyl diacylgycerol (MGDG and DGDG), sulfphoquinovosyl (SQ), and phosphatidylglycerol (PG). Values are the mean of three replicates ±SD. *represents significantly different values from wild-type and iPCC1 leaves with a *P*-value <0.05 by Student's *t*-test.

Table 1. *Fatty acid composition of different polar lipid class for wild-type and iPCC1 leaves*

	16:0		16:1		16:2		16:3		18:0		18:1		18:2		18:3	
	Col-0	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**	**Col-0**	**iPCC1**
PI	51.8±0.2	44.2±3.2*	ND	ND	ND	ND	ND	ND	32.0±2.5	14.3±3.2*	2.0±1.3	4. 9±1.5*	6.6±0.1	18.9±1.7*	7.7±1.0	17.1±1.1*
PS	43.5±1.9	32.3±6.8*	ND	ND	ND	ND	ND	ND	38.1±1.3	31.8±4.1*	2.6±0.8	4.2±2.4*	6.9±0.4	16.7±3.6*	9.0±1.1	15.1±3.9*
PC	27.2±0.8	24. 8±0.6	0.5±0.1	0.4±0.1	0.1±0.1	ND	ND	ND	6.1±0.6	5.3±0.46	7.3±0.7	7.1±1.2	29.5±0.4	32.7±0.5*	28.6±1.0	28.9±1.3
PE	32. 6±2.1	31.4±1.4	0.3±0.5	0.2±0.2	ND	ND	ND	ND	6.4±0.7	5.7±0.7	3.2±0.4	3.39±0.1	34.2±0.6	36.3±1.8	22.3±1.3	22.0±0.2
PA	34.1±5.5	34.4±4.0	ND	ND	ND	ND	ND	ND	22.3±2.0	29.0±7.8	6.3±0.5	4.9±1.3	20.4±4.3	17.8±1.6	16.9±2.5	13.9±0.8
MGDG	2.2±0.2	2.3±0.4	1.6±0.1	1.3±0.1	2.0±0.1	1.6±0.2	26.5±2. 5	28.0±1.9	1.2±0.3	0. 7±0.2	2.0±0.4	1.4±0.1	3.9±0.4	2. 9±0.2	60.7±2.4	61.6±2.5
DGDG	16.9±1.7	17.7±0.9	0.1±0.1	0.2±0.1	0.6±0.1	0.5±0.1	2.0±0.4	2.0±0.4	3.5±0.4	2. 7±0.4	2.0±0.3	1.7±0.1	5.6±0.4	4.9±0.1	69.0±2.5	69.7±1.0
SQ	19.7±3.8	24.0±2.2	1.0±1.4	ND	ND	ND	ND	ND	27.0±2.4	20.0±3. 7	8.7±6.7	5.4±1.4	21.1±4.0	26.3±4.7	22.6±2.7	24.3±2.5
PG	29.5±4.1	28.1±1.1	24.5±1.1	25.4±1.1	ND	ND	ND	ND	7. 6±4.2	3.7±0.4	8.0±0.1	7.1±0. 9	7.6±1.6	8.4±0.7	22.3±5.7	26.7±1.5

Values, expressed as a percentage of the fatty acids analysed, are the mean of three replicates ± SD. Not detected (ND) means values were below the detection limit.
* represents significantly different values from wild-type and iPCC1 leaves with a *P*-value <0.05 by Student's *t*-test.

