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Running title: ACD5 in cold stress response

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Abstract

Although sphingolipids emerged as important signals for plant response to low temperature, investigations have been limited so far to the function of long-chain base intermediates. The formation and function of ceramide phosphates (Cer-P) in chilled Arabidopsis was explored. Cer-P were analysed by TLC following *in vivo* metabolic radiolabelling. Ceramide kinase activity, gene expression and growth phenotype were determined in unstressed and cold-stressed WT and Arabidopsis ceramide kinase mutant *acd5*. A rapid and transient formation of Cer-P occurs in cold stressed WT Arabidopsis plantlets and cultured cells, that is strongly impaired in *acd5* mutant. Although concomitant, Cer-P formation is independent of long-chain base phosphate (LCB-P) formation. No variation of ceramide kinase activity was measured *in vitro* in WT plantlets upon cold stress but the activity in *acd5* mutant was further reduced by cold stress. At the seedling stage, *acd5* response to cold was similar to that of WT. Nevertheless *acd5* seed germination was hypersensitive to cold and ABA, and ABA-dependent gene expression was modified in *acd5* seeds when germinated at low temperature. Our data involve for the first time Cer-P and ACD5 in low temperature response and further underline the complexity of sphingolipid signalling operating during cold stress.

Keywords: *Arabidopsis thaliana*; chilling; ceramide phosphate; ceramide kinase; ACCELERATED CELL DEATH5; ABA.

Introduction.

Cold is a major abiotic stress that restrains plant growth and development and affects plant species distribution. Plants from temperate zones are globally tolerant to non-freezing temperatures (chilling) and exhibit different degree of sensitivity when exposed to negative temperature (freezing). Nevertheless, freezing tolerance might be enhanced after acclimation, i.e. pre-treatment at chilling temperature. The use of Omic-based approaches has recently helped identify major genes, proteins and metabolites involved in chilling and/or freezing responses (Knight & Knight 2012). These data highlighted a global modification of plant metabolism in cold-stressed plants, which at least partly relies on the modifications of gene expression and counterbalances cell dysfunctions under low temperature (Ruelland et al. 2009; Knight & Knight 2012).

Concurrently with the analysis of the cold-activated molecular programme, a range of cell signals involved in cold transduction has been identified. Alongside calcium and protein kinase/phosphatases, lipid second messengers emerged as important elements of the cold signalling network. This was first evidenced for phosphatidic acid (PdtOH), a ubiquitous signalling molecule derived from membrane phospholipids. Significantly rapid and transient increases of PtdOH production are observed in chilled plants, which are required for proper expression of cold-regulated genes (Ruelland *et al.*, 2002; Vergnolle *et al.*, 2005; Arisz et al., 2013). Furthermore modifications of PtdOH metabolism impact plant tolerance to cold (Rajashekar *et al.*, 2006; Li *et al.*, 2011; Delage et al., 2012). More recently, the implication of sphingolipids (SL) in cold transduction pathway has been questioned. SL constitute a family of lipids all sharing a common hydrophobic core, i.e a ceramide (Cer) that results from the condensation of a Long Chain Base (LCB) with a long or a very long chain fatty acid ((V)LCFA)-CoA. Cer are substituted by hydrophilic head groups, giving rise to a tremendously diverse family of complex-SL (Hannun & Obeid, 2011). By extension, the biosynthesis intermediates, LCB and Cer, are also usually named SL. LCBs and Cer can also be phosphorylated by the activities of LCB and Cer kinases leading to

LCB-P and Cer-P formation, respectively. The roles of SL as signalling molecules are now well recognized. In particular, studies carried out in animals evidenced the crucial signalling function of LCB and Cer as well as LCB-P and Cer-P (Bornancin, 2011; Kawabori *et al.*, 2013; Maceyka & Spiegel, 2014). Noteworthy it appears that LCB/Cer act antagonistically to their phosphorylated counterparts in regulating cell processes as diverse as cell division, differentiation or apoptosis (Mendelson *et al.*, 2014; Aguilera-Romero *et al.*, 2014). The control of the equilibrium between phosphorylated and unphosphorylated forms *via* the regulation of the specific kinase and phosphatase activities is therefore crucial to maintain cell fitness and determine cell fate (Hoeferlin *et al.*, 2013; Payne *et al.*, 2014). In plants, a growing hand of data indicates that comparable signalling roles could be endowed by SL in response to biotic and abiotic stress (Ng *et al.*, 2001; Coursol *et al.*, 2003, 2005; Worrall *et al.*, 2008; Markham *et al.*, 2013; Wu *et al.*, 2015). Pioneering works determined that treatments with LCB-P or LCB kinase inhibitors alter abscisic acid (ABA) responses (Ng *et al.*, 2001; Coursol *et al.*, 2003). Further studies showed that four putative isoforms of LCB kinases exist in *Arabidopsis thaliana* (Coursol *et al.*, 2005; Worrall *et al.*, 2008; Guo *et al.*, 2011), and illustrated the implication of some of these isoforms in response to ABA (Worrall *et al.*, 2008; Guo *et al.*, 2012). In plants Cer-K activity and Cer/Cer-P balance have been essentially involved in programmed cell death (PCD) and associated to plant-pathogen interactions (Brandwagt *et al.*, 2000; Greenberg *et al.*, 2000; Coursol *et al.*, 2003; Liang *et al.*, 2003; Townley *et al.*, 2005; Raffaele *et al.*, 2008; Bi *et al.*, 2014). As observed in most organisms, a unique gene designated *ACCELERATED CELL DEATH5* (*ACD5*) codes for a Cer-K in Arabidopsis. The sole mutant of this gene described to date is the point mutant *acd5* which presents a reduced Cer-K activity. The *acd5* mutant displays a spontaneous PCD that occurs at late development stages, together with an enhanced sensitivity to pathogen attack (Greenberg *et al.*, 2000; Liang *et al.*, 2003; Bi *et al.*, 2011, 2014).

Recently, several reports have also associated SL with plant response to low temperature (Cantrel *et al.*, 2011; Chen *et al.*, 2012; Dutilleul *et al.*, 2012; Degenkolbe *et al.*, 2012; Guillas *et al.*, 2012). Beyond the functions of membrane SL in cold acclimation (Steponkus & Lynch, 1989; Uemura *et al.*, 1995; Minami *et al.*, 2009, 2010), a role for SL as signals has also been evidenced. Indeed, the rapid and transient formation of phytosphingosine-phosphate (PHS-P) has been observed in cold-treated Arabidopsis plantlets (Dutilleul *et al.*, 2012). In this context, the formation of PHS-P required the activity of a particular isoform of LCB kinase designated LCBK2. Interestingly, *lcbk2* mutants are affected in specific aspects of cold response such as root growth modulation and cold-responsive gene expression. Together with PHS-P, *in vivo* labelling experiments indicated the formation of an additional, more hydrophobic SL-related species that we proposed as a putative Cer-P (Cantrel *et al.*, 2011). In the present study, the formation and possible function of this SL during Arabidopsis response to low temperature were further investigated. Analyses of the mutant *acd5* highlight that this particular SL is important for seed germination at low temperature.

Material and methods

Chemicals

Radioactive [^{33}P]orthophosphate and [^{33}P]ATP were purchased from Hartmann Analytic (Braunscheig, Germany). C8-Ceramide was purchased from Biomol (TEBU, Le Perray-en-Yvelines, France). Ceramide kinase inhibitor K1 was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). Phytosphingosine (PHS) was purchased from Enzo Life Science (Villeurbanne, France). Gibberellic acid, abscissic acid and protease inhibitors were purchased from Sigma-Aldrich (Lyon, France).

Plant and cell cultures

Experiments were performed using *Arabidopsis thaliana* L. Heynh. wild type (WT) in the Columbia (Col-0) background as cultured cells and seedlings. The *acd5* mutant (Greenberg *et al.*, 2000) was kindly provided by Pr J. Greenberg (Uni. Chicago, USA).

Cell suspensions were cultivated and cold-treated as described by Ruelland et al. (2002). For *in vitro*-grown seedling cultivation, Arabidopsis seeds were sterilized, stratified for 2 days at 4°C, and sown on basic ½ MS medium (M0221, Sigma-Aldrich, Lyon, France) adjusted at pH 5.7, 0,8% agar. When specified, 1% sucrose was added to the medium. Unless stated otherwise, plates were then placed in a growth chamber under continuous illumination (230 µE.m^{-2}.s^{-1}) at 22°C and 56 % humidity. (Ruelland *et al.*, 2002; Dutilleul *et al.*, 2012).

