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1 **Evidence for ACD5 Ceramide Kinase activity involvement in Arabidopsis response to cold**
2 **stress.**

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4 **Running title: ACD5 in cold stress response**

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22

23 **Abstract**

24

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

40

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

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48 **Introduction.**

49 Cold is a major abiotic stress that restrains plant growth and development and affects plant
50 species distribution. Plants from temperate zones are globally tolerant to non-freezing temperatures
51 (chilling) and exhibit different degree of sensitivity when exposed to negative temperature
52 (freezing). Nevertheless, freezing tolerance might be enhanced after acclimation, i.e. pre-treatment
53 at chilling temperature. The use of Omic-based approaches has recently helped identify major
54 genes, proteins and metabolites involved in chilling and/or freezing responses (Knight & Knight
55 2012). These data highlighted a global modification of plant metabolism in cold-stressed plants,
56 which at least partly relies on the modifications of gene expression and counterbalances cell
57 dysfunctions under low temperature (Ruelland et al. 2009; Knight & Knight 2012).

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

73 LCB-P and Cer-P formation, respectively. The roles of SL as signalling molecules are now well
74 recognized. In particular, studies carried out in animals evidenced the crucial signalling function of
75 LCB and Cer as well as LCB-P and Cer-P (Bornancin, 2011; Kawabori *et al.*, 2013; Maceyka &
76 Spiegel, 2014). Noteworthy it appears that LCB/Cer act antagonistically to their phosphorylated
77 counterparts in regulating cell processes as diverse as cell division, differentiation or apoptosis
78 (Mendelson *et al.*, 2014; Aguilera-Romero *et al.*, 2014). The control of the equilibrium between
79 phosphorylated and unphosphorylated forms *via* the regulation of the specific kinase and
80 phosphatase activities is therefore crucial to maintain cell fitness and determine cell fate (Hoeflerlin
81 *et al.*, 2013; Payne *et al.*, 2014). In plants, a growing hand of data indicates that comparable
82 signalling roles could be endowed by SL in response to biotic and abiotic stress (Ng *et al.*, 2001;
83 Coursol *et al.*, 2003, 2005; Worrall *et al.*, 2008; Markham *et al.*, 2013; Wu *et al.*, 2015). Pioneering
84 works determined that treatments with LCB-P or LCB kinase inhibitors alter abscisic acid (ABA)
85 responses (Ng *et al.*, 2001; Coursol *et al.*, 2003). Further studies showed that four putative isoforms
86 of LCB kinases exist in *Arabidopsis thaliana* (Coursol *et al.*, 2005; Worrall *et al.*, 2008; Guo *et al.*,
87 2011), and illustrated the implication of some of these isoforms in response to ABA (Worrall *et al.*,
88 2008; Guo *et al.*, 2012). In plants Cer-K activity and Cer/Cer-P balance have been essentially
89 involved in programmed cell death (PCD) and associated to plant-pathogen interactions (Brandwagt
90 *et al.*, 2000; Greenberg *et al.*, 2000; Coursol *et al.*, 2003; Liang *et al.*, 2003; Townley *et al.*, 2005;
91 Raffaele *et al.*, 2008; Bi *et al.*, 2014). As observed in most organisms, a unique gene designated
92 *ACCELERATED CELL DEATH5 (ACD5)* codes for a Cer-K in *Arabidopsis*. The sole mutant of this
93 gene described to date is the point mutant *acd5* which presents a reduced Cer-K activity. The *acd5*
94 mutant displays a spontaneous PCD that occurs at late development stages, together with an
95 enhanced sensitivity to pathogen attack (Greenberg *et al.*, 2000; Liang *et al.*, 2003; Bi *et al.*, 2011,
96 2014).

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

111

112 **Material and methods**

113

114 *Chemicals*

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

121

122 *Plant and cell cultures*

123 Experiments were performed using *Arabidopsis thaliana* L. Heynh. wild type (WT) in the
124 Columbia (Col-0) background as cultured cells and seedlings. The *acd5* mutant (Greenberg *et al.*,
125 2000) was kindly provided by Pr J. Greenberg (Uni. Chicago, USA).