PCC1-modulated transcriptome

Despite its demonstrated involvement in defence and development, the molecular function and the mode of action of PCC1 protein in *Arabidopsis* remain largely unknown. To explore the way PCC1 functions, the effect of reduced expression of the *PCC1* gene on the genome-wide transcriptome of *Arabidopsis thaliana* was explored. Affymetrix microarray experiments were performed comparing three different iPCC1 linees, with very low *PCC1* expression (Segarra *et al.*, 2010), versus three biological replicates of non-transformed wild-type Col-0 seedlings grown under a long-day photoperiod for 12 d. Samples for RNA extraction were harvested at 12 h after dawn when *PCC1* expression was highest in wild-type plants (Segarra *et al.*, 2010). A total of 1037 genes were differentially expressed in seedlings with reduced *PCC1* expression after applying a cut-off for the *P*-value corrected for FDR (false discovery rate) values of <0.05 and fold values >1.5 or less than –1.5. Among them, 517 and 520 genes were up- and down-regulated, respectively (Supplementary Table S1 at *JXB* online). To validate the levels of transcripts observed in the microarray analysis, a randomly selected group of genes were analysed by qRT-PCR, and a good correlation between the levels of transcripts detected by both techniques was observed (Supplementary Fig. S3), thus suggesting that the results derived from the microarray-based transcriptomic analysis are robust and useful for further biological interpretation.

GO analysis of terms over-represented among differentially expressed genes led to an enrichment (FDR values <0.03) of lipid localization, responses to ABA, glucosinolate biosynthesis and metabolism, sulphur metabolism, and organic acid transmembrane transporter activity among up-regulated genes (Table 2). The functions significantly over-represented with down-regulated genes were associated with molecular transducer and transmembrane receptor activities as well as defence responses and cell death (Table 2). Several of these functional categories including lipid localization, responses to ABA, and defence responses and cell death are in agreement with the above-described phenotypes characterized for iPCC1 plants, suggesting that there is a good correlation between the reduced function of PCC1 and the trancriptomic and phenotypical alterations.

Analysis of promoter sequences of up- and down-regulated genes in iPCC1 plants

An *in silico* analysis of the 1000 bp promoter sequence upstream the initiation codon of genes that were up- or down-regulated in iPCC1 (Supplementary Table S1 at *JXB* online) allowed identification of 8-mer motifs that were significantly over-represented. Six motifs present in at least 15% of the promoter sequences of up-regulated genes were identified (Supplementary Table S2). A GO analysis of those genes points to their involvement in lipid localization and responses to ABA. The motifs A(G)AAAT(G)AAA, TAAATAA(GC)A and AGA(G)TAA(G)A(G)T(G), which were represented in >80, 35, and 20%, respectively, of the promoters

Table 2. *Enrichment of GO categories in the PCC1-regulated transcriptome*

GO term	Ontology	Description	No. input	No. BG/Ref.	*P*-value	FDR
Up-regulated genes in iPCC1 lines						
GO:0010876	P	Lipid localization	9	24	1.1e-11	1.1e-08
GO:0019761	P	Glucosinolate biosynthesis	6	41	1.4e-05	0.0017
GO:0019760	P	Glucosinolate metabolism	7	62	1.5e-05	0.0017
GO:0009737	P	Responses to abscisic acid	17	378	1.2e-05	0.0017
GO:0006790	P	Sulphur metabolic process	10	220	0.00064	0.027
GO:0005342	F	Organic acid transmembrane transporter activity	7	78	6.9e-05	0.008
Down-regulated genes in iPCC1 lines						
GO:0006952	P	Defence response	51	766	2.5e-20	2.5e-17
GO:0008219	P	Cell death	26	286	2.1e-14	7.1e-12
GO:0050832	P	Defence response to fungus	8	108	8.3e-05	0.0055
GO:0004888	F	Transmembrane receptor activity	13	171	4.4e-07	0.00011
GO:0060089	F	Molecular transducer activity	17	422	4.8e-05	0.0024

