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1 Original Article

2

3 **Identification of salicylic acid-independent responses in an Arabidopsis**
4 **phosphatidylinositol 4-kinase beta double mutant**

5

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22

23 **Running title: Salicylic acid-independent responses in PI4K mutants**

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26 **Abstract**

27

28 **Background and Aims**

29 We have recently shown that an *Arabidopsis thaliana* double mutant of type III
30 phosphatidylinositol-4-kinases (PI4Ks), *pi4kβ1β2*, constitutively accumulated a high level of
31 salicylic acid (SA). By crossing this *pi4kβ1β2* double mutant with mutants impaired in SA
32 synthesis (such as *sid2* impaired in isochorismate synthase) or transduction, we demonstrated
33 that the high SA level was responsible for the dwarfism phenotype of the double mutant. Here
34 we aimed at distinguishing between the SA-dependent and -independent effects triggered by
35 the deficiency in *PI4Kβ1* and *PI4Kβ2*.

36 **Methods**

37 To achieve this, the *sid2pi4kβ1β2* triple mutant was a tool of choice. High-throughput
38 analyses of phytohormones were performed on this mutant together with *pi4kβ1β2* and *sid2*
39 mutants and wild-type plants. Responses to pathogens, namely *Hyaloperonospora*
40 *arabidopsidis*, *Pseudomonas syringae* and *Botrytis cinerea*, but as well to the non-host fungus
41 *Blumeria graminis*, were also determined. Callose accumulation was monitored in response to
42 flagellin.

43 **Key Results**

44 We show here the prominent role of high SA levels in influencing the concentration of many
45 other tested phytohormones, including abscisic acid and its derivatives, the Aspartate-
46 conjugated form of indole-3-acetic acid and some cytokinins such as *cis*-zeatin. We show that
47 the increased resistance of *pi4kβ1β2* plants to the host pathogens *Hyaloperonospora*
48 *arabidopsidis*, *Pseudomonas syringae* pv. *tomato* DC3000 and *Bothrytis cinerea* is dependent
49 on accumulation of high SA level. In contrast, accumulation of callose in *pi4kβ1β2* after

50 flagellin treatment was independent of SA. Concerning the response to *Blumeria graminis*,
51 both callose accumulation and fungal penetration were enhanced in the *pi4kβ1β2* double
52 mutant compared to wild-type plants. Both of these processes occurred in a SA-independent
53 manner.

54 **Conclusions**

55 Our data extensively illustrate the influence of SA on other phytohormone levels. The
56 *sid2pi4kβ1β2* triple mutant allowed to uncover the role of PI4Kβ1/β2 *per se*, thus showing the
57 importance of these enzymes in plant defence responses.

58

59 **Key words:** pi4kβ1β2 / PI4Ks, callose, salicylic acid, phytohormones, isochorismate synthase
60 1, biotic stress, pathogens, *Arabidopsis thaliana*

61

62

63 INTRODUCTION

64 Salicylic acid (SA) is a phytohormone playing a role in many plant physiological processes
65 although its role is mainly documented in plant response to biotic stress when SA accumulates
66 within tissues, both at the site of attack and in a systemic manner (Vlot et al. 2009; Janda
67 2015). In plants, SA is biosynthesized via two pathways. One is dependent on phenylalanine
68 ammonia-lyase (PAL; EC 4.3.1.24) which catalyses the conversion of phenylalanine to *trans*-
69 cinnamic acid. In the other pathway, the key enzyme is ISOCHORISMATE SYNTHASE
70 (ICS; EC 5.4.4.2) which catalyses the isomerization of chorismate into isochorismate
71 (Dempsey et al. 2011). The ICS-dependent pathway was shown to be responsible for most SA
72 accumulation upon pathogen attack. In *A. thaliana*, two ICS isoforms exist, but the major role
73 in SA biosynthesis is played by ICS1. The *ICS1* mutant is known as *sid2* for *salicylic acid*
74 *induction deficient 2* (Wildermuth et al. 2001; Wagner et al. 2013; Cui et al. 2017). When SA
75 levels increase, downstream signalling events are triggered, and the best described molecular
76 pathway is dependent on NONEXPRESSOR OF PATHOGENESIS RELATED 1 (NPR1).
77 Upon SA action, homo-oligomeric NPR1 protein undergoes dissociation by reduction and the
78 resulting monomers move into the nucleus where they interact with TGA-transcription factors
79 to induce the expression of SA responsive genes. A NPR1 independent pathway also exists in
80 response to SA (Janda 2015). The activation of SA signalling pathways leads to robust
81 changes in the plant transcriptome, including defence related genes (Seyfferth and Tsuda
82 2014). Among the immune responses affected by changes in SA levels or by SA treatments is
83 the accumulation of callose (Antignani et al. 2015; Dong et al. 2008; Kohler et al. 2002), a
84 (1,3)- β -Glucan occurring in plant cell walls.

85 The signaling pathways triggered by SA are still the subject of current research. We have
86 shown that phosphoinositides, the phosphorylated derivatives of phosphatidylinositol (PI), are
87 involved in SA transduction. Indeed, PI can be phosphorylated at the D4 position of the

88 inositol ring by phosphatidylinositol-4-kinases (PI4K) thus leading to phosphatidylinositol 4-
89 phosphate (PI4P); which can be phosphorylated further into phosphatidylinositol-4,5-
90 bisphosphate (PI-4,5-P₂). There are two types of PI4Ks according to their primary sequences
91 and pharmacological sensitivities. Type II PI4Ks are inhibited by adenosine while type III
92 PI4Ks are inhibited by micromolar concentrations of wortmannin, a steroid metabolite
93 produced by the fungus *Penicillium funiculosum* (Nakanishi et al. 1995, Balla 2007, Krinke et
94 al. 2007). From the *A. thaliana* genome, twelve putative PI4K isoforms have been identified.
95 Eight belong to type-II (AtPI4K γ 1-8), and four belong to type-III (AtPI4K α 1 and α 2,
96 AtPI4K β 1 and β 2) (Delage et al. 2012; Janda et al. 2013). We have shown that type III-PI4Ks
97 are activated when *A. thaliana* suspension cells respond to SA, thus leading to an increase in
98 PI4P and PI-4,5-P₂ (Krinke et al. 2007). PI-4,5-P₂ can act as a cofactor to some phospholipase
99 Ds (Pappan et al. 1998). Interestingly, there is an overlap between SA responsive genes
100 controlled by PI4Ks and those controlled by PLDs, leading to the working model that in
101 response to SA, PI4P and PI-4,5-P₂ are produced with PI-4,5-P₂ acting as a cofactor for a
102 PLD, whose product, PA, will trigger a signalling cascade (Krinke et al. 2009; Kalachova et
103 al. 2016).

104 In order to better characterize the role of PI4Ks in the response to SA, we have used *A.*
105 *thaliana* mutants altered in type III PI4Ks. We previously worked on a double mutant
106 defective in the two PI4K β genes. Surprisingly, *pi4k β 1 β 2* exhibited a constitutively high SA
107 level that resulted in constitutive high transcription of SA responsive genes such as *PR-1*
108 (PATHOGENESIS RELATED 1). Therefore, PI4Ks are not only involved in SA transduction
109 but they can also impact SA concentration. Furthermore, this double mutant exhibited
110 dwarfism and was more resistant to the bacterial pathogen *Pseudomonas syringae* pv.
111 *maculicola* ES4326 (Sasek et al. 2014). The *pi4k β 1 β 2* plant was crossed with mutants
112 impaired in components of SA synthesis (*sid2*, impaired in *ICS1* expression; *eds1*), SA

113 transduction (*npr1*) or a mutant expressing bacterial SA-hydroxylase (NahG) that degrades
114 SA to catechol. The resulting triple mutants allowed us to conclude that the dwarf phenotype
115 of *pi4kβ1β2* plants was dependent on SA accumulation and its transduction *via* the NPR1
116 pathway (Janda et al. 2014; Sasek et al. 2014).

117 In the present study, our aim was to identify amongst the cellular and physiological processes
118 affected by the deficiency of PI4Kβ1β2, those that were either SA-dependent or SA-
119 independent. To achieve this, we used the *sid2pi4kβ1β2* triple mutant that does not
120 accumulate SA and exhibits wild-type sized rosettes (Sasek et al. 2014). In this mutant, the
121 effects of PI4Kβ1 and PI4Kβ2 mutations would not be masked by high SA levels. We showed
122 that hormonal levels and pathogen resistance were mainly dependent on SA. However, we
123 could show that *sid2pi4kβ1β2* plants accumulated higher amounts of callose in response to
124 *flg22* and wounding. Interestingly, this SA-independent callose accumulation was also
125 observed during early stages of interactions with *Blumeria graminis* when penetration was
126 observed. Our data suggest that PI4Ks are involved in plant immune responses not only
127 through SA accumulation but also via SA-independent processes.

128

129 **MATERIALS AND METHODS**

130

131 **Plant material, growth conditions**

132 In this study, we used the following genotypes of *A. thaliana*: Columbia-0 (WT), *sid2-3*
133 (Gross et al. 2006), *npr1-1* (Cao et al. 1997) *NahG* (Delaney et al. 1994), *pi4kβ1β2*
134 (SALK_040479 / SALK_09069; Preuss et al. 2006), *sid2pi4kβ1β2*, *NahGpi4kβ1β2*, and
135 *npr1pi4kβ1β2* mutants previously described (Sasek et al. 2014).

136 All plants were cultivated in Jiffy 7 peat pellets at 22°C with 70% relative humidity. All
137 plants were watered without additional fertilizers. As for the light regimes, plants were

138 routinely cultivated in daily cycles of 10 h light (100-130 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 14 h dark. Plants
139 that would be used for hormonal analysis or transcription analysis were cultivated under 16 h
140 light (130-150 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 8 h dark.