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

132

133 *Cold treatment and plantlet labelling*

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

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

142

143 *Phosphorylated sphingolipid extraction and analysis*

144 Lipids were subsequently extracted by transferring the samples from liquid nitrogen to water
145 bath maintained at 60°C for 15 min incubation. Subsequently the supernatant was collected and 3
146 additional extractions were done in the same manner, i.e. N₂ frozen, 15 min at 60°C and extracted
147 twice with 3 ml chloroform/methanol (1:1, v/v) and once with 3 ml chloroform. Combined extracts
148 were dried under nitrogen and resuspended in 1 ml methanol. To hydrolyse glycerophospholipids, 1
149 ml 3N NH₄OH/methanol (1:1, v/v) was added to 1 ml total lipid extracts and samples were
150 incubated for 1 h at 50°C. The reaction was stopped by neutralization *via* the addition of 2.5 ml acid
151 acetic/water (2:1, v/v). Two successive phase extractions were done by the addition of 2.5 ml
152 chloroform. Combined chloroform phases were nitrogen-dried and sphingolipids were desalted by
153 butanol/water extraction. Briefly sphingolipids were resuspended in 500 µl water-saturated butanol.
154 Then 500 µl butanol-saturated water was added, samples were vortexed for 1 min and briefly
155 centrifuged to allow phase partitioning. A second extraction was done similarly. Pooled butanol
156 fractions were back extracted with 500 µl butanol-saturated water in the same manner i.e. 1min
157 vortexing followed by a brief centrifugation. Then incorporated radioactivity was estimated by
158 liquid scintillation counting on an aliquot of butanol fraction. Butanol fractions were dried under
159 nitrogen and resuspended in methanol. Sphingolipids were developed by thin-layer chromatography
160 on Silica 60 plates (Merck, Darmstadt, Germany) using chloroform/acetone/methanol/acetic-
161 acid/water (10:4:3:2:1; v/v) as solvent system (Wijesinghe *et al.*, 2007). Radiolabeled sphingolipids

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

166

167 *In vitro ceramide kinase assay.*

168 Fourteen day-old plantlets were prepared in flasks in liquid media as indicated for labelling
169 assay and submitted or not to cold treatment. They were subsequently harvested, soaked and
170 immediately frozen in liquid N₂. The crude plant extracts were prepared as previously described
171 (Liang *et al.*, 2003; Bi *et al.*, 2011). Approximately 50 mg of plantlets were ground in liquid N₂ and
172 placed in a 1.5 mL microfuge tube containing 0.5 mL of extraction buffer (20 mM MOPS pH 7.2, 2
173 mM EGTA, 1 mM DTT, 10% glycerol) supplemented with 1 mM PMSF, 5 μM pepstatin and 10
174 μM leupeptine. The extract was centrifuged for 10 min at 14 000 g, and the supernatant was assayed
175 for protein concentration using Bradford protein assay (Biorad, Marnes la Coquette, France). Cer-K
176 activity was assayed in a final volume of 100 μL of reaction buffer (20 mM MOPS pH 7.2
177 containing 2 mM EGTA, 1 mM DTT, 10% glycerol, 10 mM KCl, 15 mM MgCl₂, and 1 mM ATP)
178 supplemented with 5 μCi [³³P]ATP and C8-Cer to 50 μM final concentration. C8-Cer substrate
179 dissolved in methanol was vacuum-dried in each reaction tube and re-suspended by pipetting in 10
180 μL of 0.1% Triton X-100 prior to adding reaction buffer. The reaction was initiated by the addition
181 of 50 μg protein and was allowed for 30 min at 30°C. It was then stopped by the addition of 600 μL
182 chloroform/methanol (1:1, v/v) and 265 μL 2M KCl. Chloroform phase was N₂-dried and desalted
183 by butanol/water partitioning as described above. After N₂ drying, lipid extracts were dissolved in
184 20 μL methanol, spotted and developed on Silica 60 plates (Merck, Darmstadt, Germany) in
185 chloroform/acetone/methanol/acetic-acid/water (10:4:3:2:1, v/v). Radiolabelled species were
186 revealed and quantified as described above.

187 *Phenotypic analyses*

188 Germination tests were performed using surface sterilized seeds sown on sterile water
189 containing 0,8% agar. Plates were incubated at 4°C for 48h, and subsequently transferred to 22°C or
190 kept at 4°C. Transfer of control seeds to 22°C was considered as T₀. Germination was recorded at
191 different time points depending on incubation temperature. Where indicated, filter-sterilized GA or
192 ABA solutions were added to the medium at the indicated concentrations. For ABA experiments, an
193 equivalent volume of DMSO was used for the control.

194 For root growth tests, seeds were processed as described in the plant culture section. Three
195 day-old plantlets were transferred to 12x12 cm square plates containing ½ strength MS medium and
196 grown vertically. After 24h recovery at 22°C, considered as T₀, plates were transferred to 12°C or
197 kept at 22°C. Root elongation was recorded every two days over 10 (at 22°C) to 28 (at 12°C) days.

198

199 *RNA isolation and RT-PCR analyses*

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

206

207 *Statistical analysis*

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

211

212 **Results**

213 **Cold triggers the transient formation of a highly hydrophobic phosphorylated SL**