Cold treatment and plantlet labelling

Fourteen day-old plantlets (approximatly 50 +/- 5 mg FW) were transferred to 50 mL flasks containing 3 mL of ½ MS medium without sugar and agitated overnight at 22°C on an orbital shaker under continuous illumination. For labelling, 53 MBq.L^{-1} [^{33}P]-orthophosphate were added

to each flask, 60 min before cold shock. Cold shock was applied as described previously (Dutilleul *et al.*, 2012). Briefly, flasks were immerged in ice-cold water and maintained under shaking the time specified for the cold treatment. Then plantlets were harvested, briefly soaked and immediately transferred in 5 mL methanol and frozen in liquid nitrogen. Control plantlets were kept at 22°C, and harvested in the same manner.

Phosphorylated sphingolipid extraction and analysis

Lipids were subsequently extracted by transferring the samples from liquid nitrogen to water bath maintained at 60°C for 15 min incubation. Subsequently the supernatant was collected and 3 additional extractions were done in the same manner, i.e. N_2 frozen, 15 min at 60°C and extracted twice with 3 ml chloroform/methanol (1:1, v/v) and once with 3 ml chloroform. Combined extracts were dried under nitrogen and resuspended in 1 ml methanol. To hydrolyse glycerophospholipids, 1 ml 3N NH_4OH/methanol (1:1, v/v) was added to 1 ml total lipid extracts and samples were incubated for 1 h at 50°C. The reaction was stopped by neutralization *via* the addition of 2.5 ml acid acetic/water (2:1, v/v). Two successive phase extractions were done by the addition of 2.5 ml chloroform. Combined chloroform phases were nitrogen-dried and sphingolipids were desalted by butanol/water extraction. Briefly sphingolipids were resuspended in 500 µl water-saturated butanol. Then 500 µl butanol-saturated water was added, samples were vortexed for 1 min and briefly centrifuged to allow phase partitioning. A second extraction was done similarly. Pooled butanol fractions were back extracted with 500 µl butanol-saturated water in the same manner ie. 1min vortexing followed by a brief centrifugation. Then incorporated radioactivity was estimated by liquid scintillation counting on an aliquot of butanol fraction. Butanol fractions were dried under nitrogen and resuspended in methanol. Sphingolipids were developed by thin-layer chromatography on Silica 60 plates (Merck, Darmstadt, Germany) using chloroform/acetone/methanol/acetic-acid/water (10:4:3:2:1; v/v) as solvent system (Wijesinghe *et al.*, 2007). Radiolabeled sphingolipids

were revealed and quantified using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Cold standard PHS-P was visualized after fluorography. For this purpose, plates were sprayed with ninhydrin (Merck, Darmstadt, Germany) and heated to 100°C until red spots appeared. C8-Cer-P was detected with orcinol and sulfuric acid (Jork *et al.*, 1990; Reggiori *et al.*, 1997).

In vitro ceramide kinase assay.

Fourteen day-old plantlets were prepared in flasks in liquid media as indicated for labelling assay and submitted or not to cold treatment. They were subsequently harvested, soaked and immediately frozen in liquid N_2. The crude plant extracts were prepared as previously described (Liang *et al.*, 2003; Bi *et al.*, 2011). Approximately 50 mg of plantlets were ground in liquid N_2 and placed in a 1.5 mL microfuge tube containing 0.5 mL of extraction buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 1 mM DTT, 10% glycerol) supplemented with 1 mM PMSF, 5µM pepstatin and 10 µM leupeptine. The extract was centrifuged for 10 min at 14 000 *g*, and the supernatant was assayed for protein concentration using Bradford protein assay (Biorad, Marnes la Coquette, France). Cer-K activity was assayed in a final volume of 100 µL of reaction buffer (20 mM MOPS pH 7.2 containing 2 mM EGTA, 1 mM DTT, 10% glycerol, 10 mM KCl, 15 mM $MgCl_2$, and 1 mM ATP) supplemented with 5 µCi [^{33}P]ATP and C8-Cer to 50 µM final concentration. C8-Cer substrate dissolved in methanol was vacuum-dried in each reaction tube and re-suspended by pipetting in 10 µL of 0.1% Triton X-100 prior to adding reaction buffer. The reaction was initiated by the addition of 50 µg protein and was allowed for 30 min at 30°C. It was then stopped by the addition of 600 µL chloroform/methanol (1:1, v/v) and 265 µL 2M KCl. Chloroform phase was N_2-dried and desalted by butanol/water partitioning as described above. After N_2 drying, lipid extracts were dissolved in 20 µL methanol, spotted and developed on Silica 60 plates (Merck, Darmstadt, Germany) in chloroform/acetone/methanol/acetic-acid/water (10:4:3:2:1, v/v). Radiolabelled species were revealed and quantified as described above.

Phenotypic analyses

Germination tests were performed using surface sterilized seeds sown on sterile water containing 0,8% agar. Plates were incubated at 4°C for 48h, and subsequently transferred to 22°C or kept at 4°C. Transfer of control seeds to 22°C was considered as T_0. Germination was recorded at different time points depending on incubation temperature. Where indicated, filter–sterilized GA or ABA solutions were added to the medium at the indicated concentrations. For ABA experiments, an equivalent volume of DMSO was used for the control.

For root growth tests, seeds were processed as described in the plant culture section. Three day-old plantlets were transferred to 12x12 cm square plates containing ½ strength MS medium and grown vertically. After 24h recovery at 22°C, considered as To, plates were transferred to 12°C or kept at 22°C. Root elongation was recorded every two days over 10 (at 22°C) to 28 (at 12°C) days.

RNA isolation and RT-PCR analyses

Total RNAs were purified from Arabidopsis plantlets using RNeasy® Plant Mini kit (Qiagen, Courtaboeuf, France) according to manufacturer's procedure. One microgram of total RNA was treated with DNaseI and subsequently reverse-transcribed using Omniscript® RT kit (Qiagen, Valencia, CA, USA). Five time-diluted RT products were used for PCR amplification with DreamTaq® polymerase (Thermo Scientific, Illkirch, France) according to manufacturer's procedure. The primer sets used are compiled in Supplementary Table 1.

Statistical analysis

Experimental data reported represent at least three independent biological repeats. When appropriate, results are reported as mean values ± standard errors. Mean comparisons were calculated by Student test with *P* values and data sizes indicated in figure legends.

Results

Cold triggers the transient formation of a highly hydrophobic phosphorylated SL

Preliminary experiments indicated that a second phosphorylated lipid related to SL (designated lipid A) was evoked upon cold shock, in addition to PHS-P (Cantrel *et al.*, 2011). We therefore further investigated the characteristics of lipid A formation using *in vivo* labelling with [^{33}P] orthophosphate. In cultured cells lipid A was rapidly detected and migrated with an Rf of 0.67. (Fig. 1a, Supplementary figure 1a). Similarly, it was evoked in cold-treated 14 day-old plantlets (Fig. 1b). Co-migration experiments indicated that lipid A migrated at the same Rf in both models suggesting that the same molecular species was formed in the two plant materials upon cold exposure (Supplementary Fig.1b). In both plant materials, no accumulation of lipid A could be observed at 22°C within the time of the labelling experiment (Fig. 1a,b 22°C panels). Slight differences in kinetics were observed between plantlets and cultured cells. The maximum accumulation of lipid A was progressively reached after 10 min in cell culture and rapidly decreased back to initial state after 240 min (Fig. 1a,c). In plantlets, a dramatic accumulation of lipid A was observed within 5 min., slightly increasing to a maximum after 30 min and slowly decreasing thereafter (Fig. 1b,d). Our analysis also indicated differences in the proportion of lipid A abundance in the total labelled lipid spotted. Indeed it represented 30% at the highest in cultured cells and 50% in labelled plantlets (Fig. 1c,d). Altogether these data show that a strongly hydrophobic base-resistant phosphorylated lipid was transiently evoked upon cold treatment.

ACD5 Cer-K activity is responsible for the phosphorylation of lipid A.

