



HAL
open science

Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity related responses in *Arabidopsis thaliana* seedlings

Tetiana Kalachova, Romana Pospichalova, Petr Marsik, Pavel Kloucek, Olga Valentová, Eric Ruelland, Hana Leontovyčová, Oksana Iakovenko, Petr Maršík, Pavel Klouček, et al.

► To cite this version:

Tetiana Kalachova, Romana Pospichalova, Petr Marsik, Pavel Kloucek, Olga Valentová, et al.. Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity related responses in *Arabidopsis thaliana* seedlings. *Environmental and Experimental Botany*, In press, 10.1016/j.envexpbot.2019.103867 . hal-02268604

HAL Id: hal-02268604

<https://hal.science/hal-02268604>

Submitted on 21 Aug 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Interplay between phosphoinositides and actin cytoskeleton in the regulation of immunity**
2 **related responses in *Arabidopsis thaliana* seedlings**

3 Tetiana KALACHOVA^{1+*}, Hana LEONTOVYČOVÁ^{1,2,3+}, Oksana IAKOVENKO^{1,2,4+}, Romana
4 POSPÍCHALOVÁ¹, Petr MARŠÍK⁵, Pavel KLOUČEK⁵, Martin JANDA^{1,2,6}, Olga
5 VALENTOVÁ², Daniela KOCOURKOVÁ¹, Jan MARTINEC¹, Lenka BURKETOVÁ¹, Eric
6 RUELLAND^{6,7}

7 ¹Institute of Experimental Botany, The Czech Academy of Sciences, Rozvojová 263, Prague 160
8 000, CZECH REPUBLIC

9 ²University of Chemistry and Technology Prague, Department of Biochemistry and Microbiology,
10 Prague 166 28, CZECH REPUBLIC

11 ³Department of Biochemistry, Faculty of Science, Charles university, Hlavova 8, 128 43, CZECH
12 REPUBLIC

13 ⁴V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, National Academy of
14 Sciences of Ukraine, 1 Murmanska street, 02094 Kyiv, UKRAINE

15 ⁵Department of Food Quality and Safety, Faculty of Agrobiolgy, Food and Natural Resources,
16 Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, CZECH REPUBLIC

17 ⁶Ludwig-Maximilians-University of Munich (LMU), Faculty of Biology, Biocenter, Department
18 Genetics, Grosshaderner Str. 2-4, D-82152 Martinsried, GERMANY

19 ⁷CNRS, Institut d'Ecologie et des Sciences de l'Environnement de Paris, UMR 7618, Créteil,
20 FRANCE

21 ⁸Université Paris-Est, UPEC, Institut d'Ecologie et des Sciences de l'Environnement de Paris,
22 Créteil, FRANCE

23 + equally contributed

24 * corresponding author; kalachova@ueb.cas.cz

25 **Runing title:** Actin filaments, callose and phosphatidylinositol-4-kinase mutants

26 **Keywords:** Actin, Latrunculin B, Salicylic acid, Callose, Phosphatidylinositol-4-kinase

27

28 ABSTRACT

29 Actin cytoskeleton is indispensable for plant cell integrity. Besides, increasing evidences illustrate
30 its crucial role in plant responses to environment, including defence against pathogens. Recently,
31 we have demonstrated that pre-treatment with actin disrupting drugs latrunculin B (latB) and
32 cytochalasin E can enhance plant resistance against bacterial and fungal pathogens via activation
33 of salicylic acid (SA) pathway. Here, we show that actin depolymerization in *Arabidopsis thaliana*
34 seedlings not only triggers SA biosynthesis by ICS1, but also induces callose deposition via callose
35 synthase PMR4. This effect is SA-independent since still present in mutants that do not accumulate
36 SA. LatB also triggers the expression of several defence related genes. We could distinguish genes,
37 induced in a SA-dependent manner (*PR1*, *WRKY38*, *ICS1*) and those that are SA-independent
38 (*PR2*, *PAD4*, *BAPI*). As actin cytoskeleton is tightly connected with membrane trafficking, we
39 assayed the effect of latB on mutant plants invalidated in phosphatidylinositol 4-kinase beta1 and
40 beta2 (*PI4Kβ1β2*). Deficiency in *PI4Kβ1β2* enhanced latB-triggered actin filaments
41 depolymerisation. Yet, it did not lead to a stronger callose deposition or SA biosynthesis in
42 response to latB. Surprisingly, introduction of *NahG* construct or *pad4* mutation in *pi4kβ1β2*
43 background had much lower effect on SA induction and SA-dependent gene expression changes
44 than it has in wild type. We can thus conclude that actin disruption itself triggers immune-like
45 responses: there is an induction of SA *via* PAD4 coupled to ICS1; it leads to the induction of *PR1*
46 and *WRKY38*, and this requires a functional *PI4Kβ1β2* to be properly regulated. However, an
47 alternative, SA-independent, pathway also exists that leads to the enhanced expression of *PR2* and
48 to callose accumulation.