141 Pathogen **inoculation**

142 Two-week-old plants grown at high density in one pot were sprayed with *H. arabidopsidis*
143 *NoCo2* spores (~100 spores/ μl). The infected plants were cultivated in closed transparent plastic
144 boxes in high humidity for 6 days under 16 h light/8 h dark (100-130 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 19°C. For
145 analysis, leaves collected from one pot were considered as one sample (for each genotype, 11
146 samples were analysed). Spores were counted under a microscope using a Bürker chamber and
147 expressed as relative spore number in [%], where relative spore number for a given control
148 genotype (WT or *sid2*) was set to 100%. The spores were counted as spores per mg of tissue fresh
149 weight. The experiments [WT vs *pi4k β 1 β 2*] and [*sid2* vs *sid2pi4k β 1 β 2*] were conducted
150 independently.

151 Inoculation with *Pseudomonas syringae* was performed according to (Katagiri et al. 2002)
152 with modifications. Bacteria were cultivated overnight on King's B medium plates containing
153 rifampicin (50 $\mu\text{g}/\mu\text{l}$). *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and
154 *Pseudomonas syringae* pv. *tomato* DC3000 AvrRpt2 (*Pst* DC3000 AvrRpt2) were taken from
155 the respective plate and resuspended in 10mM MgCl_2 to give an $\text{OD}_{600}=0.001$. Four-week-old
156 plants were infiltrated with this suspension.

157 One disc (6 mm) from one leaf, three leaves at a similar developmental stage from one plant
158 and three plants were collected as one sample of one genotype at 0 dpi and 3 dpi (3 dpi only
159 for *Pst* DC3000; 2 dpi for *Pst* DC3000 AvrRpt2). Leaf discs were ground in 10mM MgCl_2
160 and decimal dilutions were performed. Colony forming units were counted.

161 Four-week-old *A. thaliana* plants were treated with 6 μl drops containing *Botrytis cinerea*
162 BMM spores ($5 \cdot 10^4$ spores/ml) by applying a single drop to each leaf, with three leaves at a

163 similar developmental stage inoculated for each plant. Treated plants were placed into closed
164 plastic boxes and kept in low light (16h light/8h dark, 21°C; 10-20 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 56 hpi.
165 *Blumeria graminis* f. sp. *hordei* (*Bgh*) was cultivated continuously on fresh barley (Golden
166 promise) grown under short day conditions (19°C, 10/14 h, 50% humidity, at a light intensity
167 of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Plants, approximately 4 weeks old, were inoculated by spreading spores
168 from infected barley onto the adaxial side of their leaves (from leaf to leaf). The 5th - 6th
169 leaves were cut off at selected hpi and cleared with 96% ethanol or chloral hydrate. For
170 penetration rate, fungal structures were stained with 250 mg/ml trypan blue in a
171 lactophenol/ethanol solution (Vogel and Somerville 2000). Stained leaves were observed by
172 classical epifluorescence microscopy or bright-field microscopy using a Zeiss AxioImager
173 ApoTome2 (objective 100x).

174

175 **Callose deposition**

176 Four-week-old *A. thaliana* plants were treated for 24 h with 100 nM flg22 or infiltrated with
177 *Pst DC3000*. Distilled water infiltration was used as a control (mock) treatment. Infiltrated
178 leaves were decolorized in ethanol:glacial acetic acid (3:1 v/v). The leaves were then rehydrated
179 in successive baths of 70% ethanol (at least 1 h), 50% ethanol (at least 1 h), 30% ethanol (at
180 least 1 h) and water (at least 2h). Leaves were stained for 4 h with 0.01 % aniline blue in 150
181 mM K_2HPO_4 , pH 9.5. Callose deposition was observed by fluorescence microscopy using a
182 Zeiss AxioImager ApoTome2 (objective 10x). In the case of *Bgh* infection analysis, we
183 calculated only callose spots using the high circularity function of the measurement settings at
184 an interval of 0.5-1 which allowed us to distinguish only the cells with the size exclusion limit
185 for spots corresponding to either encased haustoria or enormous papilla. Callose deposition
186 was observed by fluorescence microscopy using a Zeiss AxioImager ApoTome2 (objective

187 10x). Images were processed with ImageJ software. At least four leaves from three
188 independent plants were analysed for each variant.

189

190 **RNA extraction and qPCR analysis**

191 Plant tissues were homogenised in 2-ml screw-cap tubes containing 1 g of 1.3-mm diameter
192 silica beads using a FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA, USA). Total
193 RNA was isolated using a Spectrum Plant Total RNA kit (Sigma-Aldrich, USA) and treated
194 with a DNA-free kit (Ambion, USA). Subsequently, 1 µg of RNA was converted to cDNA
195 with M-MLV RNase H–Point Mutant reverse transcriptase (Promega Corp., USA) and an
196 anchored oligo dT21 primer (Metabion, Germany). Gene transcription was quantified by
197 qPCR using a LightCycler 480 SYBR Green I Master kit and a LightCycler 480 (Roche,
198 Switzerland). The PCR conditions employed were 95°C for 10 min followed by 45 cycles of
199 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s. Melting curve analyses were then carried out.
200 Relative transcription was normalized to the housekeeping genes *SAND* or *TIP41*
201 (Czechowski et al. 2005). Primers were designed using PerlPrimer v1.1.21 (Marshall, 2004).
202 The primers used were CalS1_FP, AAGAGCGGAGGGTCACTTTG;
203 CalS1_RP, GGCGACACGAATAGACGGAT; CalS12_FP,
204 TTTACTCCGTTTTCCCGAGG; CalS12_RP, GGAGAGAGACGCATCTGAGC.

205

206 **Analysis of plant hormones**

207 Plant hormones were extracted from 100 mg of frozen tissues and their concentrations were
208 determined as previously described (Dobrev and Vankova 2012; Dobrev and Kaminek 2002)
209 after the addition of appropriate internal standards. Hormone analysis was carried out on four
210 samples, each taken from three plants. Briefly, samples were homogenized in tubes with 1.3
211 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). Samples were then

212 extracted with a methanol/H₂O/formic acid (15:4:1, v:v:v) mixture, which was supplemented
213 with stable isotope-labeled phytohormone internal standards (10 pmol per sample) in order to
214 check recovery during purification and to validate the quantification. The clarified
215 supernatants were subjected to solid phase extraction using Oasis MCX cartridges (Waters
216 Co., USA). The eluates were evaporated to dryness and the generated solids were dissolved in
217 30 µl of 15% (v/v) acetonitrile in water. Hormones were separated and quantified by Ultimate
218 3000 high-performance liquid chromatography (Dionex, Bannockburn, IL, USA) coupled to a
219 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied
220 Biosystems, Foster City, CA, USA) as described by (Dobrev et al. 2017). Metabolite levels
221 were expressed in pmol/g fresh weight (FW).

222

223 **Statistical analysis**

224 At least three independent biological replicates were performed for all experiments. Statistical
225 analysis was conducted by paired *t*-test or ANOVA with Tukey honestly significant
226 difference (HSD) multiple mean comparison *post hoc* test. The number of analysed samples
227 was specified for each condition. The correlation matrix for hormonal levels was prepared
228 using R-software *Hmisc* and *corrplot* packages based on the Pearson correlation (Team 2014).

229

230 **RESULTS**

231

232 ***The pi4kβ1β2 double mutant has altered phytohormonal levels***

233 Our goal was to identify SA-dependent and SA-independent processes triggered by the double
234 *pi4kβ1β2* mutation. To do so, we used a *sid2pi4kβ1β2* triple mutant. If a process triggered by
235 the double *pi4kβ1β2* mutation is SA-dependent, then it should disappear in the *sid2pi4kβ1β2*

236 triple mutant. On the other hand, if a process triggered by the double *pi4kβ1β2* mutation is
237 SA-independent, then it should still be observed in the *sid2pi4kβ1β2* triple mutant.

238 Since the main effect of the double *pi4kβ1β2* mutation was on the level of SA, we decided to
239 quantify a broad spectrum of phytohormones in the *sid2pi4kβ1β2* triple mutant. The hormone
240 levels obtained were compared to those of *pi4kβ1β2*, *sid2* and WT plants. A first look allowed
241 us to establish that the *pi4kβ1β2* double mutation does not impact only SA levels. Many
242 hormone-related metabolites showed significantly different levels in *pi4kβ1β2* plants when
243 compared to WT plants while most of them remained at WT levels in *sid2pi4kβ1β2*
244 (supplemental Table S1). In our previous study, we created an additional triple mutant by
245 crossing *pi4kβ1β2* with a *NahG* mutant impaired in SA accumulation: *NahGpi4kβ1β2* (Sasek
246 et al. 2014). To confirm and to strengthen the results obtained with *sid2pi4kβ1β2*, we also
247 quantified phytohormones in *NahGpi4kβ1β2*, together with their corresponding single
248 mutants (Supplemental table S1). From these data, we built a correlation matrix (Fig. 1).
249 Many hormone levels were correlated to higher SA levels (Pearson correlation higher than
250 0.7) as seen for the abscisic acid (ABA) derivatives such as 9-hydroxy-ABA (9OH-ABA),
251 phaseic acid (PA) and dihydrophaseic acid (DPA). However, the high levels of DPA, PA and
252 9OH-ABA observed in *pi4kβ1β2* versus WT were no longer seen in the triple mutants with
253 low SA; therefore, these metabolite levels were controlled by SA in the *pi4kβ1β2* double
254 mutant (Fig. S1A).

255 Since the main genetic pathway of SA response is controlled by NPR1, we investigated
256 whether the hormonal control of SA was NPR1-dependent. This was achieved using a
257 previously generated *npr1pi4kβ1β2* triple mutant where the SA level was 30-fold that of WT
258 and 4-fold that of *pi4kβ1β2*. Interestingly, levels of DPA, PA and 9-OH were still high in
259 *npr1pi4kβ1β2*, showing that the SA effect on these metabolites was only partially NPR1-
260 dependent. It should be noted that ABA levels did not correlate with SA (Pearson correlation

261 0.3), thus indicating that the action of SA on ABA-derivatives is probably not directly
262 connected with the biosynthesis of ABA, but with its metabolism.