The fact that lipid A was base-resistant linked it up to sphingolipids. Moreover its Rf of +/- 0.67 identified it as a putative Cer-P (Bielawska *et al.*, 2001, Wijesinghe *et al.*, 2007). To fully establish the identity of lipid A, labelling experiments on mutants affected in ACD5 ceramide Kinase were performed. We identified two heterozygous ACD5 insertion lines available at Nask (*Line N582554,*

Line N629619). Nevertheless no homozygous plants from these lines could be obtained, suggesting that *acd5* KO mutants were lethal. Therefore, for these analyses, the *A. thaliana acd5* knock down mutant exhibiting a G412R point mutation already described (Greenberg *et al.*, 2000) was used. As for the experiments presented in Figure 1, 14 day-old *in vitro* grown plantlets were used. At this stage *acd5* plantlets are phenotypically similar to WT (Fig. 2a). Furthermore, *PR1* gene was not expressed in *acd5* 14 days old mutant plantlets exposed or not to cold (Fig. 2b), indicating that plantlets were not undergoing PCD at this stage of development (Greenberg *et al.*, 2000). Therefore, this material appeared suitable for subsequent *in planta* labelling and cold response monitoring. The [^{33}P] labelled lipid A was quantified after 3 min of cold shock. In these conditions lipid A increased by 60%, ie. 1.6 ± 0.21 fold, in WT plantlets (8.6 ± 0.10% to 14.5 ± 0.12% of total lane radioactivity) whereas it only increased by 20%, ie. 1.2 ± 0,08, fold in the *acd5* mutant (8.75 ± 0.07% to 10.5 ± 0.14% of the total lane radioactivity) (Fig. 2c). These results showed that the lipid A formation required ACD5 Cer-K activity and thereby established that it was a *bona fide* Cer-P. Therefore, the designation Cer-P will be used thereafter instead of lipid A.

Cold-evoked Cer-P and PHS-P formation occurs independently.

We previously reported that PHS-P accumulated in response to cold and that this accumulation was defective in the LCBK mutant *lcbk2* (Dutilleul *et al.*, 2012). As Cer-P and PHS-P appeared within the same time frame, a possible interdependence of the formation of these two species was investigated. In this view, PHS-P and Cer-P have been quantified in *acd5* and *lcbk2* plantlets, respectively (Fig 3). As shown in Figure 3a, PHS-P appearance was not affected by *acd5* mutation. Conversely, Cer-P accumulation was comparable in cold-stressed WT and *lcbk2* plantlets (Fig. 3b). As shown on Figure 3b, similar results were obtained in two other Arabidopsis LCBK mutants, *lcbk1* and *sphk1* (Worrall *et al.*, 2008). These observations indicated that PHS-P and Cer-P formation, whilst concomitant, occurred independently.

Evaluation of the ACD5 activity modulation in response to cold.

To investigate whether the Cer-P accumulation observed during cold response was due to an enhanced Cer-K activity, Cer kinase activity *in vitro* using crude protein extracts from plantlets exposed or not to cold was measured. Previous studies using *Arabidopsis thaliana* crude protein extract showed that C8-Cer was the preferred substrate for Cer-kinase activity *in vitro* (Liang *et al.*, 2003). Therefore we monitored the formation of [^{33}P]-C8-Cer-P using crude protein extracts from WT plantlets formerly exposed to cold or maintained at 22°C. In both case a strong Cer kinase activity was observed (Fig. 4a,b, and supplemental Fig 2). As control, no signal corresponding to C8-Cer-P could be observed in absence of C8-Cer substrate or of plant extract (Fig. 4b). In addition, activity measurements in the presence of the Cer-K inhibitor K1 which partially and specifically inhibits recombinant rice Cer-K activity *in vitro* (Bi *et al.*, 2011) was performed. The treatment with K1 inhibitor led to a 40% inhibition of Cer-P phosphorylation (Fig. 4a,b). These results evidenced that the difference in Cer phosphorylation observed *in vivo* upon cold shock was not correlated with a modification of the Cer-K catalytic activity as measured *in vitro*. The same experiment was carried out using *acd5* plantlet protein extracts. Cer-K activity measured in extracts of unstressed *acd5* plantlets was 20% lower than that of unstressed WT plantlets protein extract (Fig. 4a,b). Surprisingly, an additional decrease of 20% was observed when the assay was performed with extracts from cold-stressed *acd5* plantlets that consequently retained only 60% of the activity of WT plantlet extracts (Fig. 4a,b), similarly to previous observations (Bi *et al.*, 2011). These data suggested that cold stress exacerbated the consequences of the mutation on ACD5 Cer-K activity.

Previous works have shown that *ACD5* expression was up regulated in response to pathogen infection suggesting that such regulation may account for ACD5 function in plant defence (Liang *et al.*, 2003; Bi *et al.*, 2014). The possibility that such regulation may occur in response to cold was assessed. Within 4h of cold exposure no change in *ACD5* transcript abundance was observed in WT plantlets, whereas a slight increase was observed in the *acd5* mutant (Fig. 4c).

Altogether these results suggest a complex array of regulation for ACD5 activity *in planta* upon cold stress.

Effect of Cer-P accumulation defect on the response of *acd5* plantlets to cold.

To get insights into the involvement of Cer-P accumulation during cold stress, the phenotypic and molecular responses of *acd5* mutant to cold was analysed. Due to the pleiotropic effects of *acd5* mutation at later developmental stages (i.e. spontaneous cell death, necrosis, *PR1* activation, Cer accumulation) (Greenberg *et al.*, 2000; Bi *et al.*, 2014), all the analysis were carried out for no longer than four weeks. As the monitoring of root growth at 4°C required at least 6 weeks (Dutilleul *et al.*, 2012) it could not be performed with *acd5* mutant. Therefore, the root growth was analyzed at 12°C. With regard to root growth, no significant differences were observed between WT and *acd5* plantlets, neither at 22°C nor at 12°C (Fig. 5a and supplemental Fig 3). In addition, the expression of cold-responsive transcription factors (*CBF1-3*, *Zat10*, *Zat12*) was assessed in WT and *acd5* plantlets. As expected, transcripts of these genes were more abundant following a 4h cold treatment in WT plantlets (Fig. 5b and supplemental Fig 3). No significant difference was observed in cold-stressed *acd5* plantlets as compared to WT (Fig. 5b). These data showed that the defect in Cer-P accumulation observed in *acd5* plantlets did not affect the expression of major cold responsive markers. Yet we observed that the induction of *NOI3*, previously identified as a cold-responsive gene (Dutilleul *et al.*, 2012), was defective in *acd5* (Fig. 5b). As *NOI3* gene was also found as a target for PHS signalling, this result further evidenced the link between *NOI3* expression and SL signalling in cold response. These observations also suggested that the control of *NOI3* expression was operated independently by both PHS-P and Cer-P upon cold shock.

Effect of *acd5* mutation on seed germination at low temperature.

311 Several reports have evidenced that cold delays the emergence of radicle and globally slows
312 down the germination process in a temperature-dependent manner (Xu *et al.*, 2011, Jung *et al.*,
313 2015). To further investigate the response of *acd5* mutant to cold, its germination rate was
314 compared with that of WT seeds at 22°C, 12°C and 4°C. At 22°C, germination was complete 3 days
315 after sowing for both genotypes, indicating that *acd5* seeds were fully germinative under optimal
316 conditions (Fig. 6a). In contrast, at 12°C and 4°C, *acd5* seeds germination was significantly and
317 proportionally reduced compared to WT. Thus, as compared to WT, *acd5* germination was lowered
318 by 12% and 49% respectively at 12°C and 4°C fourteen days after sowing (Fig. 6a). Acd5 seed
319 germination therefore appeared as hypersensitive to low temperature.
320 Germination is a complex process controlled by multiple factors, and in particular by the
321 two antagonistic hormones, ABA and GA. Several studies suggested a link between these two
322 regulators and plant response to cold (Chung & Parish, 2008; Wang *et al.*, 2014, Sasaki *et al.*,
323 2015). Thus the analysis of the expression of genes involved in ABA and GA metabolism or
324 signalling in WT and *acd5* seeds germinated for 14 days at 4°C was done. *NCED3*, *6* and *9* encode
325 enzymes of the ABA biosynthesis pathway, whereas *ABI5* codes for an ABA-responsive
326 transcription factor repressing germination (Lopez-Molina *et al.*, 2001). *RGL2* encodes a member of
327 the DELLA family controlled by the GA pathway, and is considered as the main DELLA involved
328 in germination repression (Lee *et al.*, 2002; Tyler *et al.*, 2004). Both *RGL2* and *ABI5* are
329 upregulated by ABA (Lopez-Molina *et al.*, 2001), and RGL2 itself stimulates ABA synthesis and
330 therefore ABI5 accumulation, (Piskurewicz *et al.*, 2008; Achard & Genschik, 2009). *NCED3* and *9*
331 transcripts were accumulated in WT and *acd5* mutant seeds at similar level. In contrast, *NCED6*
332 transcripts were not detected in WT seeds but accumulated in *acd5* seeds (Fig. 6b and supplemental
333 Fig 3). Similarly *ABI5* and *RGL2* transcripts were more abundant in *acd5*. Altogether these results
334 indicated that the hypersensitivity of *acd5* germination at low temperature was correlated with an
335 impairment of ABA and GA dependent-gene expression. As shown on Figure 6c, the addition of

GA in the medium restored *acd5* germination rate at 4°C to the level of WT seeds. Finally, a hypersensitivity of *acd5* seeds to ABA was observed (Fig 7). Indeed, the germination rate of *acd5* seeds at 22°C was 45%, 65% and 86% lower than that of WT seeds in the presence of 5, 10 and 15 µM ABA, respectively. Taken together, these data suggest that defects in the ABA/GA balance linked to Cer-K activity impairment might be responsible for *acd5* seed hypersensitivity to cold.