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

231

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

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

237 *Line N629619*). Nevertheless no homozygous plants from these lines could be obtained, suggesting
238 that *acd5* KO mutants were lethal. Therefore, for these analyses, the *A. thaliana acd5* knock down
239 mutant exhibiting a G412R point mutation already described (Greenberg *et al.*, 2000) was used. As
240 for the experiments presented in Figure 1, 14 day-old *in vitro* grown plantlets were used. At this
241 stage *acd5* plantlets are phenotypically similar to WT (Fig. 2a). Furthermore, *PR1* gene was not
242 expressed in *acd5* 14 days old mutant plantlets exposed or not to cold (Fig. 2b), indicating that
243 plantlets were not undergoing PCD at this stage of development (Greenberg *et al.*, 2000).
244 Therefore, this material appeared suitable for subsequent *in planta* labelling and cold response
245 monitoring. The [³³P] labelled lipid A was quantified after 3 min of cold shock. In these conditions
246 lipid A increased by 60%, ie. 1.6 ± 0.21 fold, in WT plantlets ($8.6 \pm 0.10\%$ to $14.5 \pm 0.12\%$ of total
247 lane radioactivity) whereas it only increased by 20%, ie. 1.2 ± 0.08 , fold in the *acd5* mutant ($8.75 \pm$
248 0.07% to $10.5 \pm 0.14\%$ of the total lane radioactivity) (Fig. 2c). These results showed that the lipid
249 A formation required ACD5 Cer-K activity and thereby established that it was a *bona fide* Cer-P.
250 Therefore, the designation Cer-P will be used thereafter instead of lipid A.

251

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

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

262 **Evaluation of the ACD5 activity modulation in response to cold.**

263 To investigate whether the Cer-P accumulation observed during cold response was due to an
264 enhanced Cer-K activity, Cer kinase activity *in vitro* using crude protein extracts from plantlets
265 exposed or not to cold was measured. Previous studies using *Arabidopsis thaliana* crude protein
266 extract showed that C8-Cer was the preferred substrate for Cer-kinase activity *in vitro* (Liang *et al.*,
267 2003). Therefore we monitored the formation of [³³P]-C8-Cer-P using crude protein extracts from
268 WT plantlets formerly exposed to cold or maintained at 22°C. In both case a strong Cer kinase
269 activity was observed (Fig. 4a,b, and supplemental Fig 2). As control, no signal corresponding to
270 C8-Cer-P could be observed in absence of C8-Cer substrate or of plant extract (Fig. 4b). In addition,
271 activity measurements in the presence of the Cer-K inhibitor K1 which partially and specifically
272 inhibits recombinant rice Cer-K activity *in vitro* (Bi *et al.*, 2011) was performed. The treatment with
273 K1 inhibitor led to a 40% inhibition of Cer-P phosphorylation (Fig. 4a,b). These results evidenced
274 that the difference in Cer phosphorylation observed *in vivo* upon cold shock was not correlated with
275 a modification of the Cer-K catalytic activity as measured *in vitro*. The same experiment was
276 carried out using *acd5* plantlet protein extracts. Cer-K activity measured in extracts of unstressed
277 *acd5* plantlets was 20% lower than that of unstressed WT plantlets protein extract (Fig. 4a,b).
278 Surprisingly, an additional decrease of 20% was observed when the assay was performed with
279 extracts from cold-stressed *acd5* plantlets that consequently retained only 60% of the activity of
280 WT plantlet extracts (Fig. 4a,b), similarly to previous observations (Bi *et al.*, 2011). These data
281 suggested that cold stress exacerbated the consequences of the mutation on ACD5 Cer-K activity.

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

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

289

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

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

309

310 **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

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

341

342 **Discussion.**

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

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

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

375 In contrast with the absence of phenotype at the plantlet stage, *acd5* seed germination
376 appeared hypersensitive to low temperature. Germination is regulated by ABA and GA, two
377 hormones that function antagonistically (Finkelstein *et al.*, 2002; Schwechheimer & Willige, 2009).
378 We found that *acd5* seed germination was hypersensitive to ABA, suggesting that the germination
379 defect of *acd5* seeds at low temperatures might involve ABA-dependent processes. As no
380 germination defect is observed at 22°C, it is unlikely that dry *acd5* seeds present higher endogenous
381 ABA levels. Furthermore GA treatment reverted *acd5* germination phenotype at 4°C pointing
382 towards dysfunctions of GA/ABA regulation at low temperature in *acd5* seeds. Supporting this
383 hypothesis, the expression of *NCED6*, *ABI5* and *RGL2* remained high in *acd5* seeds germinated at
384 4°C and could afford for repressing germination (Lopez-Molina *et al.*, 2001). As a whole, it
385 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-

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

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

416

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424

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588

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 ³³P-
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 T₀
599 and the other time points (**: *P*<0.01). R_f 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 ³³PO₄ 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 Salicylic 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

613 **Figure 3: PHS-P and Cer-P accumulation in sphingolipid kinase mutants in response to cold**
614 **shock.** 14-days old plantlets were labeled as described in Material and Methods section. PHS-P and
615 Cer-P abundances were compared after $^{33}\text{PO}_4$ labelling *in planta* and expressed as fold increase
616 between cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) plantlets. Results represent means
617 \pm SD of three independent biological repeats. (a) Cold-induced increase of PHS-P abundance in WT
618 and *acd5* mutant. (b) Cold-induced increase of Cer-P abundance in WT and LCB kinase mutants.