263

264 Other hormones with high Pearson correlations to SA were the Asp conjugated form of
265 indole-3-acetic acid (Asp-IAA) and some cytokinins. The increased level of IAA-Asp
266 observed in *pi4kβ1β2* was no longer observed in the triple mutants with low SA. However, it
267 was still visible in the *npr1pi4kβ1β2* triple mutant thus suggesting that this metabolite is
268 controlled by SA but it is only partially NPR1-independent (Fig. S1B). The pattern of IAA
269 was different from that of Asp-IAA, suggesting that SA control of Asp-IAA is on aspartate
270 conjugation. As for cytokinins, the increase in *cis*-zeatin (*cZ*), *cis*-zeatin-riboside (*cZR*), *cis*-
271 zeatin-7-N-glucoside (*cZ7G*), *cis*-zeatin-riboside-*O*-glucoside (*cZROG*) and *trans*-zeatin-*O*-
272 glucoside (*tZOG*) and the decrease of *trans*-zeatin-7-N-glucoside (*tZ7G*) and *trans*-zeatin -9-
273 N-glucoside (*tZ9G*) in the *pi4kβ1β2* double mutant was SA-driven. In contrast to the other
274 hormones tested, SA action on *tZROG* appeared to be partially independent of NPR1 (Fig.
275 S1C).

276

277 We identified one hormone for which its level was altered in *pi4kβ1β2* plants independently
278 of SA. Indeed, the increase of oxIAA-GE observed in *pi4kβ1β2* was still visible in the triple
279 mutants with low SA. It should be noted that oxIAA did not follow the same pattern (Fig. S2).

280

281

282 **Pathogen resistance, including a necrotroph, of *pi4kβ1β2* plants is SA-dependent**

283

284 At the hormonal level our data show that the major change induced by the *pi4kβ1β2* double
285 mutation was an increase in SA, with this change determining the levels of many other

286 hormones. Since a major role of SA is related to biotic stress responses, we reasoned that
287 processes related to biotic stress, dependent or not on SA, might also be altered in the double
288 mutant.

289 Whether the PI4K double mutation *per se* was accompanied by an enhanced resistance to
290 pathogens was investigated. Comparing resistance in *sid2pi4kβ1β2* plants to that in *pi4kβ1β2*
291 or *sid2* would allow us to distinguish between the effectiveness of SA-dependent and
292 independent responses. Therefore, different pathogens with different lifestyles (biotrophs,
293 hemibiotrophs and necrotrophs) were tested (Glazebrook 2005). The *pi4kβ1β2* double mutant
294 plants were more resistant to the biotroph *H. arabidopsidis NoCo2* compared to WT.
295 However, *sid2pi4kβ1β2* resistance was similar to that of *sid2* plants (Fig. 2A). We then
296 studied resistance to the hemibiotroph *Pseudomonas syringae* pv. *tomato* DC3000 in its wild
297 type (*Pst* DC3000) or AvrRpt2 expressing form (*Pst* DC3000 AvrRpt2). *Pst* DC3000
298 AvrRpt2 leads to a strong effector triggered immunity (ETI) response compared to *Pst*
299 DC3000. With both forms, pathogen development was reduced in *pi4kβ1β2* plants compared
300 to the WT while *sid2pi4kβ1β2* resistance was comparable to that of *sid2* and lower than WT
301 plants (Fig. 2B, Fig. S3). Unexpectedly, the double mutant also showed an increased
302 resistance to the necrotroph *B. cinerea* which was also SA-dependent since *sid2pi4kβ1β2*
303 resistance was similar to that of *sid2* and WT plants (Fig. 2C). For each pathogenic assay,
304 triple mutant resistance was similar to *sid2*, indicating that SA-dependent pathways were
305 dominant in the immune response. Putative mechanisms regulated by PI4K activity alone
306 were not sufficient to establish pathogen resistance.

307

308 **The enhanced level of basal callose deposition in *pi4kβ1β2* is mainly SA-dependent while**
309 **stress-induced callose accumulation is not**

310 SA levels modulate numerous processes associated with immune responses including the
311 strengthening of leaf tissues and particularly cell walls around the infection site by
312 lignification and callose accumulation (Voigt 2014). Interestingly, SA pre-treatment also has a
313 positive effect on flagellin-induced callose accumulation (Yi et al. 2014). We therefore
314 studied callose levels, accumulated in leaf tissues in response to treatment with the flagellin
315 epitope (flg22). At first, we studied callose accumulation in the absence of flg22 treatment
316 (control; Fig. 3A). The *pi4kβ1β2* double mutant exhibited a constitutively high level of callose
317 deposition, as previously shown (Antignani et al. 2015). We were able to show that a spatial
318 pattern in callose accumulation existed, since the examination of different regions of interest
319 (ROI) indicated a higher accumulation in the upper part of the leaf edges (Fig. 3B).

320 Callose accumulation was then assessed in either mock-infiltrated or flg22-treated plants
321 (Fig.4). In this case, callose accumulation was much higher in *pi4kβ1β2* when compared to
322 WT plants (Fig. 4A, B). The *sid2pi4kβ1β2* triple mutant was used to investigate whether high
323 callose deposition in the double mutant depended on its high SA level. For mock treatments,
324 callose deposition was much lower in *sid2pi4kβ1β2* compared to *pi4kβ1β2* plants. This
325 provides arguments for a SA-dependent higher basal callose deposition. This was confirmed
326 by the response to flg22. In WT plants, flg22 induced 20-times more callose compared to
327 control mock infiltrations. Again, the callose level in *pi4kβ1β2* was higher (2-fold) compared
328 to WT plants. This increase was reduced when the *sid2* mutation was introduced into the
329 *pi4kβ1β2* double mutant as the level in the triple mutant was *circa* 2-fold lower than in the
330 double mutant and in the same range as the WT level. This indicated that SA was a major
331 inducer of callose accumulation in the *pi4kβ1β2* genotype context. Yet, the *sid2pi4kβ1β2*
332 triple mutant exhibited a higher callose deposition than *sid2* plants. Therefore, the *pi4kβ1β2*
333 double mutation *per se* had a role in the high callose accumulation observed in the *pi4kβ1β2*
334 double mutant.

335 We also studied callose accumulation in leaf tissues in response to mechanical wounding (Fig.
336 S4). Again, in response to wounding, the PI4K double mutation enhanced callose
337 accumulation via a SA-dependent pathway.

338 Whether callose overaccumulation correlated with the transcription of callose synthases
339 (*CalSs*) was then investigated. Among 12 callose synthases, *CalS1*, *CalS12* have been shown
340 to be related to SA and/or biotic stresses (Dong et al. 2008). The transcript levels of *CalS1*
341 and *CalS12* were tested by qPCR in WT, *pi4kβ1β2*, *sid2* and *sid2pi4kβ1β2* plants treated or
342 not with flg22. No correlation was observed between *CalS1* and *CalS12* transcript levels and
343 callose accumulation (Fig. S5).

344

345 ***pi4kβ1β2* has altered non-host resistance that is SA-independent**

346 The establishment of non-host resistance is based on different mechanisms, involving
347 vesicular secretion as well as callose accumulation (Collins et al. 2003, Assaad et al. 2004,
348 Takemoto et al. 2006, Bohlenius et al. 2010, Lee et al., 2017). To study the role of PI4K in
349 such responses, we tested penetration success and callose production in response to the non-
350 host pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*). In *Bgh/A. thaliana* interactions, callose
351 accumulates in defensive papillae and haustorial encasements or around dead cells (Assaad et
352 al. 2004, Ellinger et al. 2013, Jacobs et al., 2003). The enhanced number of plant cells with
353 developed haustoria or dead cells reflects the penetration success of fungal hyphae (Fig. 5A).
354 In our experiments a higher penetration correlated with greater callose accumulation in the
355 plant tissue. In particular the *pi4kβ1β2* double mutant showed an enhanced successful
356 penetration of *Bgh* 24 h post inoculation (hpi), as seen by the enhanced number of haustoria
357 and dead cells. A similar defect in penetration resistance was seen in *sid2/pi4kβ1β2*, thus
358 revealing the SA-independent character of this phenomenon (Fig. 5B). Both, *pi4kβ1β2* and
359 *sid2/pi4kβ1β2* accumulated more callose (Fig. 5C) and over larger areas compared to WT

360 plants (Fig. 5C, D). Thus, the lower penetration resistance accompanied by callose
361 accumulation in *pi4kβ1β2* was independent of the SA pathway.

362

363 **DISCUSSION**

364 The aim of this study was to investigate SA-dependent and -independent processes caused by
365 the *pi4kβ1β2* double mutation. As previously shown (Sasek et al. 2014), this mutant
366 accumulates a constitutively high level of SA. In *pi4kβ1β2*, SA biosynthesis is dependent on
367 ISOCHORISMATE SYNTHASE 1 (ICS1), demonstrated by an absence of SA accumulation
368 in the *sid2pi4kβ1β2* triple mutant with impaired *ICS1* transcription (Sasek et al. 2014). This
369 was confirmed with the hormone analysis described in the present study. The *sid2pi4kβ1β2*
370 triple mutant lacking high SA was used as a tool to distinguish between SA-dependent and
371 SA-independent effects caused by the double mutation in *pi4kβ1β2*.

372 We first measured phytohormonal levels in fully developed leaves of four-week-old *A.*
373 *thaliana* WT, *pi4kβ1β2*, *sid2* and *sid2pi4kβ1β2* plants. According to our knowledge, this is
374 the broadest phytohormonal study carried out with a SA over-accumulating mutant (in our
375 case *pi4kβ1β2*) and its comparison with a plant having the same background but with an
376 impaired SA pathway (*sid2pi4kβ1β2*). The level of 15 hormone derivatives (excluding SA)
377 was altered in *pi4kβ1β2* compared to WT leaves. For 13 of these, this was SA-dependent. We
378 could identify two metabolites, *cis*-zeatin-riboside-5'-monophosphate and glucosylesters of
379 oxindole-3-acetic acid, for which the *pi4kβ1β2* double mutation effect was SA-independent.
380 The same conclusions were also reached with another triple mutant, *NahGpi4kβ1β2*. Amongst
381 the hormones controlled by SA were ABA derivatives such as DPA, PA and 9OH-ABA. As
382 ABA levels did not correlate with SA, the action of SA on ABA-derivatives did not appear to
383 act on ABA biosynthesis but on its metabolism. Such an effect of SA on ABA catabolism is
384 poorly described. A slight induction of ABA 8'-hydroxylase expression was observed after a

385 24 h SA treatment of rice seedlings (Mega et al. 2015). In an *A. thaliana cpr22* mutant
386 (*constitutive expressor of PR genes 22*), the increase of SA and ABA levels due to a high-low
387 humidity shift was also followed by the SA-dependent expression of genes encoding ABA
388 metabolising enzymes (Mosher et al. 2010).