of up-regulated genes in iPCC1 plants, were contained in a consensus 3AF1 binding site tetramer sequence previously characterized as important for the light-responsive promoter of several genes including rbcS-3A from pea (Terzaghi and Cashmore, 1995). Moreover, the first one was also contained in characterized consensus sequences for regulating starch-branching enzyme I gene expression in maize endosperm (Kim and Guiltinan, 1999) and the leghaemoglobin lbc3 gene from soybean (Jensen *et al.*, 1988). The third most represented motif, CTTCT(C)TCT(G), was identified in >25% of the up-regulated genes and it was contained in a previously identified TL1 consensus sequence in the promoter of NPR1-responsive genes (Wang *et al.*, 2005) and also in the TCA-1 (tobacco nuclear protein 1) binding site of SA-inducible genes (Goldsbrough *et al.*, 1993). A CTCTCTCT(CG) motif identified in 15% of the up-regulated genes was present in a CT-rich motif found in the S1 region downstream of the transcription start site of the *Cauliflower mosaic virus* 35S promoter, and it may work as an expression enhancer (Pauli *et al.*, 2004). This motif was also present in a consensus sequence described to be involved in phytochrome A-regulated gene expression (Hudson and Quail, 2003). Finally, the motif C(G)A(G)CGTGT(G)C, which was identified in 15% of the up-regulated genes, contains the CACGTG G-box motif extensively present in both induced and repressed phyA-responsive promoters (Hudson and Quail, 2003) as well as the ABRE (ABA-responsive element ACGTGG/TC) motif that has been identified in the promoter region of ABA-inducible genes (Busk and Pagès, 1998; Nakashima *et al.*, 2006) and seems to bind the GBF family of bZIP transcription factors (Menkens *et al.*, 1995; Jakoby *et al.*, 2002).

On the other hand, common motifs in the promoter sequences of down-regulated genes were also found. Nine of them were present in >15% of the promoters of down-regulated genes, and the corresponding genes were mostly involved in defence responses and cell death (Supplementary Table S3 at *JXB* online). The most represented motif, AAATTTT(G)A(G), which was present in 62% of the promoters of down-regulated genes, is contained in a previously characterized motif (CAAAATTTTGTA) that participates in the HSR203-responsive element (HSRE), which is involved in the hypersensitive response to pathogens in tobacco (Pontier *et al.*, 2001). The second most represented motif, A(C)CAA(C)A(G)AAT, which was present in 55% of the promoters, is part of the consensus sequence GATACANNAATNTGATG characterized as a methyl jasmonate (JA)-responsive element in tomato lipoxygenases (Beaudoin and Rothstein, 1997). A third motif, GA(G)A(C)GA(G)A(C)GA(CG), was identified in 35% of the down-regulated genes and it was contained in the TL1 consensus sequence CTGAAGAAGAA identified in 13 NPR1-responsive endoplasmic reticulum-resident genes (Wang *et al.*, 2005) and also in the TCA-1 binding site of SA-inducible genes (Goldsbrough *et al.*, 1993).

Discussion

The way in which a small protein such as PCC1 with either no domains typically present in transcription factors or nuclear localization signals regulates several phenotypes in *Arabidopsis* is challenging. This work shows that reduced expression of the *PCC1* gene leads to profound changes in both metabolic and signalling events that together or independently lead to altered developmental and defence-related processes. Previously reported data from bioinformatic analysis (Venancio and Aravind, 2010) and from massive proteomic identification of plasma membrane proteins (Marmagne *et al.*, 2004), as well as from unpublished experimental results, suggest that PCC1 is associated with the plasma membrane. Data presented in this work are consistent with PCC1 participating in lipid-related events in membranes. iPCC1 plants displayed alterations in the lipid content and composition as well as changes in the transcript levels of genes coding for the production, storage, mobilization, and signalling of lipids. First, iPCC1 plants have strongly reduced levels of PI, and to a lesser extent of PS, PE, and PC, as well as an enriched content of desaturated fatty acids of PI, PS, and PC (Fig. 4, Table 1). Secondly, a significant enrichment of the lipid localization GO category was observed among up-regulated genes in iPCC1 plants (Table 2). Those lipid-related genes