619
620 **Figure 4: Cold affects ACD5 activity and gene expression in *acd5* plantlets.** (a) Crude protein
621 extracts from cold-stressed (3 min, 4°C) and unstressed (3 min, 22°C) WT and *acd5* plantlets were
622 used for evaluation of *in vitro* Cer-K activity. Cer-K activity was assayed for 30 min, as described
623 in Material and Methods section, using C8-Cer and $^{33}\text{PO}_4$ as a substrate, in the absence or presence
624 of 50 μM Cer-K K1 inhibitor. As controls, assays were carried in the absence of added lipid
625 substrate (C8-Cer) or protein extract. Figure shows a representative TLC obtained after
626 development of the radiolabelled sphingolipids. (b) The amount of ^{33}P -C8-Cer formed by Cer-K
627 activity *in vitro* was determined after TLC development using a Storm PhosphorImager. Results
628 represent the ratio between ^{33}P -C8-Cer formed in the different assays and that formed when extract
629 from unstressed control (WT/22°C) is used. Data are given as mean \pm SD of six independent
630 biological repeats. Different letters indicate statistical differences determined by Student's test
631 ($P < 0.05$). (c) *ACD5* transcript abundance was analyzed by semi-quantitative RT-PCR in RNA
632 extracted from unstressed (1h, 22°C) and cold-stressed (1h, 4°C) WT and *acd5* plantlets. *S19*
633 transcripts were used as an internal standard.

634
635 **Figure 5: *acd5* plantlet phenotypes upon cold exposure.** (a) *acd5* and WT seedlings were grown
636 vertically at 22°C or 12°C as described in Material and Methods section. Primary root elongation
637 was recorded every two days over 8 to 28 days. Root elongation is expressed in cm/day and

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

644
645 **Figure 6: *acd5* seed germination at low temperature.** (a) *acd5* and WT seeds were germinated at
646 4, 12 or 22°C in the dark. Germination rates were monitored after the indicated times (d: days)
647 according to the temperature applied. Results are means \pm SD of 4 repeats ($n > 200$ seeds). Asterisks
648 indicate statistic difference determined by Student's test between *acd5* and WT in the same
649 conditions (*: $P < 0.05$, **: $P < 0.01$). (b) *NCED3-9*, *ABI5* and *RGL2* gene expression was analysed
650 by semi-quantitative RT-PCR in WT and *acd5* seeds germinated for 14 days at 4°C. Results are
651 representatives of 3 independent repeats. *18S* gene expression was used as a standard. (c) *acd5* and
652 WT seed germination was assayed at 4°C in obscurity, in presence or absence of 5 μ M GA, and
653 germination rates were compared after 14 days. Results are means \pm SD of 4 repeats ($n > 200$ seeds).

654
655 **Figure 7: *acd5* germination sensitivity to ABA.** WT and *acd5* seed germination was assayed 48h
656 at 22°C, in presence or absence of the indicated amount of ABA. Results are the means \pm SD of 5
657 repeats ($n > 250$ seeds). Asterisks indicate statistic difference determined by Student's test between
658 *acd5* and WT in the same conditions (*: $P < 0.05$, **: $P < 0.01$).

659
660 **Supplemental Figure 1: (a) evaluation of the lipid A Rf.** 14 day-old plantlets were labelled with
661 ^{33}P -orthophosphate at 22 or 4°C for 30 min. Extracted lipids were developed on TLC and revealed
662 by autoradiography. Migration was stopped before migration front reached the top of the plate. Rf

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

669
670 **Supplemental Figure 2: Cold affects ACD5 activity and gene expression in *acd5* plantlets.**

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

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

679
680 **Supplemental Figure 3: Relative expression levels of genes analyzed by RT-PCR.** Relative
681 intensities of the bands of interest presented in Figure 4c (a), Figure 5c (b) and Figure 6b (c) has
682 been determined by image analysis using ImageJ software. It is expressed as the ratio between the
683 intensity of the band of interest and that of the corresponding reference gene, after subtraction of
684 background intensity.