49

50 INTRODUCTION

51 Plants are always confronted to changes in their environment. This can concern physical
52 parameters such as light intensity and/or quality, temperature or water availability, or interactions
53 with other organisms, including bacteria or fungi. Some of the interactions can lead to diseases.
54 Nowadays, plant responses to the environmental challenges, either abiotic or biotic, are an object
55 of an intense research. A novel aspect of such a research is the investigation of the roles of
56 processes that were so far mainly (or uniquely) considered as important for the cell structure. Such
57 a process is the dynamics of cytoskeleton. Cytoskeleton is comprised of microfilaments and
58 microtubules. Actin filaments (F-actin) are highly dynamic structures formed from monomers (G-
59 actin) (Drobak et al, 2004). Even in basal conditions, filaments constantly grow at one end and
60 depolymerize at the opposite one (Li et al, 2017). The filaments are indispensable for cell shaping,
61 growth and polarity (Andreeva et al, 2010), cytokinesis (Brill et al, 2000), but also for intracellular
62 trafficking of vesicles (Geitmann & Nebenführ, 2015). Yet, the direct mechanisms regulating actin
63 dynamics in regards to vesicle trafficking are still waiting to be revealed. A particular role has been
64 proposed for phospholipids, especially phosphoinositides (Ebine & Ueda, 2015).
65 Phosphoinositides are phosphorylated forms of phosphatidylinositol (PI). From the position and
66 number of the phosphate groups on the inositol ring, we can distinguish PI-3-phosphate (PI3P),
67 PI-4-phosphate (PI4P) and PI-4,5-bisphosphate (PIP₂). Phosphoinositides play a key role in vesicle
68 trafficking (Delage et al, 2013) and also interact with cytoskeleton: for instance, F-actin capping
69 protein interaction with actin monomers is regulated by PIP₂ (Pleskot et al, 2012). This makes
70 phosphoinositides potential central players in the regulation of cytoskeleton dynamics versus
71 vesicle trafficking. Phosphatidylinositol-4 kinases (PI4Ks) are the first enzymes committing PI
72 into phosphoinositides phosphorylated at position 4 of inositol. The study of these enzymes and of
73 their corresponding mutants can be useful to investigate the regulation of the vesicle trafficking
74 together with cytoskeleton dynamics. In *Arabidopsis thaliana*, the isoforms AtPI4K β 1 and
75 AtPI4K β 2 appear to be located in the endomembrane system (Antignani et al, 2015; Kang et al,
76 2011). They participate in cytokinesis in *A.thaliana* roots by targeting actin filaments to the cell
77 plate (Lin et al, 2019).