code for proteins involved in the transport/localization, storage, and signalling of lipids (Supplementary Table S4 at *JXB* online). Because of the plasma membrane-associated location of PCC1, lipid-related signalling is particularly relevant to explain its widespread effect on plant physiology. iPCC1 plants contained up- and down-regulated genes coding for GDSL-like lipases (Supplementary Table S4), which have been characterized as important components in triggering responses to biotic and abiotic stress factors (Oh *et al.*, 2005; Hong *et al.*, 2008) as well as in hormone-related control of plant growth and development (Cao *et al.*, 2006). Moreover, iPCC1 plants might have an altered PI metabolism and transport as suggested by the reduced levels of PI (Fig. 4B, Table 1) and also by the up-regulation of genes coding for PI-related kinases and phosphatases as well as the down-regulation of genes coding for transporters of the SEC14 family (Supplementary Table S4). On the other hand, iPCC1 plants showed enhanced levels of the chloroplast lipid MGDG. Although this is a mere hypothesis, PCC1 might affect chloroplast lipid composition through interaction with signalling events that either end with the modulation of transcription of genes coding for precursor chloroplast proteins or interfere with the lipid trafficking from the endoplasmic reticulum to the chloroplast. Determination of whether PCC1 modulates early or late events and localization or metabolism of lipids in lipid-related signalling would require more work.

A complex pattern of alterations was detected in the defence responses of iPCC1 plants against pathogens with different lifestyles. iPCC1 plants were more susceptible than wild-type plants to the hemibiotrophic oomycete *P. brassicae* and more resistant to the necrotrophic fungus *B. cinerea*. Since iPCC1 plants were slightly hypersensitive to ABA (Figs 1, 2), the responses to pathogens of different lifestyles might be related to alterations in ABA signalling. However, it has been recently reported that *Arabidopsis* mutant plants defective in either the biosynthesis or signalling of ABA were more resistant to the necrotrophic fungus *Plectosphaerella cucumerina* (Sánchez-Vallet *et al.*, 2012), thus suggesting that there is not a direct connection between PCC1 and ABA signalling in controlling defensive responses to different pathogens. In turn, the enhanced susceptibility of iPCC1 plants to *Phytophtora* (Fig. 3) is consistent with the previously reported enhanced resistance of *PCC1*-overexpressing plants to the downey mildew *P. parasitica* (Sauerbrunn and Schlaich, 2004). iPCC1 plants had down-regulated expression of *RPP4*, *RPP5*, and *RPP13* resistance genes to *Hyaloperonospora arabidopsidis* (Reignault *et al.*, 1996; Knoth *et al.*, 2007). RPP4 and RPP5 are targets of the modulation exerted by Suppressor of *npr1-1* Constitutive 1 (SNC1). Microarray data presented in this work indicate that *SNC* expression is strongly down-regulated (–13.1-fold) in iPCC1 plants (Supplementary *Table S1* at JXB online), providing a possible link between the innate immunity and the ubiquitin pathway in *Arabidopsis* (Goritschnig and Zhang, 2007). Interestingly, iPCC1 plants showed a strong down-regulation of the genes coding for UBQ11, UBQ10, and the ubiquitin-conjugating enzyme 37 (UBC37) (Supplementary Table S1) which may be indicative of altered function of the ubiquitin pathway in iPCC1 plants. A PCC1-mediated effect on protein ubiquitination could also be an efficient way to exert regulation on many different targets, thus allowing a wide display of PCC1-regulated phenotypes.

The functional roles of PCC1 in regulating defence responses of *Arabidopsis* against a wide array of pathogens must be complex enough to explain the enhanced susceptibility of iPCC1 plants to *P. brassicae* and the enhanced resistance to *B. cinerea*. These two pathogens have different lifestyles and their interactions with the plant are governed by different signalling pathways involving regulatory molecules such as JA, ethylene (ET), and SA. Regarding this, the transcriptome of iPCC1 plants showed a large over-representation of down-regulated genes coding for components involved in SA-mediated signalling. Interestingly, *PCC1* gene expression is up-regulated by SA (Segarra *et al.*, 2010), and by using *pPCC1::GUS* plants it was found that its expression is spread throughout seedlings (Supplementary Fig. S4 at *JXB* online). SA-related down-regulated genes include EDS1 that, together with PAD4, has been considered an essential regulatory hub for the establishment of basal resistance to biotrophic and hemi-biotrophic pathogens, by activating SA-mediated resistance and by regulating the antagonism with JA- and ET-based defence responses to pathogens (Wiermer *et al.*, 2005). Also the *NPR1* gene coding for the ankyrin repeat-containing central regulator of SA-activated resistance to pathogens (Dong, 2004a) was down-regulated in iPCC1 plants (Supplementary Table S1). In connection with the potential membrane-related site of action for PCC1 discussed above, the *ACD6* gene coding for a membrane-associated protein involved in controlling cell death and SA-mediated defence to pathogens (Dong, 2004b; Lu *et al.*, 2005) was also severely down-regulated in iPCC1 plants (Supplementary Table S1). Enhanced resistance of iPCC1 plants to necrotrophic pathogens such as *B. cinerea* might be explained by the down-regulation of the SA-related defence signalling and the proposed functional antagonism of that pathway with JA/ET-related defence (Thaler *et al.*, 2012).