Discussion.

In the present report we further investigated the implication of sphingolipids in plant response to cold, with a focus on Cer-P. Our results show that a sphingolipid species related to Cer-P is rapidly and transiently evoked in Arabidopsis plantlets and in cultured cells upon cold shock. In addition to its base-resistance and Rf characteristics, which strongly identifies this lipid as being a Cer-P (Wijesinghe *et al.*, 2007), its formation was impaired in the *acd5* mutant. ACD5 encodes the unique Cer-K in Arabidopsis and has been previously involved in plant response to pathogens (Liang *et al.*, 2003; Bi *et al.*, 2014). Using *in vivo* labelling of phosphorylated lipids, a 70% decrease of cold-evoked Cer-P formation in the *acd5* mutant after cold exposure as compared to WT was observed. The level of Cer-P formed following cold exposure in *acd5* plantlets is therefore in good accordance with a lower Cer-K activity of this mutant already measured *in vitro* using *acd5* plant extracts (Liang *et al.*, 2003, Bi *et al.*, 2011). Moreover, these data directly linked ACD5 activity to the cold-evoked Cer-P accumulation by the detection of Cer-P *in vivo* and suggest that this enzyme might be regulated under cold stress. Such modulations of Cer-K activity *in vivo* have been evidenced in mammal cells (Rovina *et al.*, 2010) and the present study suggests that they also take place in plants for transiently modifying Cer-P abundance. A series of reports have recently illustrated modifications of SL metabolism during cold response at the level of LCB/LCB-P and complex SL (Cantrel *et al.*, 2011; Chen *et al.*, 2012; Dutilleul *et al.*, 2012; Guillas *et al.*, 2012). The analysis of mutants of Cer-P and LCB-P metabolism suggests that the transient formations of PHS-

P and Cer-P upon cold stress are not interdependent. Further investigations are now required to unravel how these events are coordinated and integrate in the overall stress response.

The characteristics of cold-triggered Cer-P formation, i.e. rapid and transient, suggested a role for this molecule as a signal during cold response. Nevertheless neither short nor long term responses to cold were affected in *acd5* mutant at the vegetative stage. In good agreement with our phenotypic observations, the expression of canonical cold-responsive genes such as *CBF*s or *Zat12* was not impaired in stressed *acd5* plantlets. This might be due to the residual Cer-P formed by *acd5* mutant that would be sufficient for triggering cold responses. It might also indicate that additional signals compensate for Cer-P defect. Finally the lack of phenotype might reflect that Cer-P is not a major determinant of cold acclimation in plantlets. Nevertheless, the induction by cold of *NOI3*, a gene of unknown function that we identified as cold-regulated (Dutilleul *et al.*, 2012), was strongly impaired in *acd5* mutant indicating that the impairment of Cer-P might affect specific cold responses. As *NOI3* is also regulated by PHS-P, it might constitute a generic target for SL signalling in plants.

In contrast with the absence of phenotype at the plantlet stage, *acd5* seed germination appeared hypersensitive to low temperature. Germination is regulated by ABA and GA, two hormones that function antagonistically (Finkelstein *et al.*, 2002; Schwechheimer & Willige, 2009). We found that *acd5* seed germination was hypersensitive to ABA, suggesting that the germination defect of *acd5* seeds at low temperatures might involve ABA-dependent processes. As no germination defect is observed at 22°C, it is unlikely that dry *acd5* seeds present higher endogenous ABA levels. Furthermore GA treatment reverted *acd5* germination phenotype at 4°C pointing towards dysfunctions of GA/ABA regulation at low temperature in *acd5* seeds. Supporting this hypothesis, the expression of *NCED6*, *ABI5* and *RGL2* remained high in *acd5* seeds germinated at 4°C and could afford for repressing germination (Lopez-Molina *et al.*, 2001). As a whole, it suggests that ABA-dependent mechanisms leading to germination inhibition are exacerbated in

386 *acd5* seeds, in good correlation with the observed phenotypes. How ACD5 CerK activity
387 participates in ABA signalling in seeds is currently unknown. Noteworthy a recent study reported
388 that *atcer1* ceramidase mutants present strongly reduced ABA-induced stomatal closure (Wu et al.,
389 2015). Together, these studies suggest new functions for Cer metabolism in ABA response.

390 Although *in vivo* analyses clearly established an ACD5-dependent *de novo* accumulation of
391 Cer-P upon cold stress, *in vitro* Cer-K activity was identical in extracts from unstressed or cold-
392 stressed plantlets. A similar discrepancy has been previously observed in Cos7 cells subjected to
393 osmotic stress and was attributed to the regulation of Cer-K *in vivo* via its translocation to plasma
394 membrane (Kim *et al.*, 2006). More generally, localisation has been proposed as a critical factor for
395 Cer-K activity and/or regulation (Carré *et al.*, 2004; Rovina *et al.*, 2009; Bornancin, 2011). Bi et al.
396 (2014) reported that ACD5 is located in different membranes, and proposed that ACD5 could
397 shuttle and thereby get regulated *in vivo* upon stress, what would be lost when cell integrity is
398 disrupted. In addition, as shown by Boath et al. (2008), Cer-P undergo a rapid turnover *in vivo.*
399 Cold could therefore also affect Cer-P level by lowering Cer-P degradation via the inhibition of
400 Cer-P phosphatases. Further investigations on this hypothesis will require the characterization of
401 putative Cer-P phosphatases that are currently unidentified in eukaryotes.

402 Our study also revealed an intriguing feature of the G412R mutated form of ACD5. When
403 extracted from unstressed *acd5* plantlets, it exhibited a 20% reduced activity compared to WT
404 ACD5, but the inhibition rose to 40% when proteins were extracted from cold-stressed plantlets.
405 This last value agrees those reported by Bi et al. (2011) when using older plants i.e. plants
406 undergoing PCD. As Cer-K activity was measured *in vitro* under controlled conditions, this further
407 reduction of activity is likely a consequence of a stable alteration of acd5 acquired *in planta* and
408 triggered by cold. Noteworthy, the G412R point mutation stands in a highly-conserved CC1 region,
409 predicted as a beta helix (Bornancin, 2011). A destabilisation of the enzyme structure caused by the
410 G412R mutation might therefore be amplified by temperature changes. In this respect the up-

regulation of *ACD5* gene transcription observed in *acd5* mutant exposed to cold could constitute a compensatory mechanism to cope with the additional loss of Cer-K activity triggered by cold.

Taken together our data bring the direct evidence for the implication of the Cer-K ACD5 in abiotic stress response. It now paves the way for unravelling the underlying regulatory mechanisms and addressing the versatility of Cer-K participation in plant response to environmental cues.

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589 **Figures Legends**

590

591 **Figure 1: A strongly hydrophobic phosphorylated sphingolipid accumulates upon cold**
592 **exposure:** (a) Cultured cells or (b) 14 day-old plantlets were exposed to 4°C in presence of ^{33}P-
593 orthophosphate, or were kept at 22°C for the indicated time. After extraction, radiolabelled
594 sphingolipids were developed on a TLC, detected by autoradiography, and quantified using a Storm
595 PhosphorImager. The TLCs presented are representative of >4 biological replicates. (c, d)
596 Quantification of the abundance of the lipid A expressed as a percentage of the total radioactivity
597 incorporated in the lane. Results represent means ± SD of at least four independent experiments.
598 Asterisks indicate statistic differences determined by Student's test between lipid A abundance at T_0
599 and the other time points (**: P<0.01). Rf were calculated from the TLC presented in
600 Supplementary figure 1a.