78 Regarding cytoskeleton and the responses to pathogens, it must be noted that soon after
79 contact between a plant cell and a pathogen, actin filaments get rapidly reordered to support
80 transport of the stress-related compounds. For instance, in the case of fungal infection, actin is

81 needed for the transport of antimicrobial compounds and callose to the penetration site (Hardham,
82 2007). This results in forming cell wall appositions that arrest pathogen growth at the invaded cell
83 (Sassmann et al, 2018). This is part of plant immunity response. In case of bacterial infection, the
84 reaction at the level of cytoskeleton is more diffuse along the leaf tissue but an increase of actin
85 filament density and bundling can nevertheless be measured (Lu & Day, 2017). Various
86 components of pathogen cells can trigger actin polymerization in plants. The most described are
87 the so-called microbe associated molecular patterns (MAMPs), like flagellin flg22, elf18, chitin,
88 oligogalacturonides (Li et al, 2017), or secreted effectors (e.g. HopG1 of *Pseudomonas syringae*,
89 (Shimono et al, 2016). Those compounds not only affect cytoskeleton, but induce other immune
90 responses, especially reactive oxygen species formation, defence genes expression, biosynthesis
91 of salicylic acid (SA) (Bigeard et al, 2015).

92 A well described response to pathogens is callose accumulation (Schneider et al, 2016).
93 Callose is a polysaccharide, β -1,3-glucan, synthesized in the apoplast and involved in various
94 processes in plants including cytokinesis (Chen et al, 2009; Thiele et al, 2009), pollen development
95 (Záveská Drábková & Honys, 2017), cell-to-cell communication through plasmodesmata (Cui &
96 Lee, 2016), and various stress responses. Arabidopsis genome encodes twelve callose synthases,
97 each of a specific function (Chen & Kim, 2009). Callose synthase 12 (also known as GLUCAN-
98 SYNTHASE LIKE 5, GSL5, of POWDERY MILDEW RESISTANT 4, PMR4) is the one
99 responsive to wounding and pathogen responses (Ellinger et al, 2014; Keppler et al, 2018). Callose
100 biosynthesis in the apoplast and its accumulation highly depend on the transport of the substrates
101 outside of the cells. Notably, distinct signaling pathways may result in callose formation. For
102 instance, wounding-induced and MAMP-triggered callose might represent different branches of
103 responses going through PMR4 (Keppler et al, 2018). We therefore wanted to investigate the
104 influence of actin depolymerization on callose accumulation. An elegant way to investigate the
105 role of cytoskeleton is the use of drugs that alter cytoskeletal dynamics. Latrunculin B (latB) is
106 such a drug. It originates from a Red Sea sponge *Latrunculia magnificans*. LatB reversely binds
107 actin monomers preventing their polymerization (Morton et al, 2000). It is often used in plant
108 biology to assess the role of actin in endo- and exocytosis (Moscatelli et al, 2012), pollen tube
109 elongation (Gibbon et al, 1999) and growth (Baluška et al, 2001). Concerning the response to
110 pathogens, it has been shown that when applied simultaneously with pathogens, latB negatively
111 affects plant resistance to them (Henty-Ridilla et al, 2013). However, latB is able to activate SA-

112 associated gene expression in *Arabidopsis thaliana* (Matoušková et al, 2014). In *Brassica napus*
113 and *Nicotiana benthamiana*, the pre-treatment of plant leaves with low concentrations of latB -
114 increased SA levels in tissues, activated defence gene expression and was sufficient to increase
115 resistance to further infection. Similar results were obtained with another actin disrupting drug,
116 cytochalasin E (Leontovyčova et al, 2019).

117 We firstly show that actin disruption by latB triggers callose accumulation in *A. thaliana*
118 seedlings through the callose synthase PMR4. Using mutants altered in SA signaling (*pad4*),
119 biosynthesis (*sid2*) and accumulation (*NahG*), we demonstrate that this callose deposition is SA-
120 independent. Furthermore, we found that latB effect on gene expression does not correspond to a
121 general transcriptome deregulation, but that specific pathways related to the response to pathogens
122 are the ones triggered by latB. Amongst the gene responses, we were also able to reveal SA-
123 dependent and SA-independent effects of latB. Interestingly, we show that deficiency in PI4K β 1 β 2
124 enhanced the susceptibility of actin filaments to latB while latB does not appear to impact
125 phosphoinositides levels on plasma membrane. To study the role of PI4K β 1 β 2 in SA-independent
126 responses to actin disruption, we have introduced *NahG* construct as well as *sid2* and *pad4*
127 mutations into *pi4k β 1 β 2* background. Surprisingly, the latB treatment still strongly induced SA in
128 *pad4pi4k β 1 β 2* and *NahGpi4k β 1 β 2* triple mutants, while SA accumulation was prevented in *pad4*
129 and *NahG* mutant plants. To conclude, here we characterize the immunity-like responses to latB,
130 showing that actin depolymerisation acts through both SA-dependent and SA-independent
131 pathways. Moreover, we reveal the existence of an unexpected cross-talk between actin filaments
132 and phosphoinositides in the regulation of those defence-related responses.