685
686 **Supplemental Table 1: Sequences of the primers used in this studies.**

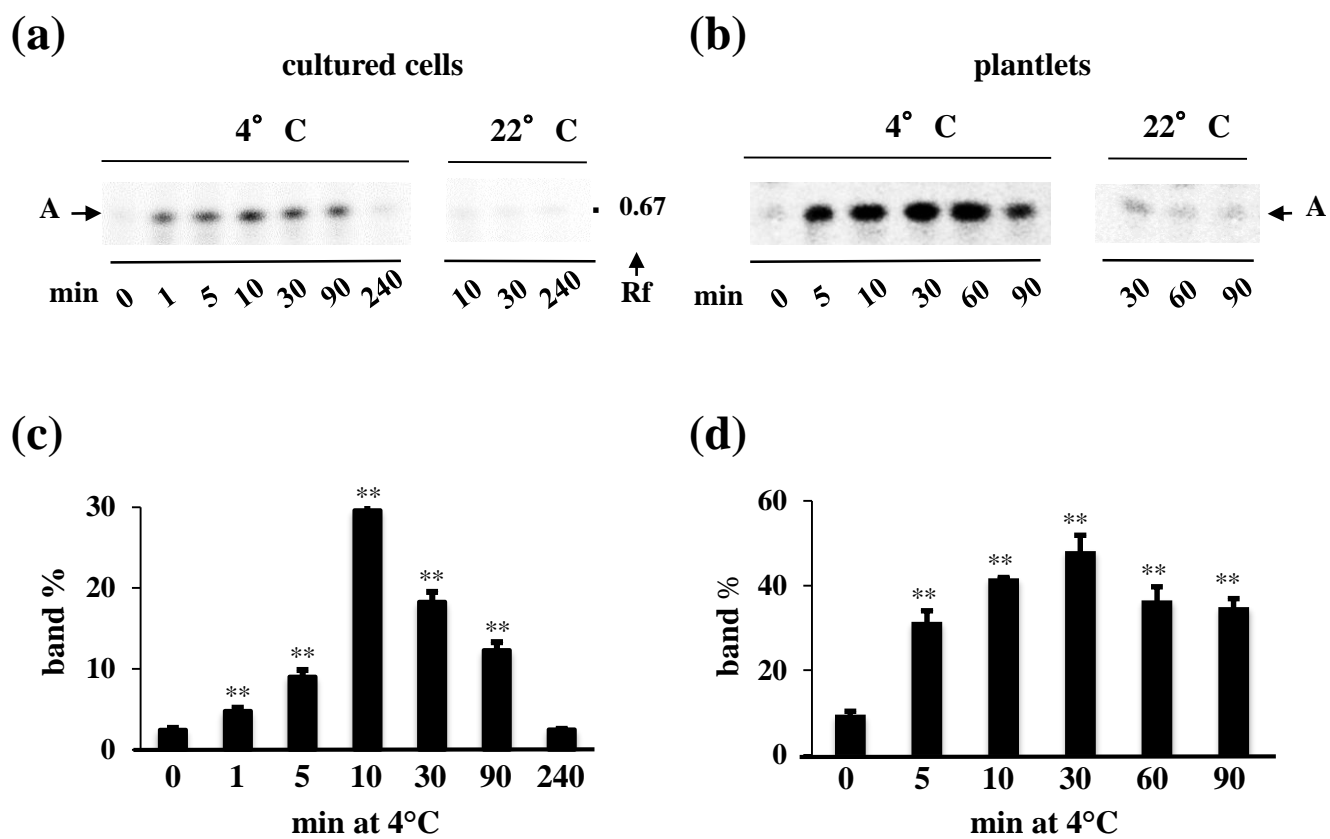


Figure 1

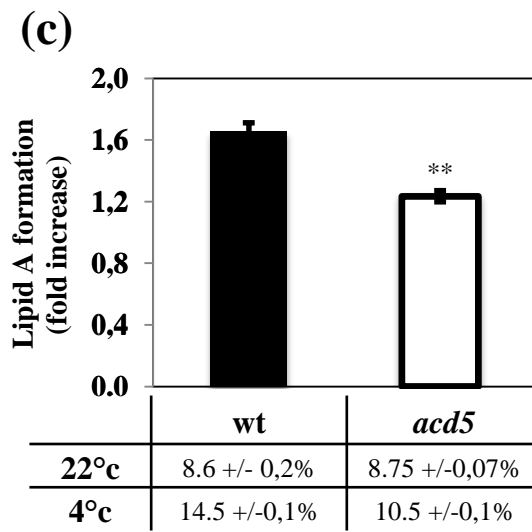
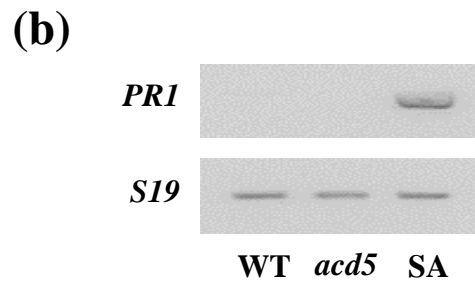
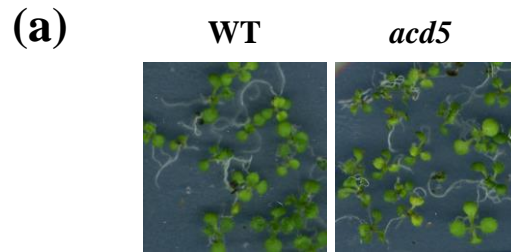
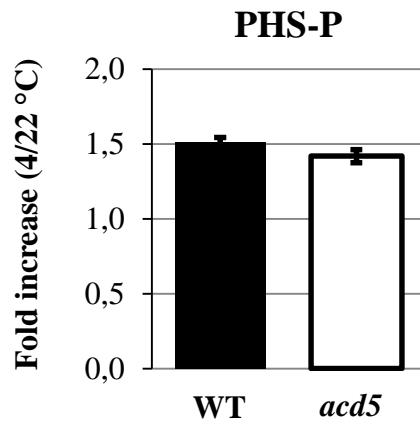