389 Other hormones displaying a high correlation to SA were IAA-Asp and some cytokinins (SA
390 positively controlled cZROG and tZOG and negatively controlled tZ7G and tZ9G). The
391 pattern of IAA was different from that of IAA-ASP, suggesting that SA control of IAA-Asp
392 was on aspartate conjugation. Aspartate conjugation is catalysed by GH3.2-GH3.6 (Normanly
393 2010), with our transcriptome data obtained with *in vitro* grown *pi4kβ1β2* seedlings (Sasek et
394 al. 2014) indicating that *GH3.3* (At2g23170) was overexpressed in the double mutant. It
395 would be interesting to investigate whether it was responsible for the IAA-Asp/SA correlation
396 in our double mutant.

397 Our results demonstrate the major role of hormonal cross-talk between SA and other
398 hormones but only a minor role of impairment of PI4Kβ1/β2 *per se*. Since a major role of SA
399 is related to responses to biotic stresses, we reasoned that other processes related to biotic
400 stress, whether SA-dependent or not, could also be altered in the double mutant. We tested the
401 resistance of WT, *pi4kβ1β2*, *sid2* and *sid2pi4kβ1β2* plants against representative biotrophic
402 (oomycete *H. arabidopsidis* NoCo2), hemibiotrophic (bacteria *Pst* DC3000) and necrotrophic
403 (fungus *B. cinerea*) pathogens. Results clearly showed that resistance to these pathogens was
404 dependent on a high SA content. Resistance to *H. arabidopsidis* NoCo2 and *Pst* DC3000 was
405 perhaps not surprising as resistance to such pathogens is generally associated with SA
406 signalling (Glazebrook 2005). On the other hand, the role of SA in regulating resistance to
407 necrotrophs is rather uncommon. Indeed, plant defence against necrotrophs is commonly
408 associated with jasmonic acid signalling (Glazebrook 2005; Ferrari et al. 2003). However,
409 Ferrari et al. (2003) showed that resistance to *B. cinerea* could be dependent on high SA

410 levels, in accordance with our observations. A similar finding was reported for defence
411 response to the necrotroph *Sclerotinia sclerotiorum* (Novakova et al. 2014). Moreover, we
412 tested *A. thaliana* ETI by using a bacterial strain highly expressing the AvrRpt2 effector (*Pst*
413 DC3000 AvrRpt2). An ETI response can induce the expression of genes commonly associated
414 with SA, such as *PR-1*, in a SA-independent manner in *A. thaliana* (Tsuda et al. 2013). Yet,
415 we found that the higher resistance of *pi4kβ1β2* plants against *Pst* DC3000 AvrRpt2 was SA-
416 dependent. In conclusion, the higher resistance of *pi4kβ1β2* mutant against all the host
417 pathogens assayed was strongly SA-dependent.

418 Non-host resistance, efficient against non-adapted pathogens, does not rely fully on the SA
419 pathway. Here we show that *pi4kβ1β2* exhibited a SA-independent defective resistance
420 towards penetration of the non-host pathogen *Bgh*.

421 Callose is a linear polysaccharide (1,3-β-glucan) occurring in plant cells where it is important
422 for many plant physiological processes such as cytokinesis (Chen and Kim 2009). Callose
423 accumulation is triggered in response to pathogens and is used as a common test of PTI upon
424 treatment with typical PAMPs such as flg22, the epitope of flagellin (Luna et al. 2011). In
425 mock inoculated *pi4kβ1β2*, callose deposition was greater than in WT leaves, thus confirming
426 findings of Antignani et al. (2015). Interestingly, this was also true for the *sid2pi4kβ1β2* triple
427 mutant when compared to *sid2* plants, thus indicating a SA-independent phenomenon.
428 Following inoculation with flg22, an increase in callose deposition was observed in all
429 genotypes tested, but this was still higher in *pi4kβ1β2* compared to WT leaves, and callose
430 deposition was higher in *sid2pi4kβ1β2* with respect to *sid2* plants. Therefore, it seems that the
431 *pi4kβ1β2* double mutation *per se* enabled higher callose deposition under biotic stress
432 conditions.

433 The biosynthesis of callose occurs outside of the cell (Ellinger and Voigt 2014).
434 Accumulation can be regulated at different levels: transcriptional, translational, or during

435 enzyme transport to the plasma membrane and out of the cell via vesicular trafficking.
436 Phosphorylation and direct translocation of callose synthase is crucial in the regulation of
437 biosynthesis, whereas transcriptional control might have only a minor role (Ellinger and Voigt
438 2014). Our data on the transcription levels of *CALS1* and *CALS12* indicate that in *pi4kβ1β2* a
439 transcriptional effect is not involved in the observed over accumulation of callose. So how is
440 it possible to explain the action on callose of the *pi4kβ1β2* double mutation *per se*? A number
441 of reports indicate that PI4Ks can impact trafficking. In *A. thaliana*, PI4Kβ1 was shown to be
442 recruited by the GTP bound Rab4b GTPase. Both RabA4b and PI4Kβ1 localize to budding
443 secretory vesicles in the trans-golgi network (TGN) and to secretory vesicles *en route* to the
444 cell surface. A *pi4kβ1β2* double mutant produces secretory vesicles of highly variable sizes
445 (Antignani et al. 2015; Preuss et al. 2006; Kang et al. 2011). The product of PI4Ks activity,
446 PI4P massively accumulates at the plasma membrane and at early endosomes / TGN and
447 Golgi (Platre and Jaillais 2016; Noack and Jaillais 2017). Therefore PI4Kβ1/β2 are important
448 in vesicle trafficking. Interestingly, inhibiting PI4K with phenylarsine oxide (PAO)
449 suppressed the salt-induced endocytosis of plasma membrane intrinsic protein 2;1 (Ueda et
450 al., 2016). Similarly, inhibiting PI4K led to the internalization of CELLULOSE
451 SYNTHASE3 from the plasma membrane (Fujimoto et al. 2015). Can the impact of PI4K
452 betas on trafficking explain the increased callose accumulation? Callose biosynthesis and
453 accumulation have been shown to be affected by vesicle trafficking (Ellinger and Voigt
454 2014). PI4Ks has been shown to play an important role in cytokinesis (Lin et al. 2019),
455 especially in the correct organization of the vesicles at the cell division plane and further
456 formation of a cell plate. During phragmoplast formation, PI4Kβ1 likely interacts with
457 MPK4, a member of the MAP65 protein family that regulates microtubule organization (Lin
458 et al., 2019). Callose is also essential for cytokinesis (Thiele et al. 2009). We can therefore
459 only speculate whether the effects of PI4Ks on callose, cytokinesis and trafficking are

460 interconnected. Interestingly, the role of SA in these processes has not been tested. Since
461 PI4K β 1/ β 2 can impact the secretory pathway, they could also impact the translocation of
462 callose synthases. Furthermore, PMR4 (CALS12) binds to small RabA4c GTPase at TGN and
463 PI4K β 1 binds to RabA4b GTPase, the most similar small GTPase to RabA4c, at TGN
464 (Böhlenius et al., 2010). It should also be noted that the impact of PI4K betas on trafficking
465 could also explain our non-host resistance data. The *syp121* mutant altered in a SNARE
466 protein involved in trafficking has been reported to accumulate SA and also display defective
467 non-host resistance (Collins et al., 2003).

468

469 In conclusion (Fig. 6), the *pi4k β 1 β 2* double mutant constitutively accumulated a high SA
470 level via ICS1/SID2 and this had a big impact on other hormone levels and it was associated
471 with an increased resistance to several plant pathogens (*P. syringae*, *H. arabidopsidis*, *B.*
472 *cinerea*). The *pi4k β 1 β 2* double mutation also affected pathogen-related processes in a high
473 SA-independent manner as seen by differences in callose accumulation in response to *flg22*,
474 to *Bgh* infection, to wounding, and the higher penetration success of *Bgh*. The identification
475 of such processes directly affected by the mutation on PI4Ks will now allow us to better
476 investigate the role of these enzymes, in relation to signalling or trafficking events.

477

478

479

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489

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493

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644

645 **FIGURE CAPTIONS**

646 **Figure 1. Correlation matrix between phytohormones levels.** The matrix was built using
647 the Pearson correlation of 27 hormone-related metabolites from 24 independent samples
648 corresponding to 6 genotypes (WT; *sid2*; *NahG*; *pi4kβ1β2*; *sid2pi4kβ1β2*; *NahGpi4kβ1β2*;
649 four plants per genotype). Positive correlations are displayed in blue and negative correlations
650 in red. Colour intensity and the size of the circles are proportional to the correlation
651 coefficients. Red rectangles highlight the correlation between SA and other hormones.

652

653 **Figure 2.** Resistance to biotic stresses of *pi4kβ1β2* and *sid2pi4kβ1β2* mutants. A. Resistance
654 to biotroph *H. arabidopsidis*. B. Resistance to hemibiotroph *Pseudomonas syringae* pv.
655 *syringae* DC3000. Infiltration treatment of 4 week-old-plants, with 6 independent samples. C.

656 Resistance to the necrotroph *Botrytis cinerea*. 4-5-week-old *A. thaliana* were inoculated with
657 a 6 μ L drop containing spores of *B. cinerea* (50000 spores per mL), placed into a plastic box
658 and kept in the dark for 56 h. Statistical differences between the genotypes for B and C were
659 assessed using ANOVA, with a Tukey honestly significant difference (HSD) multiple mean
660 comparison *post hoc* test. Different letters indicate a significant difference, Tukey HSD,
661 $P < 0.05$, $n=10$ for A, $n=7$ for B and $n=27$ for C.