133

134 Materials and methods

135 *Plant material*

136 The following *Arabidopsis thaliana* genotypes were used: Columbia-0 (WT); *NahG* (Delaney et
137 al, 1994); *NahGpi4k β 1 β 2* (Šašek et al, 2014); *pad4.1* (NASC stock N3806); *pad4pi4k β 1 β 2* (this
138 study); *sid2*, *pi4k β 1 β 2*, *sid2pi4k β 1 β 2* (Kalachova et al, 2019); *pmr4*; pUBQ::PMR4-GFP in WT
139 background; pUBQ::PMR4-GFP in *pmr4* background (Kulich et al, 2018); Lifeact-GFP
140 (Cvrčková & Oulehlová, 2017); pUBQ::mCitrine-2xFAPP1 (Simon et al, 2014).

141 *Plant cultivation and treatment*

142 Seedlings were grown in MS medium, containing 4.41 g Murashige and Skoog medium including
143 vitamins (Duchefa, Netherlands) and 5 g sucrose per litre, pH adjusted to 5.7 using 1M KOH.
144 Surface-sterilized seeds were sown in 24-well plates, 4-5 seeds per well containing 400 μL of
145 medium and cultivated for 10 days before treatment under a short-day photoperiod (10 h/14 h
146 light/dark regime) at 100-130 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 22°C. On the 7th day, 200 μL of fresh medium was
147 added to the wells.

148 Latrunculin B (latB, Sigma-Aldrich, USA) was dissolved in DMSO at 2 mM stock solution. The
149 seedlings were treated 24 h with 200 nM latB and 0.01% DMSO as mock control prior to sampling.

150 *Gene expression analysis*

151 The whole seedlings (approximately 300 μg fresh weight) from three wells were immediately
152 frozen in liquid nitrogen. The tissue was homogenized in plastic Eppendorf tubes with 1 g of 1.3
153 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). Total RNA was isolated
154 using Spectrum Plant Total RNA kit (Sigma-Aldrich, USA) and treated with a DNA-free kit
155 (Ambion, USA). Subsequently, 1 μg of RNA was converted into cDNA with M-MLV RNase H⁻
156 Point Mutant reverse transcriptase (Promega Corp., USA) and an anchored oligo dT21 primer
157 (Metabion, Germany). Gene expression was quantified by qPCR using a LightCycler 480 SYBR
158 Green I Master kit and LightCycler 480 (Roche, Switzerland). The PCR conditions were 95 °C for
159 10 min followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. Melting curve
160 analysis was then conducted. Relative expression was normalized to the housekeeping gene
161 *AtTIP41*. A list of the analysed genes and primers is available in Supplemental table ST1.

162 *Trypan blue and anilin blue staining*

163 Seedlings were immersed into the staining solution (10 mL lactic acid (85%, w:w), 10 mL phenol,
164 10 mL glycerol, 10 mL distilled H₂O, 40 mg trypan blue) for 2 min (Fernández-Bautista et al,
165 2016). Seedlings were then washed twice in ethanol:glacial acetic acid (1:3, v:v) until leaves were
166 fully decoloured. Seedlings were rehydrated by incubating in ethanol solutions of decreasing
167 concentrations (70%, 50%, 30%, v:v) for at least 30 min each. They were then stained in 0.01 %
168 aniline blue in 150 mM K₂HPO₄, pH 9.5 for 4h and kept in water for the microscopy observation.