601

602 **Figure 2: *acd5* mutants are defective in cold-induced lipid A accumulation:** (a) Phenotype of
603 the 14 day-old plantlets used for $^{33}PO_4$ labelling *in planta*. Note that *acd5* plantlets are
604 phenotypically identical to WT at this stage. (b) PR1 transcript abundance was analyzed by semi-
605 quantitative RT-PCR in RNA extracted from WT and *acd5* 14 day-old plantlets as well as as
606 positive control Salicilic acid treated WT plantlets. At this stage PR1 can not be detected in *acd5*
607 plantlets, further demonstrating that they are in the same condition as WT plantlets (c) Ratio of
608 radiolabelled lipid A abundance in cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) WT and
609 *acd5* plantlets. Results represent means ± SD of four independent experiments, each composed of
610 five independent biological samples. Asterisks indicate statistic differences determined by Student's
611 test between *acd5* and WT (**: P<0.01)

612

Figure 3: PHS-P and Cer-P accumulation in sphingolipid kinase mutants in response to cold shock. 14-days old plantlets were labeled as described in Material and Methods section. PHS-P and Cer-P abundances were compared after $^{33}PO_4$ labelling *in planta* and expressed as fold increase between cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) plantlets. Results represent means ± SD of three independent biological repeats. (a) Cold-induced increase of PHS-P abundance in WT and *acd5* mutant. (b) Cold-induced increase of Cer-P abundance in WT and LCB kinase mutants.

Figure 4: Cold affects ACD5 activity and gene expression in *acd5* plantlets. (a) Crude protein extracts from cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) WT and *acd5* plantlets were used for evaluation of *in vitro* Cer-K activity. Cer-K activity was assayed for 30 min, as described in Material and Methods section, using C8-Cer and $^{33}PO_4$ as a substrate, in the absence or presence of 50 µM Cer-K K1 inhibitor. As controls, assays were carried in the absence of added lipid substrate (C8-Cer) or protein extract. Figure shows a representative TLC obtained after development of the radiolabelled sphingolipids. (b) The amount of ^{33}P-C8-Cer formed by Cer-K activity *in vitro* was determined after TLC development using a Storm PhosphorImager. Results represent the ratio between ^{33}P-C8-Cer formed in the different assays and that formed when extract from unstressed control (WT/22°C) is used. Data are given as mean ± SD of six independent biological repeats. Different letters indicate statistical differences determined by Student's test (P<0.05). (c) *ACD5* transcript abundance was analyzed by semi-quantitative RT-PCR in RNA extracted from unstressed (1h, 22°C) and cold-stressed (1h, 4°C) WT and *acd5* plantlets. *S19* transcripts were used as an internal standard.

Figure 5: *acd5* plantlet phenotypes upon cold exposure. (a) *acd5* and WT seedlings were grown vertically at 22°C or 12°C as described in Material and Methods section. Primary root elongation was recorded every two days over 8 to 28 days. Root elongation is expressed in cm/day and

represents the mean ± SE (n = 40–120). (b) Phenotypes of WT and *acd5* Arabidopsis plantlets grown at 12°C (3 week-old) and 22°C (2 week-old). In these conditions spontaneous HR-like lesions were visible on *acd5* plants after 3 weeks at 22°C and 5 weeks at 12°C (data not shown). (c) Expression of *CBFs*, *ZAT* and *NOI3* genes, analysed using semi-quantitative RT-PCR in 14-days old WT and *acd5* seedlings maintained at 22°C or transferred to 4°C for 4 h. Data are representative of at least three repeats.

Figure 6: *acd5* seed germination at low temperature. (a) *acd5* and WT seeds were germinated at 4, 12 or 22°C in the dark. Germination rates were monitored after the indicated times (d: days) according to the temperature applied. Results are means ± SD of 4 repeats (n>200 seeds). Asterisks indicate statistic difference determined by Student's test between *acd5* and WT in the same conditions (*: P<0.05, **: P<0.01). (b) *NCED3-9*, *ABI5* and *RGL2* gene expression was analysed by semi-quantitative RT-PCR in WT and *acd5* seeds germinated for 14 days at 4°C. Results are representatives of 3 independent repeats. *18S* gene expression was used as a standard. (c) *acd5* and WT seed germination was assayed at 4°C in obscurity, in presence or absence of 5 µM GA, and germination rates were compared after 14 days. Results are means ± SD of 4 repeats (n>200 seeds).

Figure 7: *acd5* germination sensitivity to ABA. WT and *acd5* seed germination was assayed 48h at 22°C, in presence or absence of the indicated amount of ABA. Results are the means ± SD of 5 repeats (n>250 seeds). Asterisks indicate statistic difference determined by Student's test between *acd5* and WT in the same conditions (*: P<0.05, **: P<0.01).

Supplemental Figure 1: (a) evaluation of the lipid A Rf. 14 day-old plantlets were labelled with ^{33}P-orthophosphate at 22 or 4°C for 30 min. Extracted lipids were developed on TLC and revealed by autoradiography. Migration was stopped before migration front reached the top of the plate. Rf

was calculated as the ratio of the distance of migration of a given molecule as compared to the migration front (1). **(b) Co-migration of radiolabelled lipid A extracted from cold-stressed cultured cells (Cc) and plantlets (P)**. Cultured cells or 14 day-old plantlets were exposed to 4°C for 5 min in presence of ^{33}P-orthophosphate. After extraction, radiolabelled sphingolipids were developed on a TLC and detected by autoradiography. The signals corresponding to lipid A (A) and phytosphingosine-P (PHS-P) are indicated with arrows, together with the origin (O) of migration.

Supplemental Figure 2: Cold affects ACD5 activity and gene expression in *acd5* plantlets. Crude protein extracts from cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) WT and *acd5* plantlets were used for *in vitro* Cer-K activity. Cer-K activity was assayed for 30 min, as described in Material and Methods section, using C8-Cer as a substrate. As controls, assays were carried in the absence of added lipid substrate. Figure shows a representative TLC obtained after development of the radiolabelled sphingolipids.

As the assays were done by addition of ^{33}P ATP on crude extracts, unidentified non-specific bands due to various kinases activities are also present, as well as a faint band probably corresponding to the phosphorylation of the endogenous Ceramide (Cer-P).

Supplemental Figure 3: Relative expression levels of genes analyzed by RT-PCR. Relative intensities of the bands of interest presented in Figure 4c (a), Figure 5c (b) and Figure 6b (c) has been determined by image analysis using ImageJ sofware. It is expressed as the ratio between the intensity of the band of interest and that of the corresponding reference gene, after subtraction of background intensity.

Supplemental Table 1: Sequences of the primers used in this studies.