Figure 2

(a)



(b)

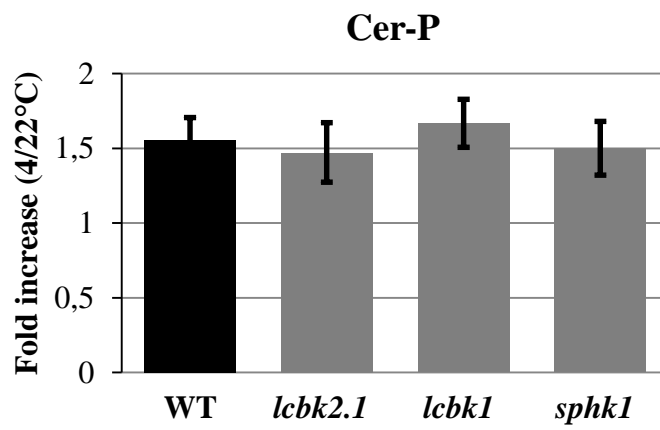
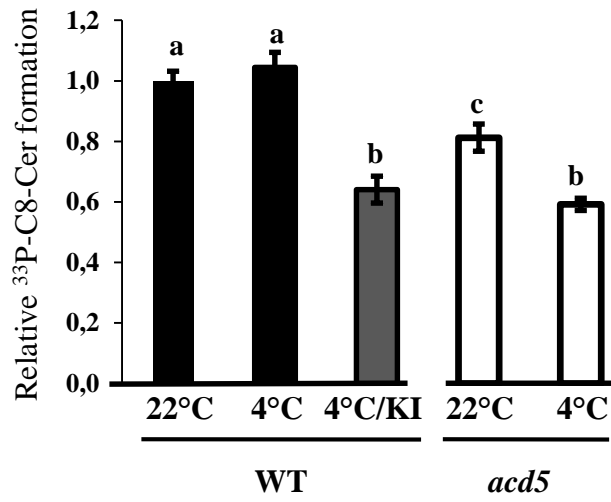
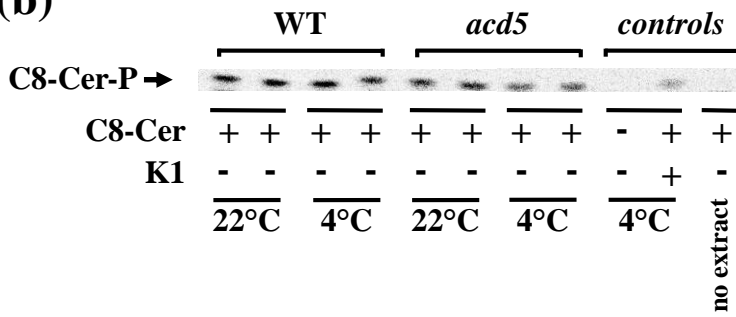


Figure 3

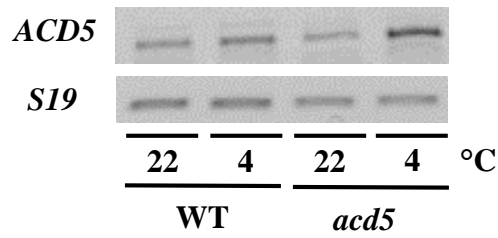
(a)



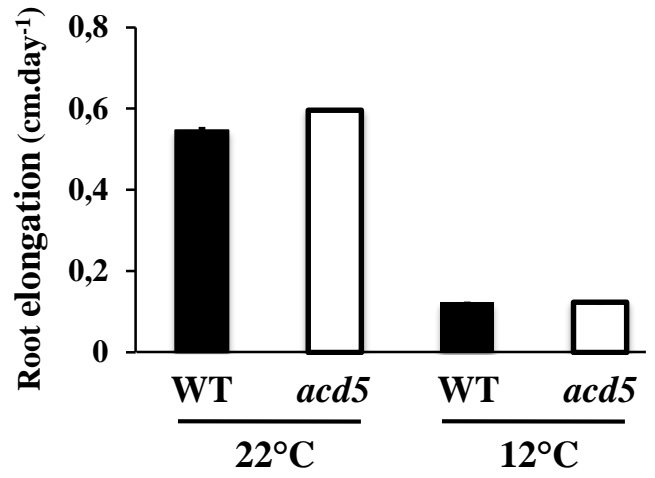
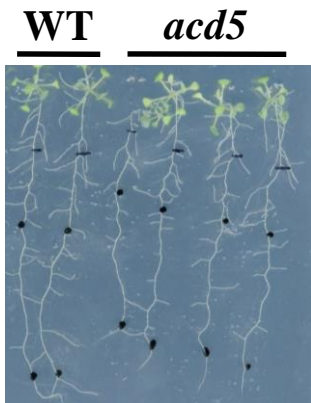
(b)