169 *Callose imaging and quantification*

170 Callose deposition was observed by fluorescence microscope using a Zeiss AxioImager
171 ApoTome2 (objective 5x). Callose accumulation was calculated using FIJI software
172 (<https://fiji.sc/>) (Schindelin et al, 2012) as the percent occupancy of aniline blue signal (spots) per
173 region of interest (ROI). One round-shaped ROI (d=1500 µm) was taken from one cotyledon, at
174 least 18 independent cotyledons were analysed per variant.

175

176 *Confocal microscopy*

177 For *in vivo* microscopy, a Zeiss LSM 880 inverted confocal laser scanning microscope (Carl Zeiss
178 AG, Germany) was used with either a 40× C-Apochromat objective (NA = 1.2 W) or a 20x Plan-
179 Apochromat objective (NA = 0.8). Fluorescence associated with actin filaments (LifeAct-GFP) or
180 phosphoinositides (mCitrine-2xFAPP1) was acquired at excitation 488 nm emission 490–540 nm
181 for GFP and excitation 516nm emission 520-552 nm for mCitrine. Images were acquired at in z-
182 stacks 20µm thickness). Actin filaments density analysis was calculated by Fiji software as the
183 percent occupancy of GFP/mCitrine signal in each Maximum intensity projection. For Lifeact-
184 GFP, image threshold was set to include all actin filaments and area fraction was measured. For
185 each variant, fluorescent intensity of at least 30 ROI (50x50µm) from 10-14 leaves was analysed.

186

187 *Salicylic acid measurement*

188 All seedlings from 3 wells were sampled as 1 sample, frozen in nitrogen and stored at -80°C until
189 the analysis. Samples were homogenized in tubes with 1.3 mm silica beads using a FastPrep-24
190 instrument (MP Biomedicals, CA, United States) with 2 x 500 µl of extraction reagent
191 methanol/water/formic acid (15:4:1, v:v:v) supplemented with stable isotope-labelled ¹³C-SA
192 internal standards, each at 100 ng per sample, to check the recovery during the purification and to
193 validate the quantification. Extract was subjected to solid phase extraction using Oasis MCX
194 cartridges (Waters Co., Milford, MA, United States) and eluted with methanol. The eluate was
195 evaporated to dryness and dissolved in 30 µl of 15% acetonitrile/water (v/v) immediately before
196 the analysis. immediately before the analysis. Quantification was performed on an Ultimate 3000