662

663 **Figure 3. Pattern of callose accumulation in *pi4k β 1 β 2* leaves.** A. Aniline blue staining, and
664 fluorescent microscopy. The bar corresponds to 500 μ m. B. Callose particles accumulated in
665 different ROI. The squares represent the ROI. Data are presented as means \pm SEM. Statistical
666 differences were assessed using a two-way ANOVA, with a Tukey honestly significant
667 difference (HSD) multiple mean comparison *post hoc* test. Different letters indicate a
668 significant difference, Tukey HSD, $P < 0.05$. $n=11$.

669

670 **Figure 4.** Callose deposition in response to flagellin. A. Representative images of callose
671 accumulated in leaves of 4-week-old *A. thaliana* plants by aniline blue staining, 24 h after
672 infiltration with 0.1 μ M flg22 or mock infiltration. The bar = 1cm. B. Quantification of
673 callose particles. Values represent an average of 5 ROI. Data are presented as means +SD.
674 For each treatment, statistical differences between the genotypes were assessed using a one-
675 way ANOVA, with a Tukey honestly significant difference (HSD) multiple mean comparison
676 *post hoc* test. Different letters indicate a significant difference, Tukey HSD, $P < 0.01$, $n=4$.

677

678

679 **Figure 5.** Resistance of *pi4k β 1 β 2* to penetration by the non-host pathogen *Blumeria graminis*
680 f. sp. *Hordei* (*Bgh*). A. Four types of interactions counted in the penetration success analysis

681 after trypan blue staining. Scale bar represents 5 μm . B. Data showing penetration success of
682 *Bgh* 24 hpi in each genotype, the mean number of cells with either haustoria or dead cells,
683 respectively. C. Data show mean area of a callose spot per mm^2 at 24hpi after interaction with
684 *Bgh* spores. D. Data show mean area of a callose spot per mm^2 at 24hpi after interaction with
685 *Bgh* spores. Data at B, C and D were processed by ANOVA, with a Tukey honestly
686 significant difference (HSD) multiple mean comparison *post hoc* test, the data represent 1
687 independent experiment, the experiment was repeated four times. The letters indicate
688 significant difference Tukey HSD, $P < 0.01$. E. Pictures demonstrating callose staining with
689 aniline blue 24 hpi with *Bgh*. Scale bar 100 μm .

690

691 **Figure 6.** Schematic representation of the effects of the *pi4k β 1 β 2* double mutation on
692 Arabidopsis plants.

693

694

695 SUPPLEMENTARY DATA

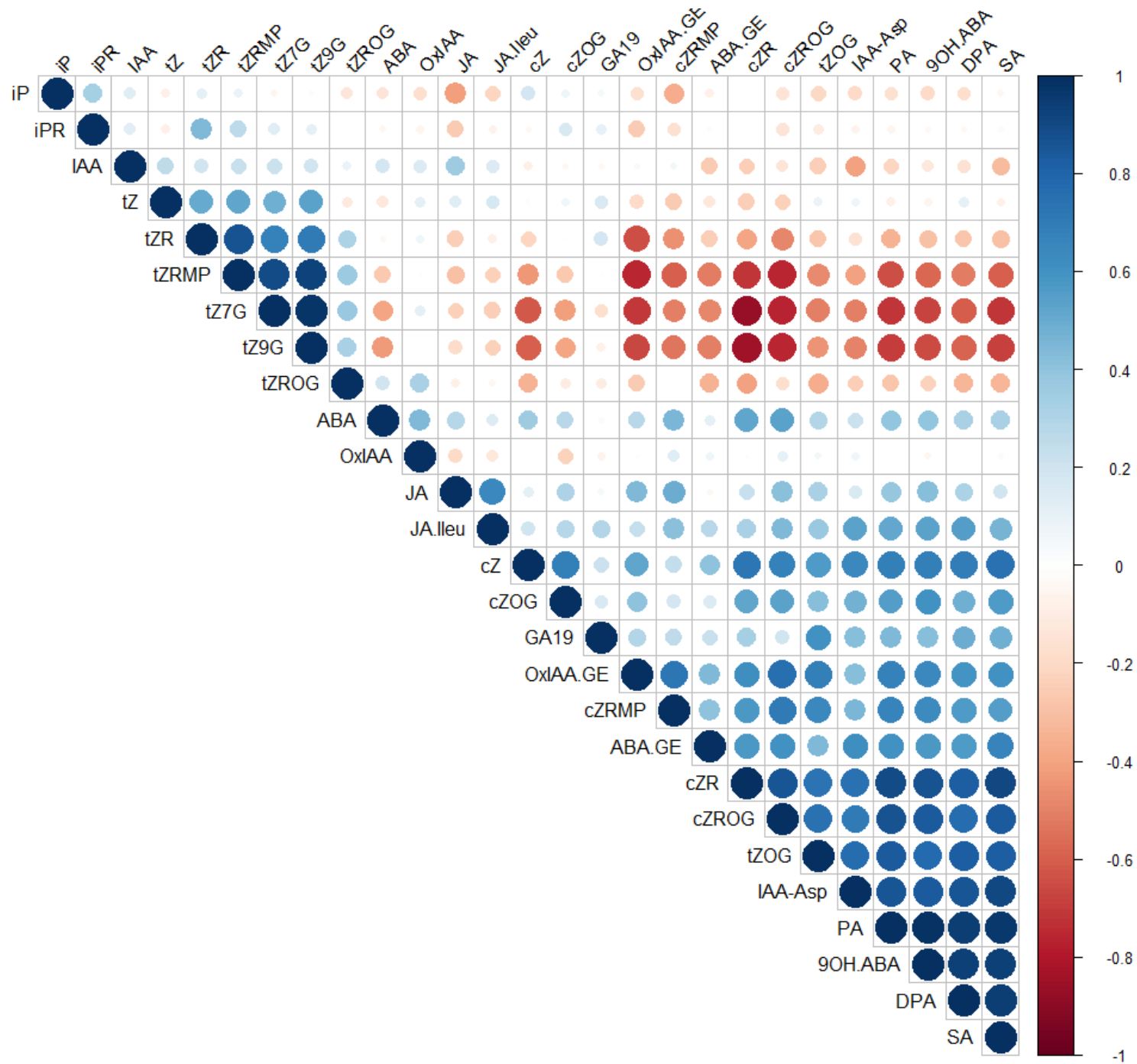
696 Supplemental Table S1. Phytohormone levels in 4-week-old plants. Values are expressed in
697 pmol/gFW. For each genotype, 4 plants were assessed.

698 Fig. S1: Levels of hormones controlled by SA in four-week-old mutant and WT plants.
699 Values represent means and SEM from four samples. For each hormone, statistical
700 differences between the genotypes were assessed using a one-way ANOVA, with a Tukey
701 honestly significant difference (HSD) multiple mean comparison *post hoc* test. Different
702 letters indicate a significant difference, Tukey HSD, $P < 0.05$. $n=4$. Note the broken y-axis
703 due to high SA in the *npr1pi4k β 1 β 2* triple mutant.

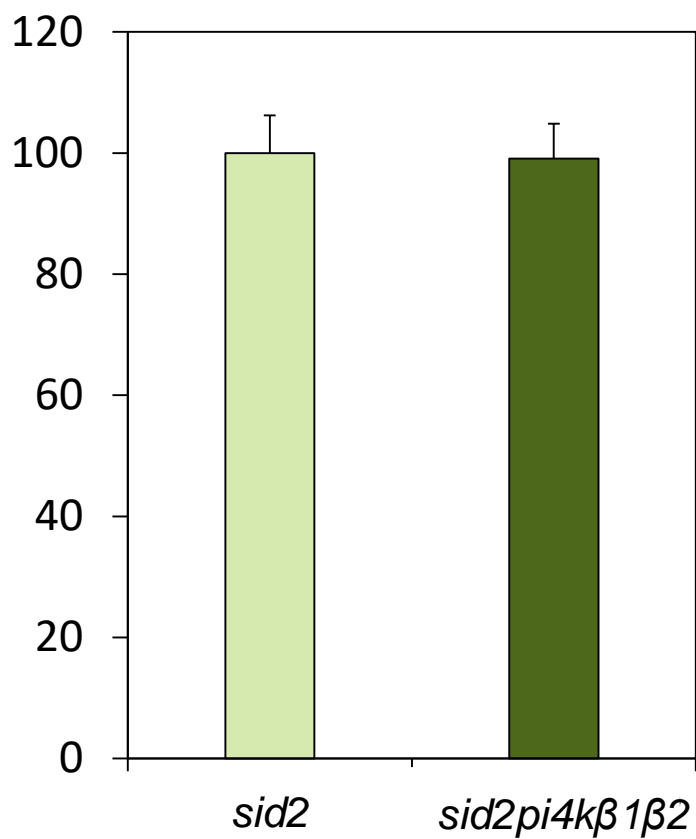
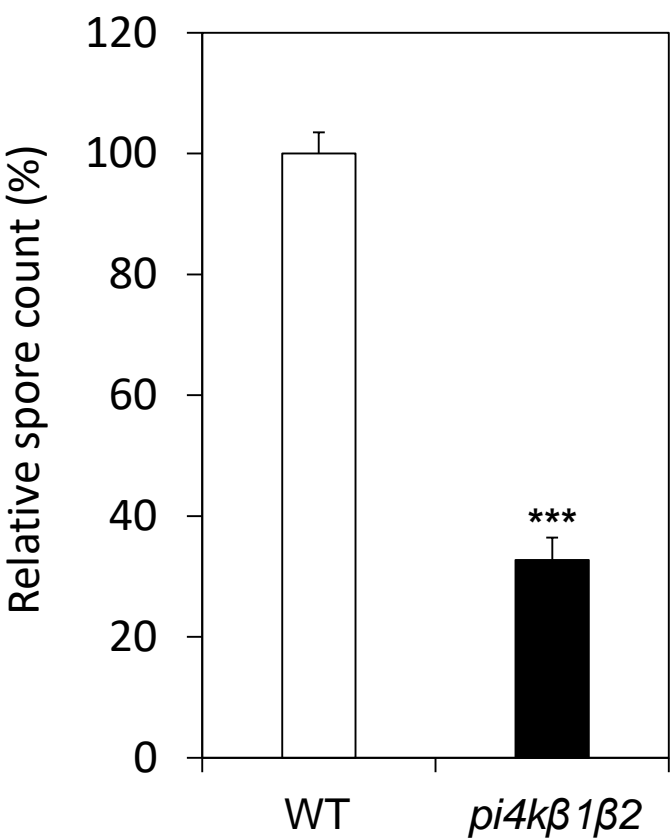
704 Fig. S2. Levels of hormones not controlled by SA in four-week-old mutant and WT plants.