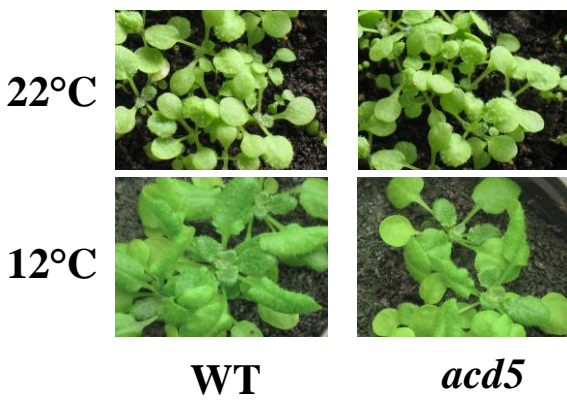
(c)



(a)



(b)



(c)

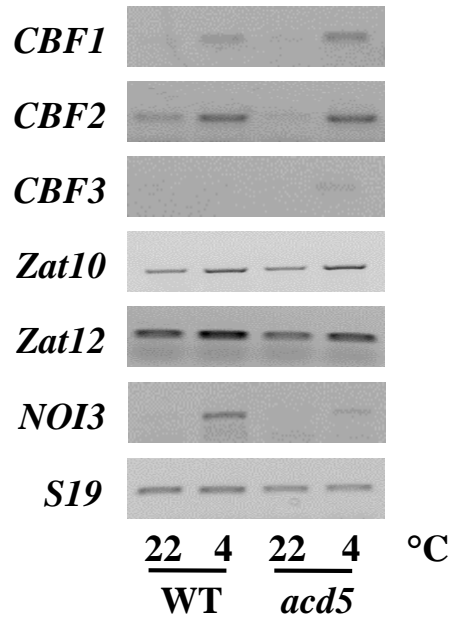
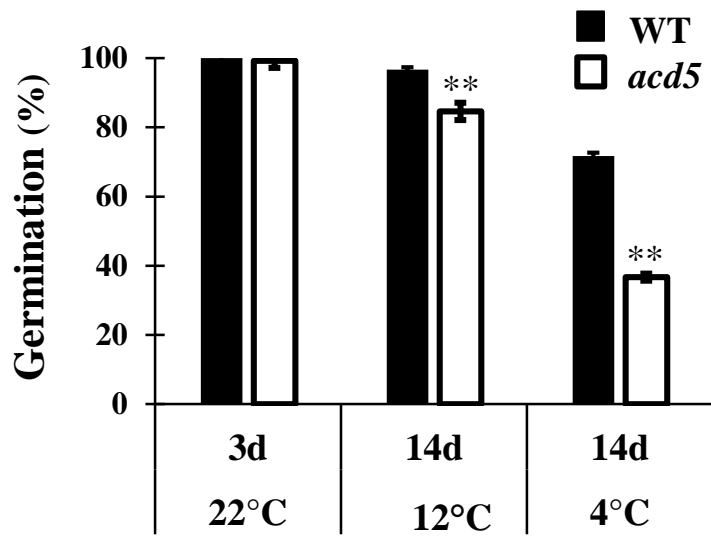
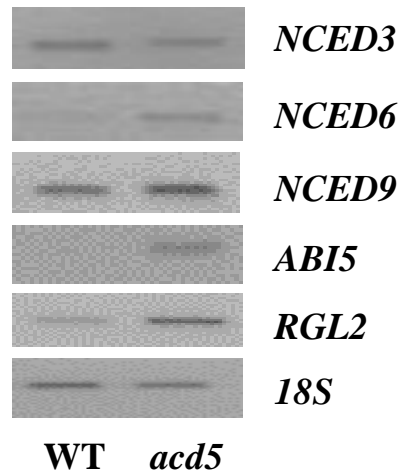


Figure 5

(a)



(b)



(c)