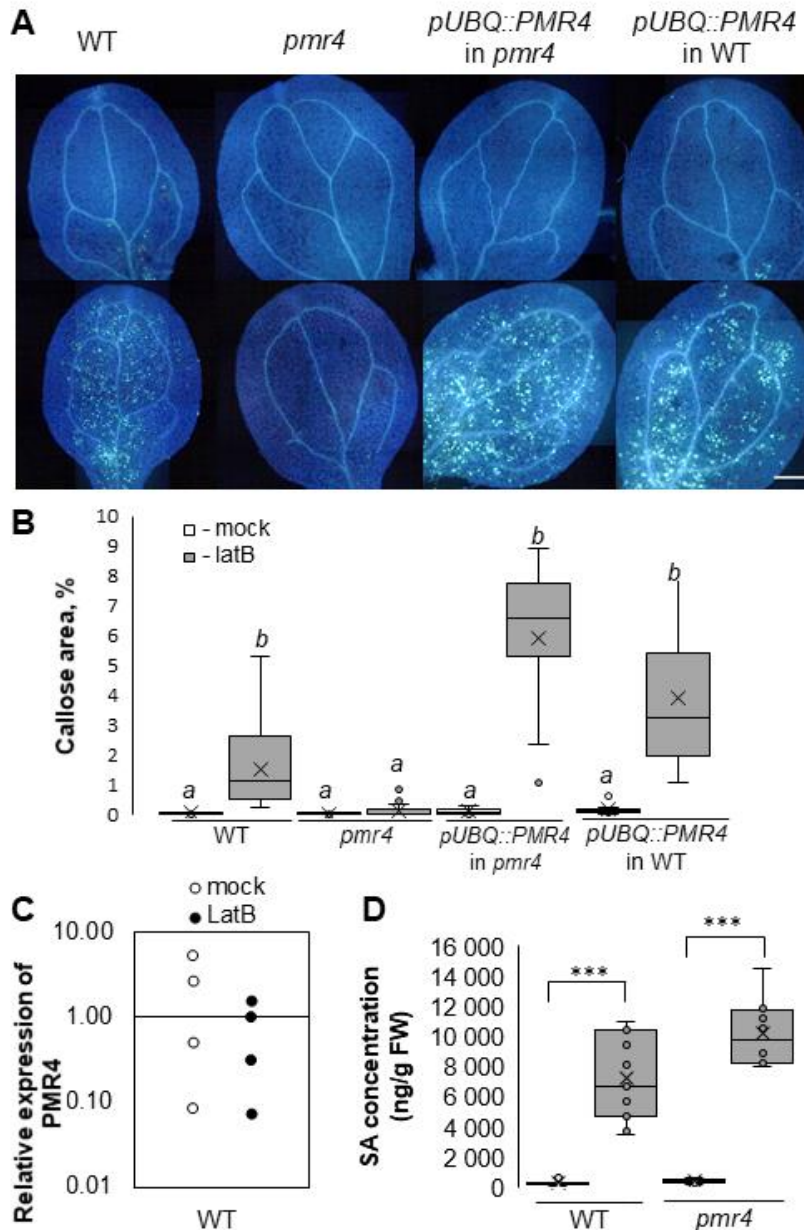
197 high-performance liquid chromatograph (UHPLC, Dionex; Thermo Fisher Scientific, Waltham ,
198 MA, United States) coupled to IMPACT II Q-TOF ultra-high resolution and high mass accuracy
199 mass spectrometer (HRAM-MS; Bruker Daltonik, Bremen, Germany). Separation was carried out
200 using column Acclaim RSLC 120 C18 (2.2 μm , 2.1x100 mm; Thermo Fisher Scientific, Waltham,
201 MA, United States) tempered at 30°C and mobile phase consisting of 0.1% formic acid (A) and
202 methanol (B) by gradient elution. The gradient started at 1%B and going to 39 % B in 3min, 60 %
203 B in 7 min, 100 % B in 8 min, followed by 3 min of washing by 100% B and 5 min equilibration
204 at initial conditions (1%B). Flow rate was changing during separation from 200 $\mu\text{l}/\text{min}$ to 300
205 $\mu\text{l}/\text{min}$ in 7 min and then kept at this rate to 16 min. Injection volume was 5 μL . The full-scan data
206 were recorded in negative electrospray ionization (ESI) mode and processed using Data Analysis
207 4.3 and TASQ 1.4 software (both Bruker Daltonik, Bremen, Germany). SA and labelled internal
208 standard ($^{13}\text{C}_6\text{-SA}$) were monitored as ions of their deprotonated molecules $[\text{M-H}]^-$ (137.0239 m/z
209 and 143.0440 m/z, respectively). SA content was expressed in ng/g fresh weight (FW).

210

211 RESULTS

212 **Latrunculin B induces callose via PMR4 callose synthase**

213 Ten-day-old *A. thaliana* seedlings were treated for 24 h with 200 nM latB. As expected,
214 latB treatment caused actin disruption, measured as the decrease of filament abundancy (fig.S1).
215 The concentration used for the drug did not lead to cell death (fig.S1). More unexpectedly, the
216 treatment also caused callose accumulation (measured as the area occupied with callose spots)
217 (fig.1A,B).