705 For each hormone, statistical differences between the genotypes were assessed using a one-

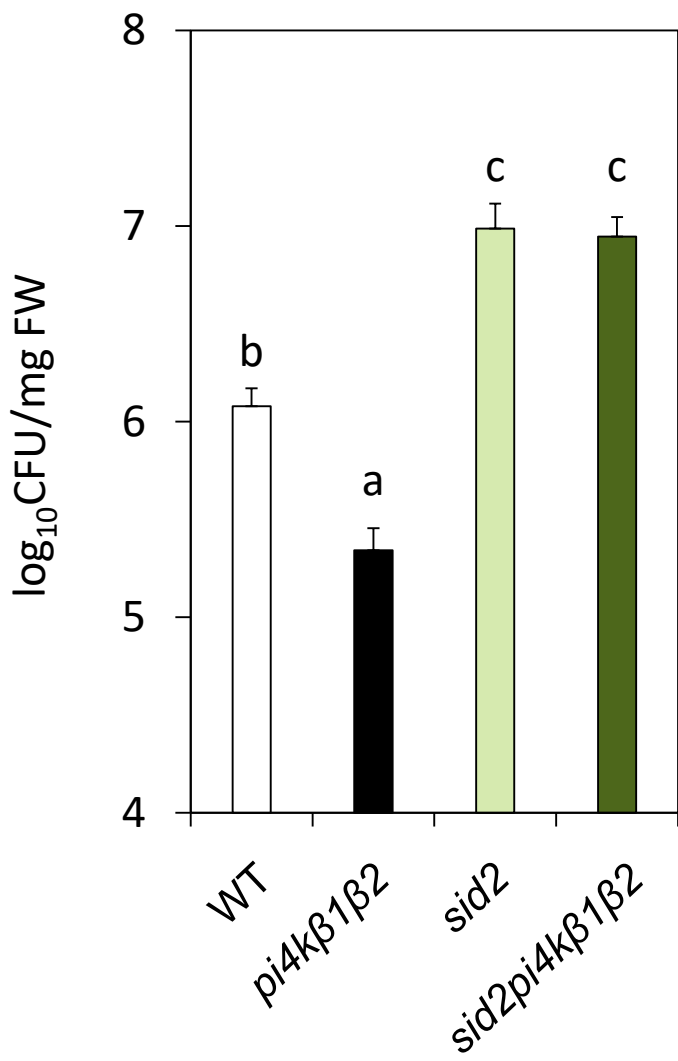
706 way ANOVA, with a Tukey honestly significant difference (HSD) multiple mean comparison
707 *post hoc* test. Different letters indicate a significant difference, Tukey HSD, $P < 0.05$. $n=4$.
708 Fig. S3. Resistance to *P. syringae* pv. *tomato* DC3000 AvrRpt2. 4-week-old *A. thaliana*
709 plants were infiltrated with a bacterial suspension. Data show infection development at 2 dpi.
710 Fig. S4. Callose deposition in response to wounding. A. Representative images of callose
711 accumulated in leaves of 4-week-old *A. thaliana* plants 24 h after infiltration with a needleless
712 syringe, leaf area outside of the place of infiltration (“mock”) or at the very place of treatment
713 (“wounding”). Aniline blue staining, bar=1cm. B. Quantification of callose particles. Data are
714 given as means +SD. For each treatment, statistical differences between the genotypes were
715 assessed using a one-way ANOVA, with a Tukey honestly significant difference (HSD)
716 multiple mean comparison *post hoc* test. Different letters indicate a significant difference,
717 Tukey HSD, $P < 0.01$, $n=4$.
718 Fig. S5. Relative transcription of some *CalS* genes in untreated rosette leaves or 24h after
719 infiltration with 0.1 μ M flg22.