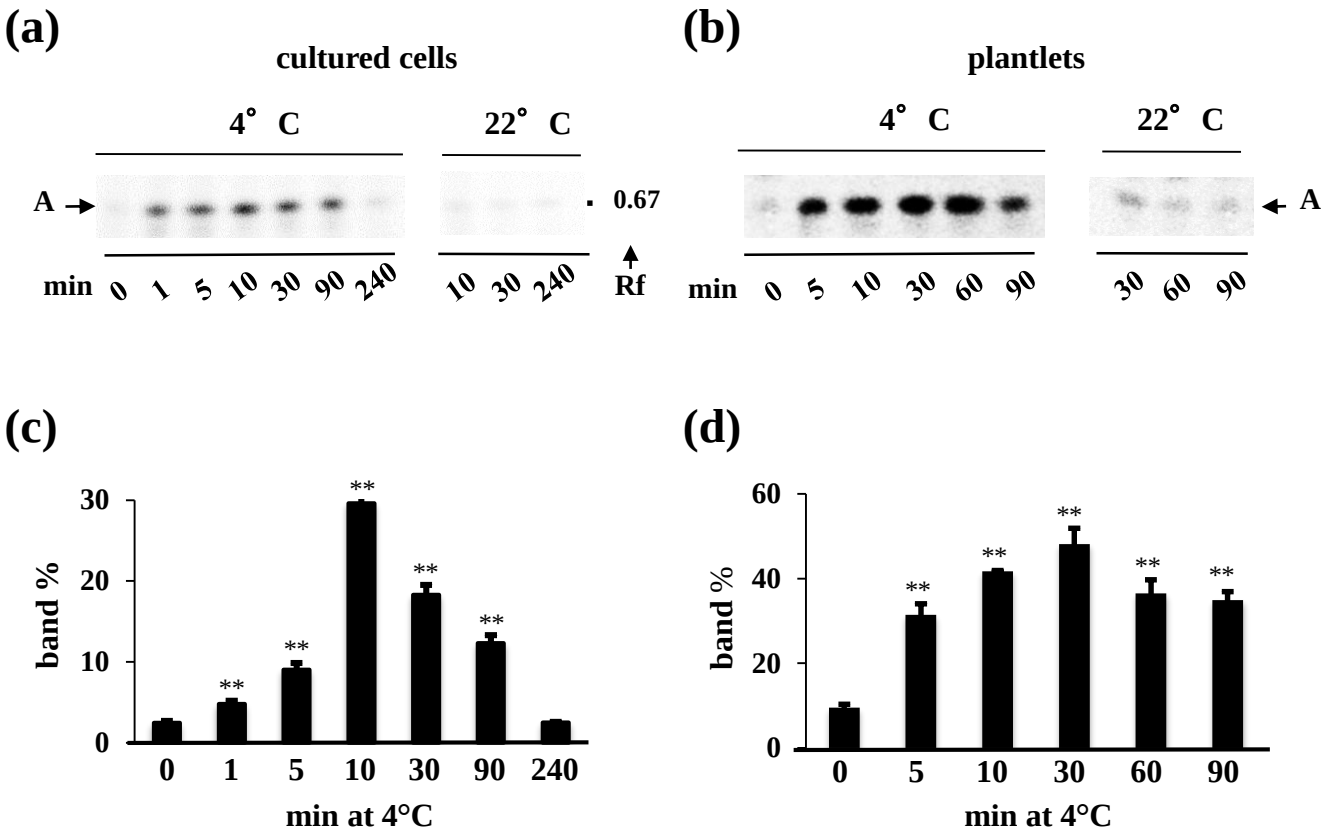
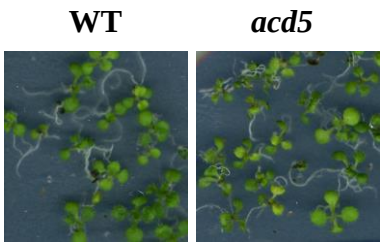
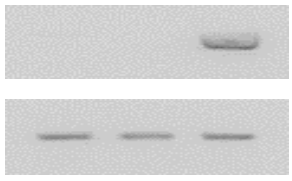

(a)
cultured cells
4° C
22° C
A
0.67
min
0 1 5 10 30 90 240
10 30 240
Rf
(b)
plantlets
4° C
22° C
A
min
0 5 10 30 60 90
30 60 90
(c)
band %
30
20
10
0
**
0 1 5 10 30 90 240
min at 4°C
(d)
band %
60
40
20
0
**
0 5 10 30 60 90
min at 4°C

Figure 1

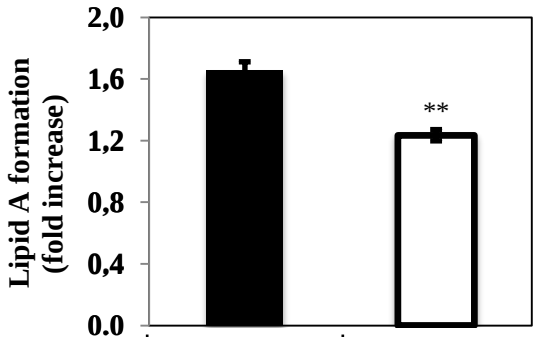
(a)



(b)



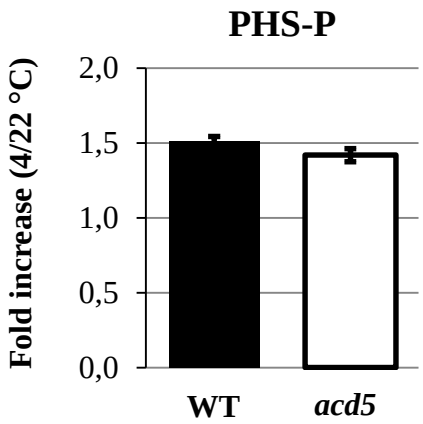
(c)



	wt	*acd5*
22°c	8.6 +/- 0,2%	8.75 +/-0,07%
4°c	14.5 +/-0,1%	10.5 +/-0,1%

Figure 2

(a)


PHS-P
Fold increase (4/22 °C)
2,0
1,5
1,0
0,5
0,0
WT
acd5

(b)

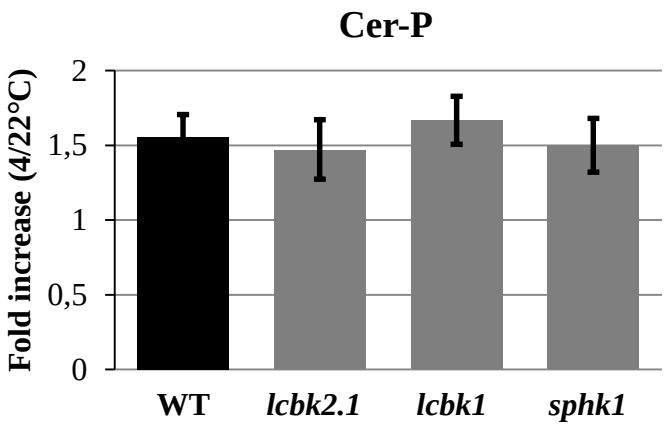
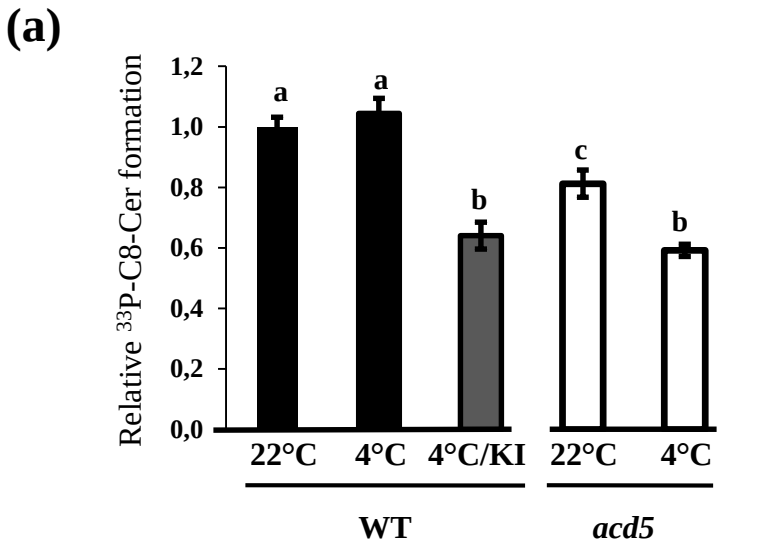
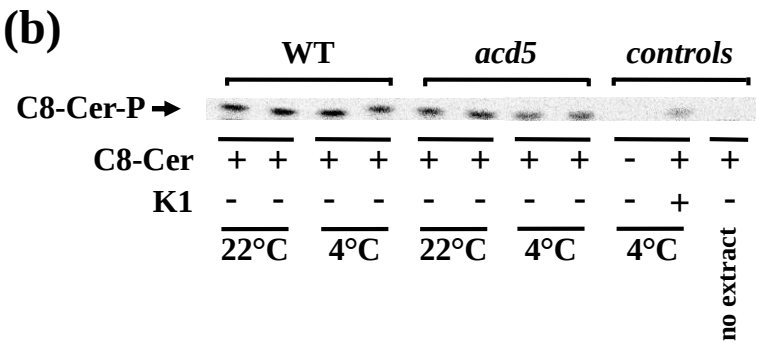

Cer-P
Fold increase (4/22°C)
2
1,5
1
0,5
0
WT
lcbk2.1
lcbk1
sphk1

Figure 3


(a)
Relative 33P-C8-Cer formation
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
a
b
c
b
22°C
4°C
4°C/KI
22°C
4°C
WT
acd5


(b)
WT
acd5
controls
C8-Cer-P
C8-Cer + + + + + + + + - + +
K1 - - - - - - - - - + -
22°C
4°C
22°C
4°C
4°C
no extract