The functional interaction between PCC1 and ABA is supported by different sets of data in this work. First, iPCC1 plants are hypersensitive to ABA in several phenotypes, including seed germination and seedling establishment (Fig. 1), as well as stomatal closure and root growth (Fig. 2). Secondly, the up-regulated transcriptome of iPCC1 plants is enriched in ABA-related genes (Table 1), and many of them contained ABA-responsive elements (Busk and Pagès, 1998; Nakashima *et al.*, 2006) in their promoters (Supplementary Table S3 at *JXB* online). Several reports in the last years have provided details on the main module of ABA signalling from hormone perception by receptors of the PYR/PYL/RCAR family to downstream components including phosphatases of the PP2C family, kinases of the SnRK2 family, and ABA-modulated transcription factors of different families (Fujii *et al.*, 2009; Ma *et al.*, 2009; Melcher *et al.*, 2009; Park *et al.*, 2009; Cutler *et al.*, 2010; Raghavendra *et al.*, 2010; Umezawa *et al.*, 2010). An overall view of the PCC1-modulated transcriptome shows that none of the genes coding for the ABA receptors was significantly altered in iPCC1 plants (Supplementary Fig. S5). In turn, five out of nine PP2C phosphatase-encoding genes of clade A were significantly up-regulated in iPCC1 plants

(Supplementary Fig. S5). Up-regulation of genes coding for ABA-related PP2Cs may be a response directed to attenuate signalling in plants showing hypersensitivity to ABA. Nevertheless, because the transcriptome analysis in this work was performed under non-stressed conditions, the profound changes detected in gene expression correlate well with iPCC1 seeds and plants displaying developmental phenotypes such as reduced seed germination, seedling establishment, and root elongation. These phenotypical alterations are all well explained by the hypersensitivity to ABA of iPCC1 plants even under non-stress conditions.

Taken together, the data presented in this work support an important role for PCC1 as a regulator and as an integrator of responses to the environment and to endogenous developmental cues. The likely membrane-associated location points to a potential double role for PCC1 as a modulator of receptor systems, including pathogen-derived molecular patterns, as well as a regulator at multiple levels of early downstream signalling. In this way, PCC1 seems to be involved in regulating both ABA-mediated developmental transitions as well as SA- and JA-triggered pathogen-related responses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Seedling establishment in osmotic media supplemented with mannitol or NaCl.

Figure S2. Water loss in wild-type and iPCC1 seedlings.

Figure S3. Comparison of transcript levels detected by microarray and qRT-PCR analysis.

Figure S4. *PCC1* expression is induced by SA.

Figure S5. Microarray-derived transcript levels of the core ABA signalling module in *Arabidopsis*.

Table S1. Comparative transcriptomic analysis of iPCC1 versus wild-type plants.

Table S2. *In silico* analysis of motifs over-represented in the promoter sequences of genes that were up-regulated in iPCC1 versus Col-0 plants.

Table S3. *In silico* analysis of motifs over-represented in the promoter sequences of genes that were down-regulated in iPCC1 versus Col-0 plants.

Table S4. Lipid-related genes that were up- and down-regulated in iPCC1 compared with wild-type plants.

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