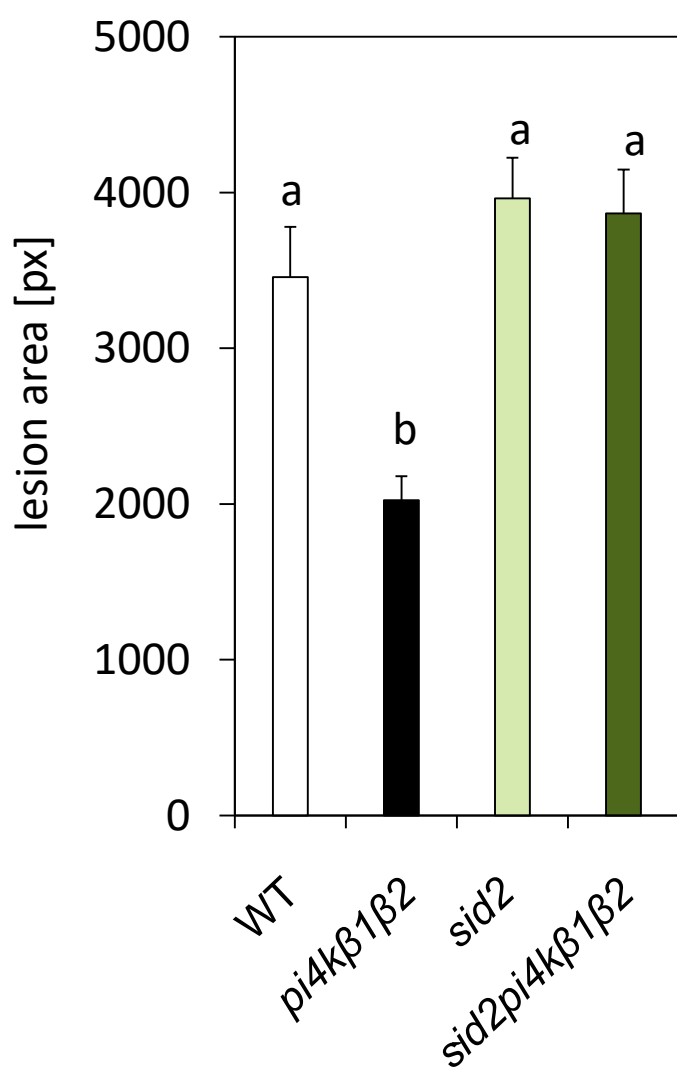
A. *H. arabidopsis*

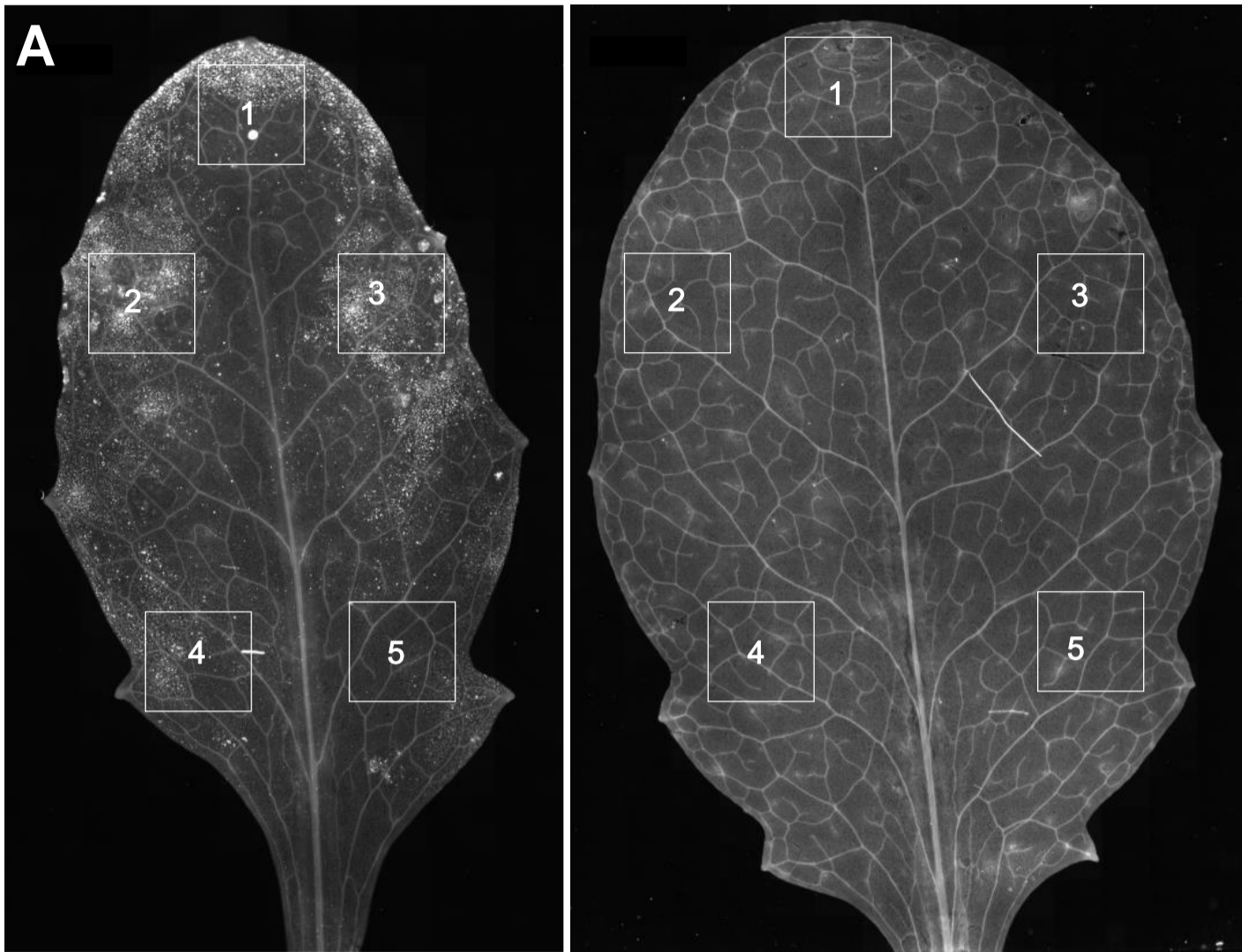


B. *Pst DC3000*



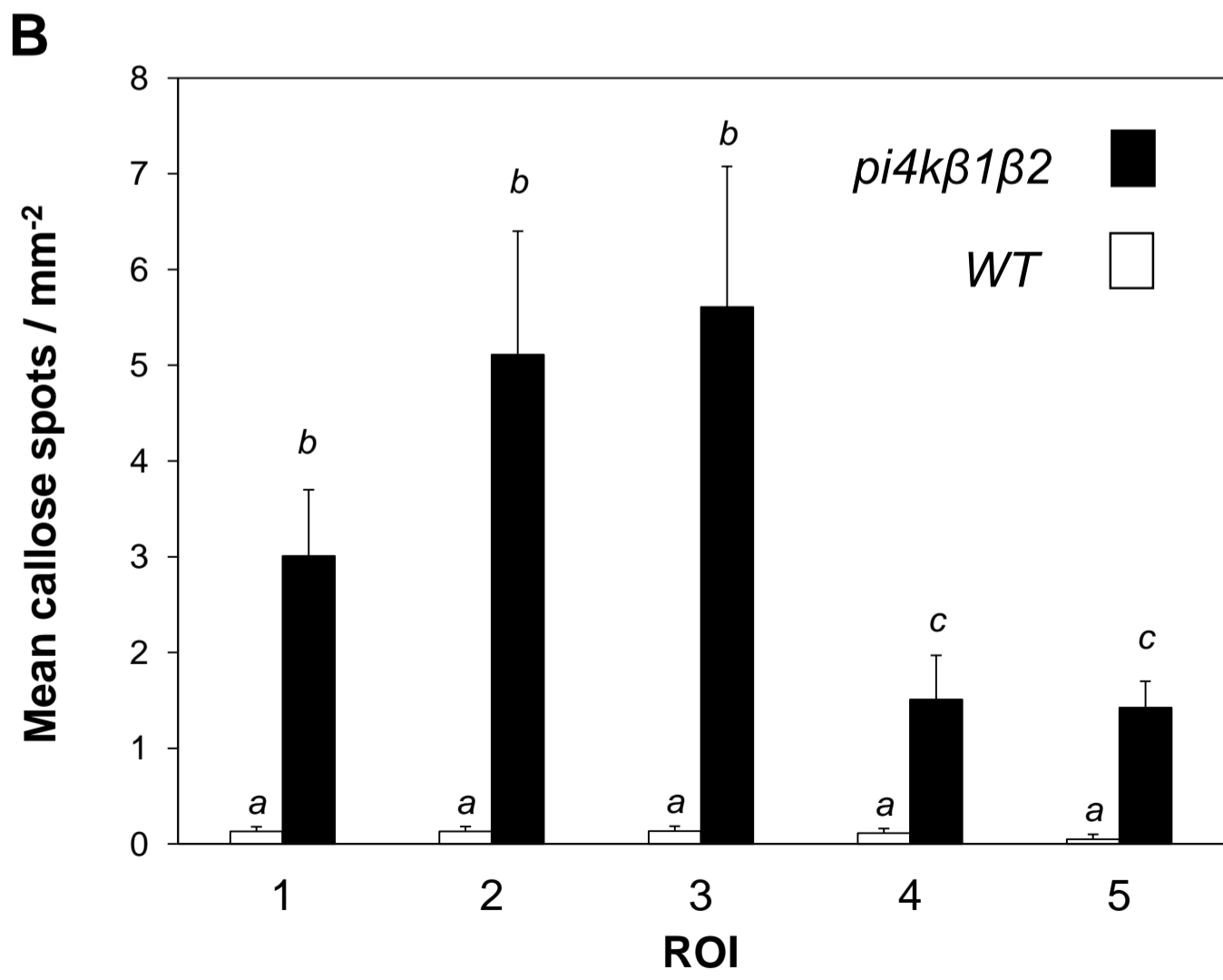
C. *B. cinerea*

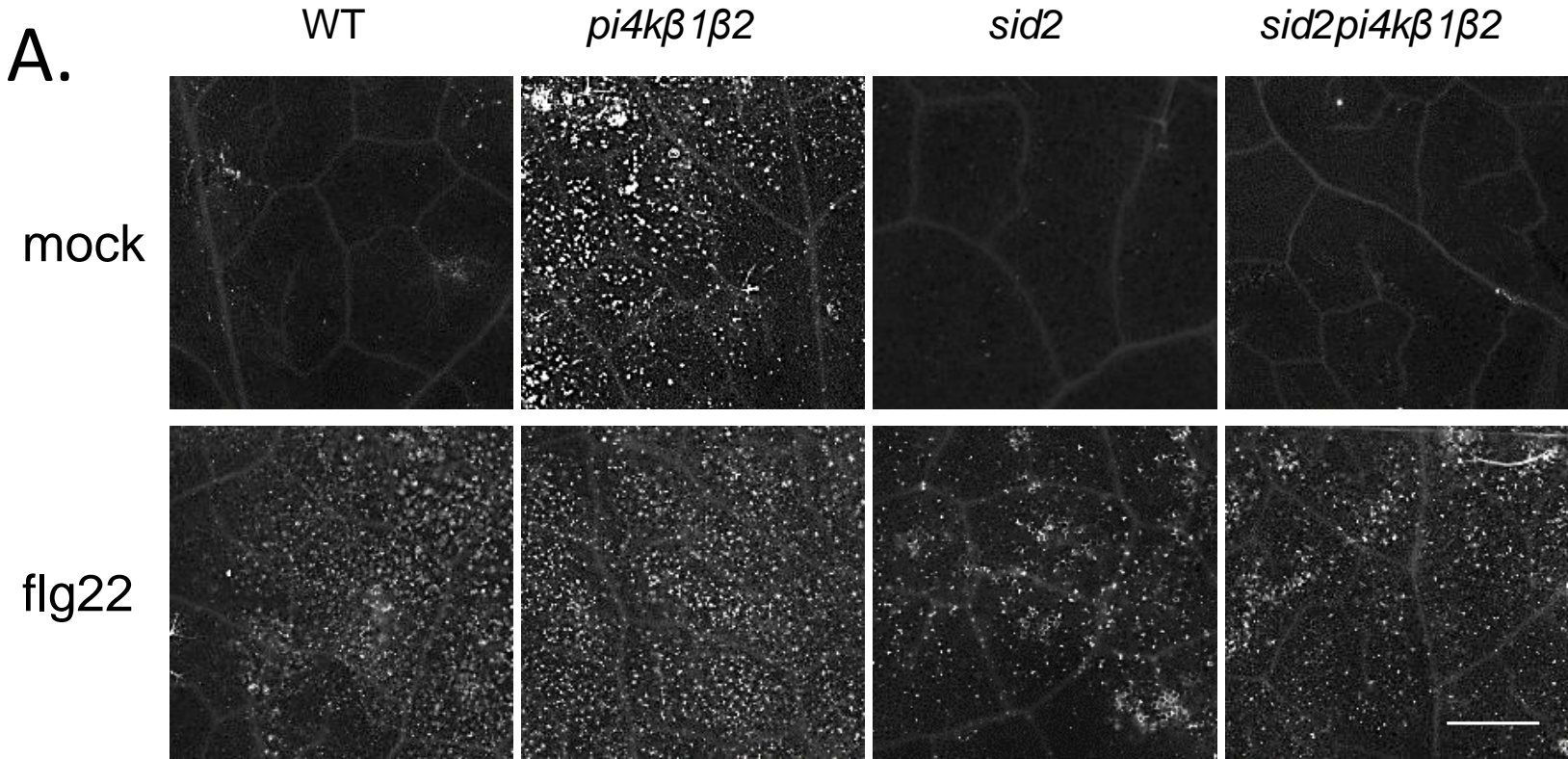




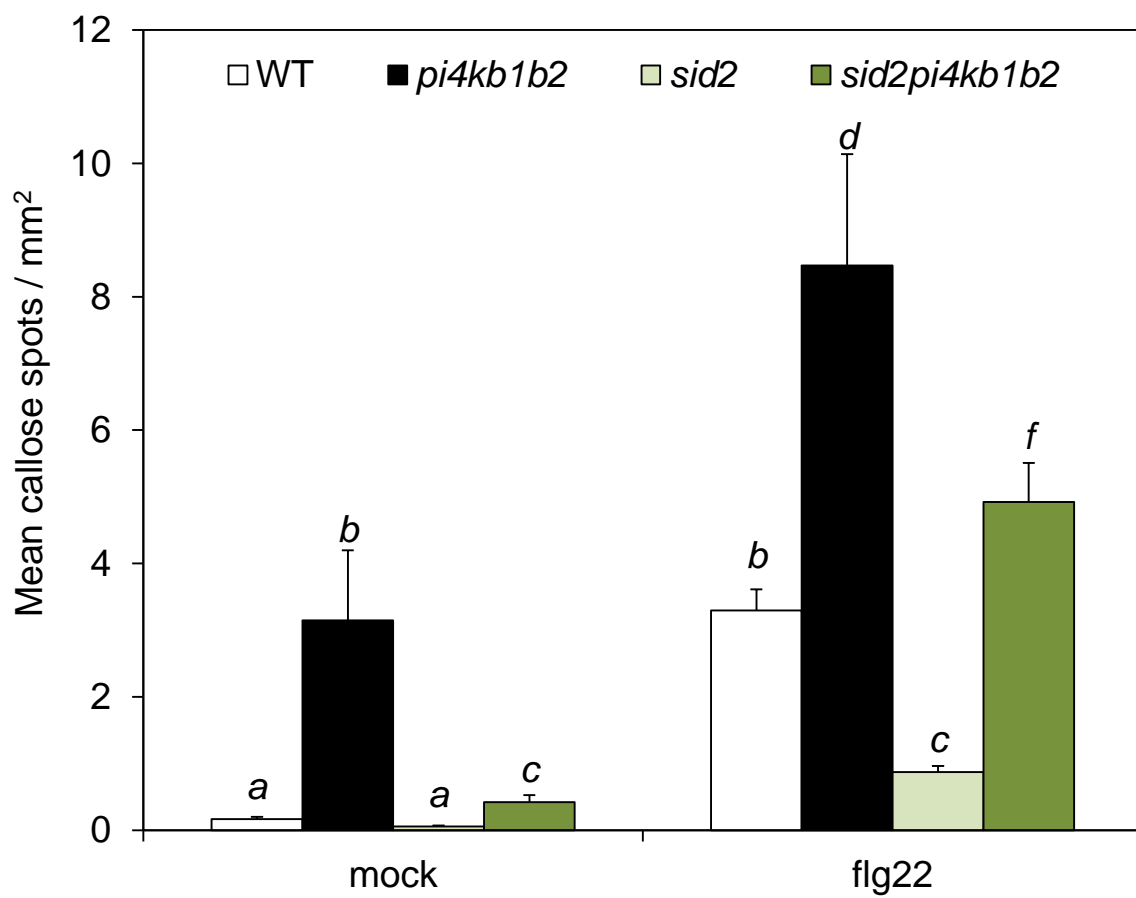