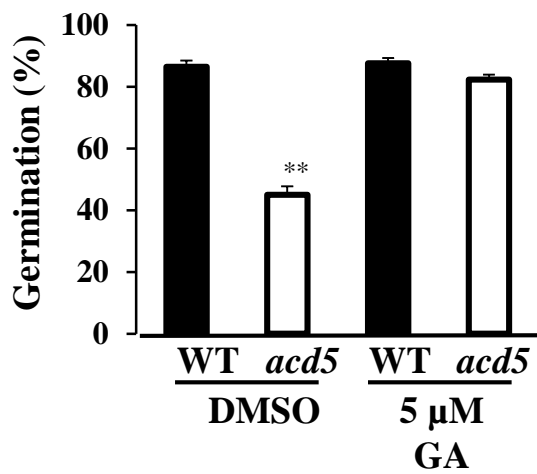


Figure 6

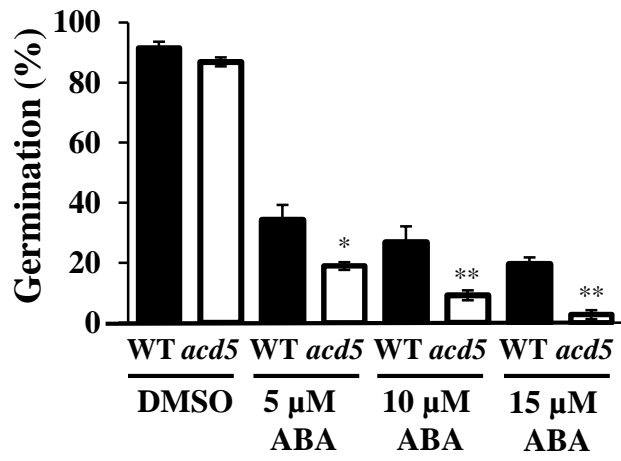
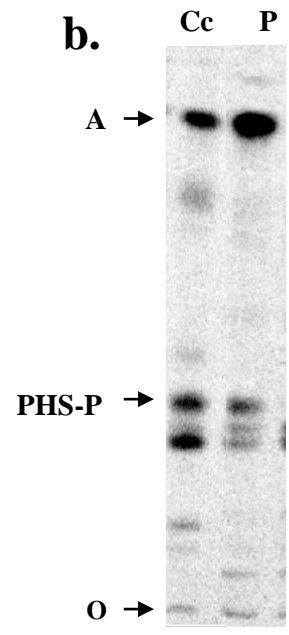
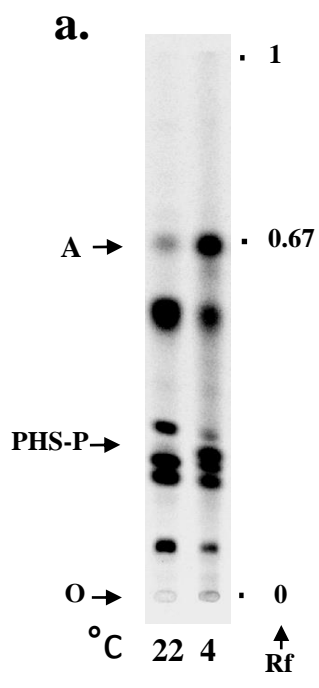
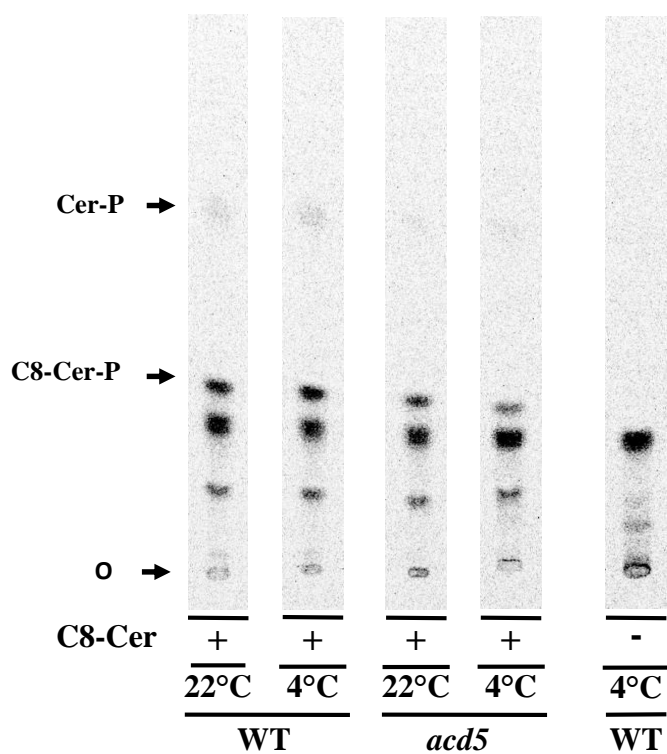


Figure 7





(a)	22°C		4°C	
	WT	<i>acd5</i>	WT	<i>acd5</i>
<i>ACD5</i>	0.91	0.93	0.90	1.18

(b)	22°C		4°C	
	WT	<i>acd5</i>	WT	<i>acd5</i>
<i>CBF1</i>	0.11	0.63	0.3	0.88
<i>CBF2</i>	0.69	1.21	0.35	1.43
<i>CBF3</i>	0.13	0.24	0.01	0.31
<i>ZAT10</i>	0.65	0.84	0.73	1.00
<i>ZAT12</i>	1.66	2.11	1.49	1.88
<i>NOI3</i>	0.13	0.85	0.18	0.34

(c)	4°C	
	WT	<i>acd5</i>
<i>NCED3</i>	1.00	1.18
<i>NCED6</i>	0.37	1.00
<i>NCED9</i>	1.88	2.36
<i>ABI5</i>	0.03	1.21
<i>RGL2</i>	0.56	2.00

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' CACTCGTTTCTCAGTTTACAAAC 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' TACTTGTAGCTCAACTTCTCCA 3'

Zat12 (At5g59820)
5' CCTTAGGAGGTCACCGTGC 3'
5' CAAGCCACTCTCTTCCCACT 3'

Supplemental Table 1