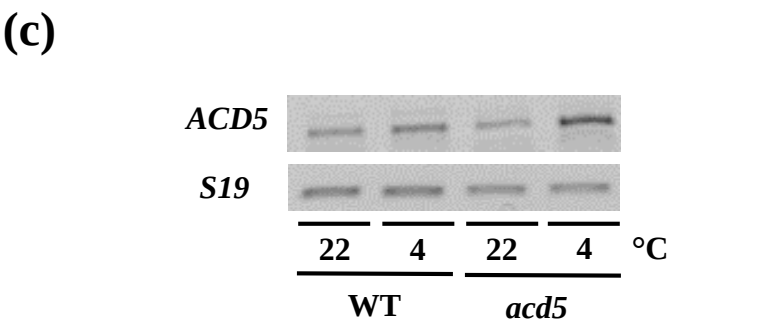

(c)
ACD5
S19
22
4
22
4
°C
WT
acd5

Figure 4

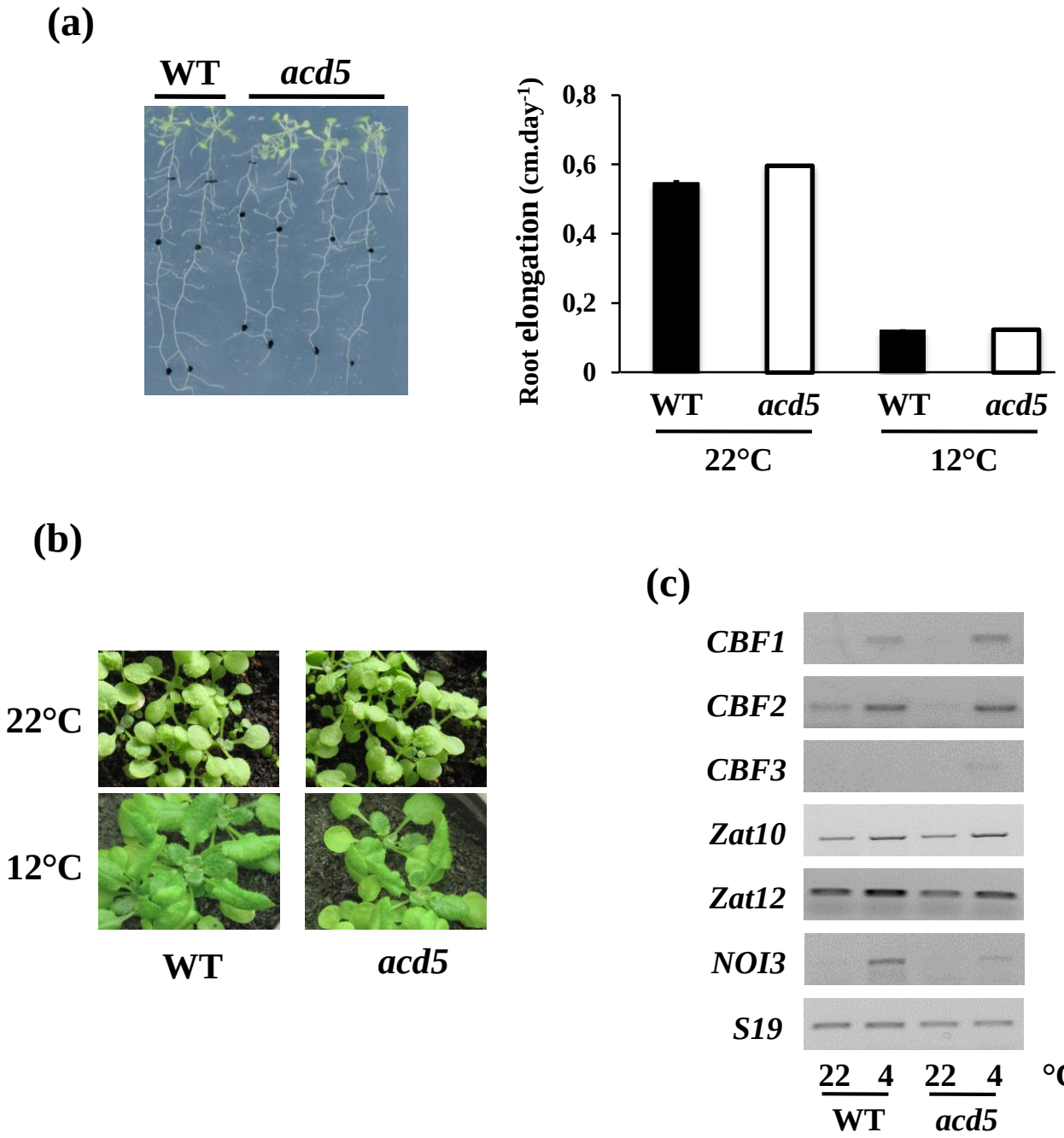

(a)
WT
acd5
Root elongation (cm.day-1)
0,8
0,6
0,4
0,2
0
WT
acd5
WT
acd5
22°C
12°C
(b)
22°C
12°C
WT
acd5
(c)
CBF1
CBF2
CBF3
Zat10
Zat12
NOI3
S19
22 4 22 4 °C
WT
acd5

Figure 5

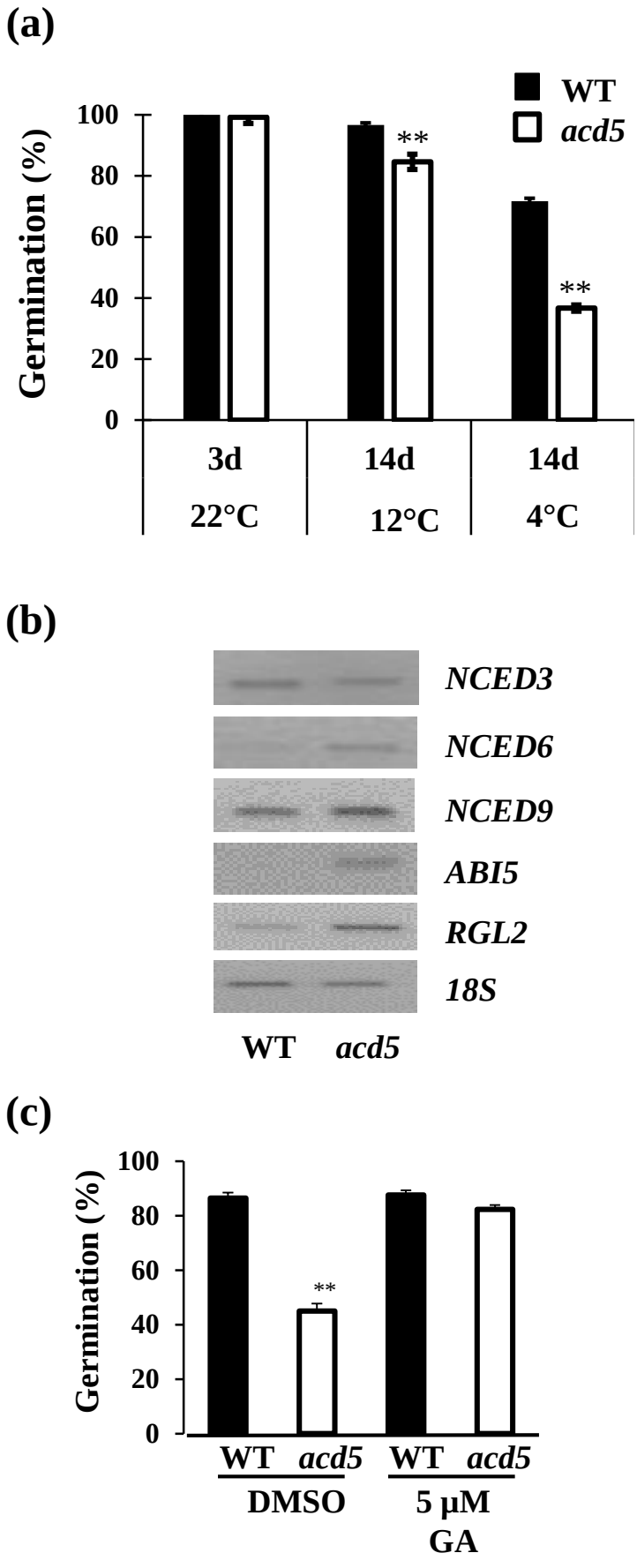

(a)
WT
acd5
Germination (%)
100
80
60
40
20
0
**
**
3d
22°C
14d
12°C
14d
4°C
(b)
NCED3
NCED6
NCED9
ABI5
RGL2
18S
WT
acd5
(c)
Germination (%)
100
80
60
40
20
0
**
WT
acd5
WT
acd5
DMSO
5 µM
GA