pi4kβ1β2

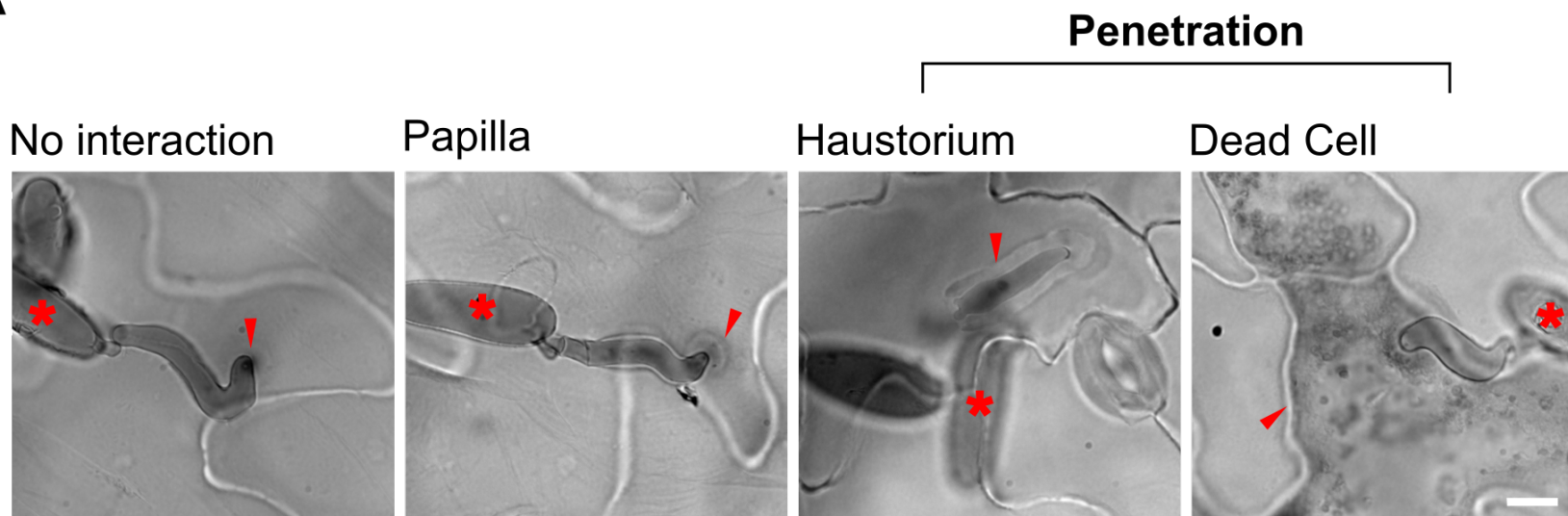
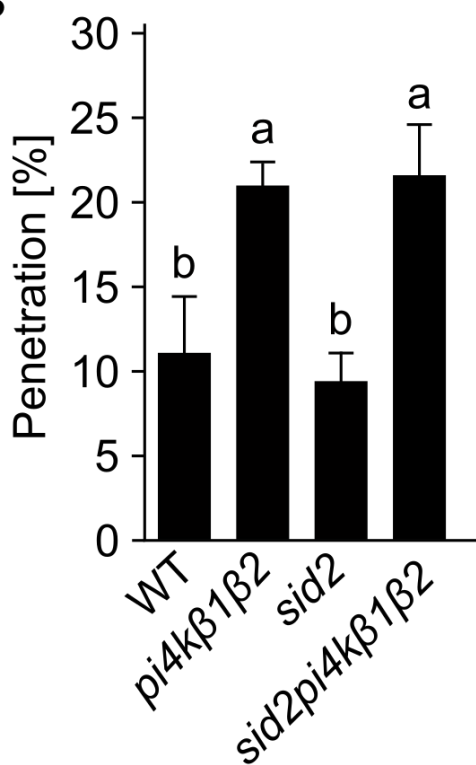
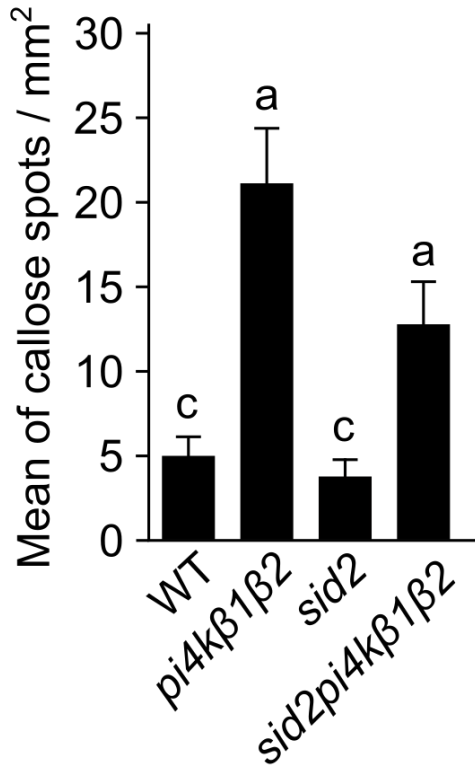
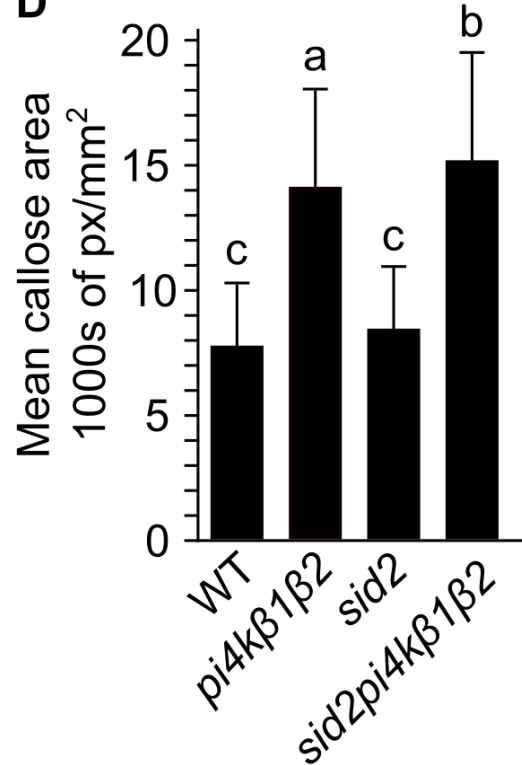
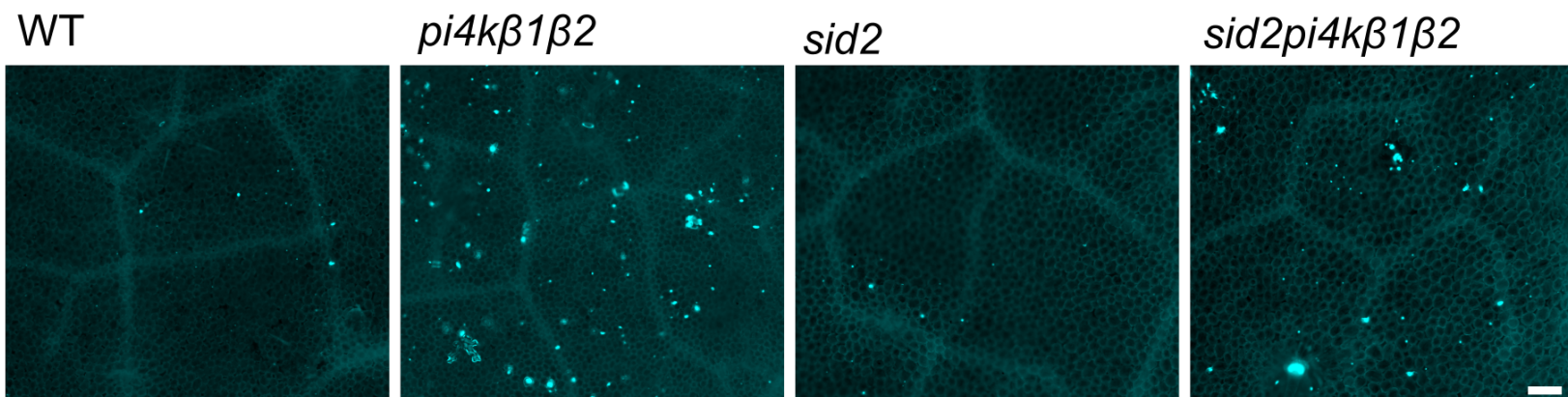
WT





B.



A**B****C****D****E**

Hormonal cross-talk