Figure 6

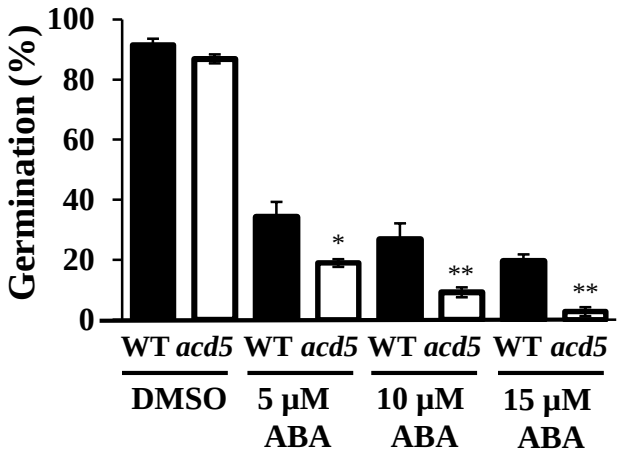
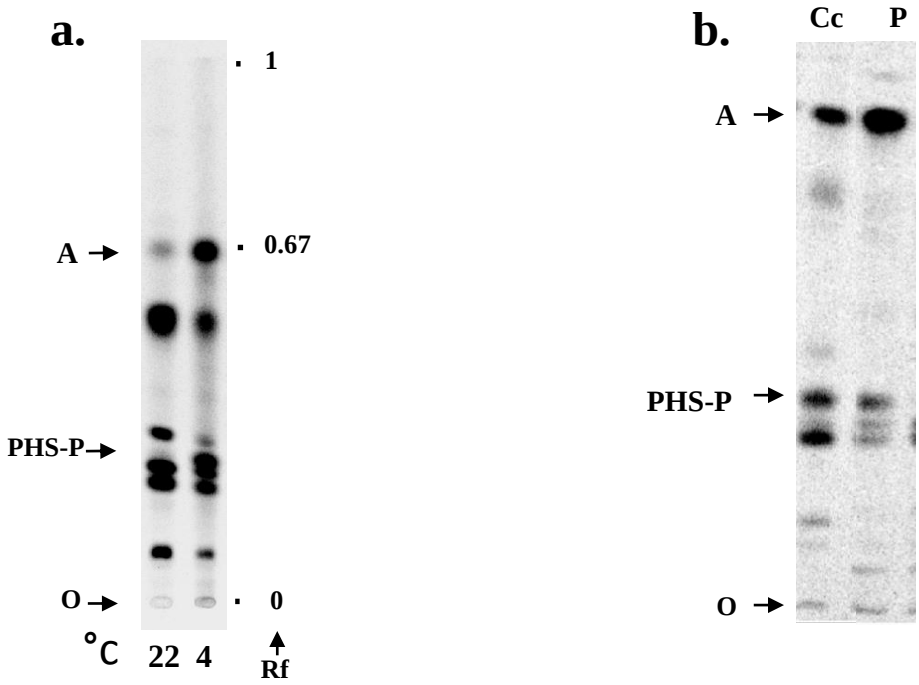
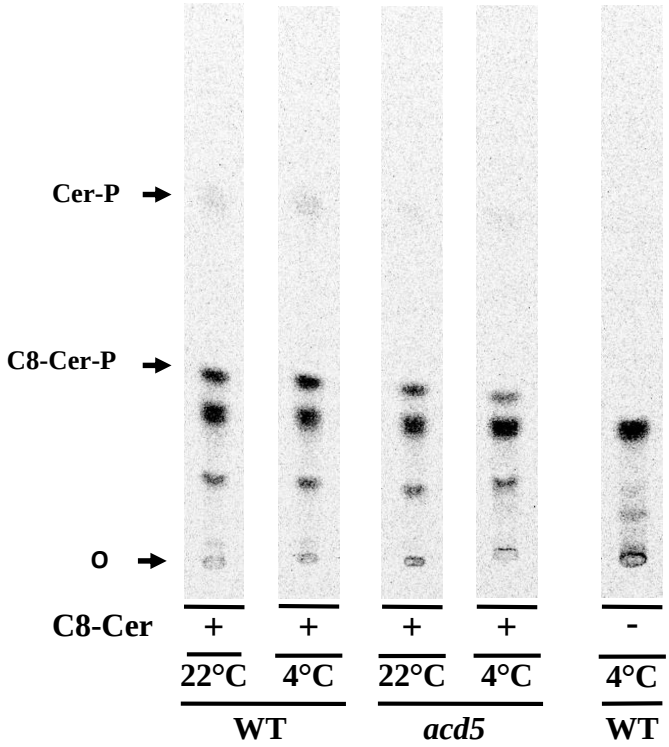

Germination (%)
100
80
60
40
20
0
*
**
**
WT acd5 WT acd5 WT acd5 WT acd5
DMSO
5 µM ABA
10 µM ABA
15 µM ABA

Figure 7



Supplemental Figure 1



Supplemental figure 2

(a)

	22°C		4°C	
	WT	***acd5***	**WT**	***acd5***
ACD5	**0.91**	**0.93**	**0.90**	**1.18**

(b)

	22°C		4°C	
	WT	***acd5***	**WT**	***acd5***
CBF1	**0.11**	**0.63**	**0.3**	**0.88**
CBF2	**0.69**	**1.21**	**0.35**	**1.43**
CBF3	**0.13**	**0.24**	**0.01**	**0.31**
ZAT10	**0.65**	**0.84**	**0.73**	**1.00**
ZAT12	**1,66**	**2.11**	**1.49**	**1.88**
NOI3	**0.13**	**0.85**	**0.18**	**0.34**

(c)

	4°C	
	WT	***acd5***
NCED3	**1.00**	**1.18**
NCED6	**0.37**	**1.00**
NCED9	**1.88**	**2.36**
ABI5	**0.03**	**1.21**
RGL2	**0.56**	**2.00**

Supplemental Figure 3

ABI5 (At2g36270)
5’ TGTCTCTATTTGGAACGCAG 3’
5’ ATAACCTGATGGGTCACCCA 3’
ACD5 (At5g51290)
5’ GCGAGAATTCCGAAACTG 3’
5’ CATCCACAACAAGCCCATCAGG 3’
CBF1 (At4g25490)
5’ CCTTATCCAGTTTCTTGAAAC 3’
5’ CGAATATTAGTAACTCCAAACGCAC 3’
CBF2 (At4g25470)
5’ CCTTATCCAGTTTCTTGAAAC 3’
5’ GACCATGAGCATCCGTCGTCATATGAC 3’
CBF3 (At4g25480)
5’ CCTTATCCAGTTTCTTGAAAC 3’
5’ CACTCGTTTCTCAGTTTTACAAAC 3’
NCED3 (At3g14440)
5’GCTGCGGTTTCTGGGAGAT 3’
5’GGCGGGAGAGTTTGATGATT 3’
NCED6 (At3g24220)
5’TTCAAGATACCGACACTTCCTG 3’
5’GGGCGATTCTGCTCCATAG 3’
NCED9 (At1g78390)
5’TCCCCTGCTATGTTTCTTCC 3’
5’AGACGGTGGTTTGAATGTCG 3’
NOI3 (At2g17660)
5’ AGCAAAGCCGGTGAAGATAA 3’
5’ CAAAGGGCTCATAAGGCTCA 3’
RGL2 (At3g03450)
5’ TGGCAGACGCGACCACTCAT 3’
5’ TACTCGTTCTCTTAACTCTCAA 3’
S19 (At3g04920)
5’ TCCAGGAAGCAGTTCGTTATTGATG 3’
5’ TCACTTCTTCTTGGCATCACCAG 3’
Zat10 (At1g27730)
5’ AGGCTCTTACATCACCAAGATTAG 3’
5’ TACACTTGTAGCTCAACTTCTCCA 3’
Zat12 (At5g59820)
5’ CCTTAGGAGGTCACCGTGC 3’
5’ CAAGCCACTCTCTTCCCACT 3’

Supplemental Table 1