218
 219 **Figure 1. Latrunculin B (latB) induces PMR4-dependent callose accumulation.** Ten days old
 220 *A. thaliana* seedlings were treated for 24h with 0.05% DMSO (mock) or 200 nM latB. A.
 221 Representative images of aniline blue stained cotyledons of WT, *pmr4*, pUBQ::PMR4-GFP in
 222 *pmr4*, and pUBQ::PMR4-GFP in WT, epifluorescent microscopy, scale bar = 500µm. B.
 223 Quantification of the relative area occupied with callose particles, n=18. Values for the mock-
 224 treated plants are presented in white boxes, latB treated - in dark grey boxes. In the plots, central
 225 line represents the median occupancy, cross represents the mean, bottom and top edges of the box
 226 are 25 and 75% of distribution and the ends of whiskers are set at 1.5 times the interquartile range.
 227 Values outside this range are shown as outliers. Different letters indicate variants that are
 228 significantly different, one-way ANOVA with Tukey-HSD test, $p < 0.05$. C. Relative expression of
 229 *PMR4*. Relative expression of *PMR4* in WT plants. Values were normalized for the untreated WT.

230 *TIP41* was used as the reference gene. D. Salicylic acid content. *** - variants are significantly
231 different, two-tailed *t*-test, $p < 0.001$, $n = 8$.

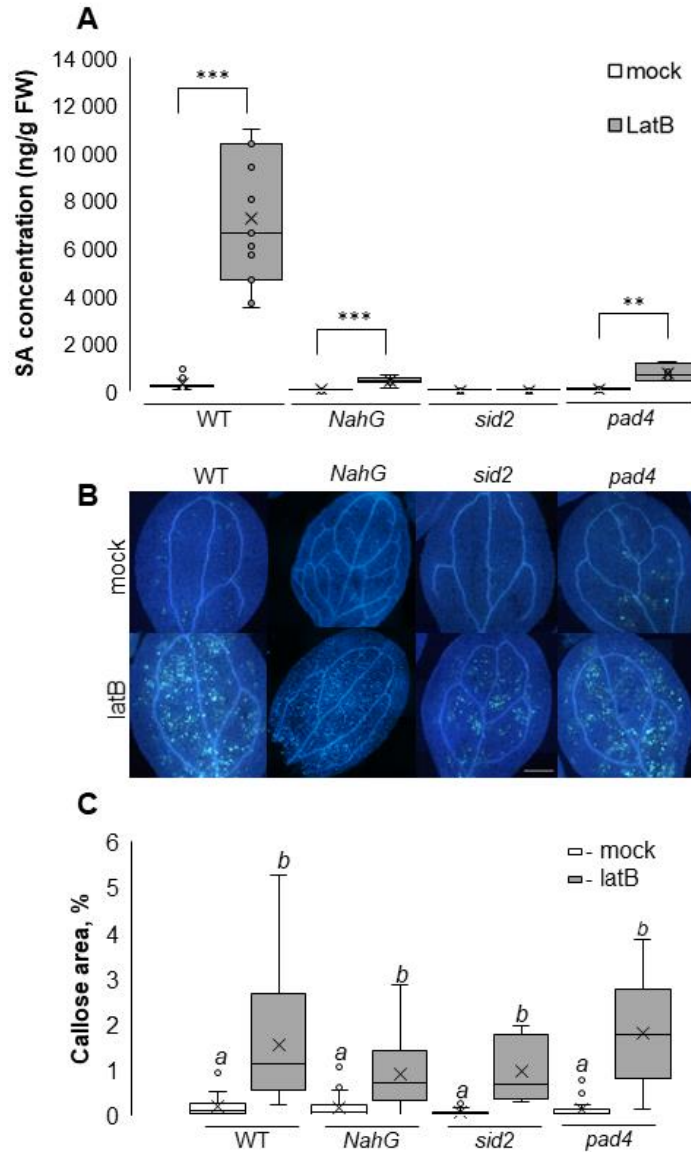
232

233 Among twelve known callose synthases in *A. thaliana*, PMR4 was shown to be a major
234 one involved to biotic stress responses (Nishimura et al., 2003; Kulich et al., 2018). We therefore
235 tested its contribution to latB-triggered callose. In the *pmr4* mutant seedlings, the callose level
236 detected after latB treatment was nearly equal to that in mock-treated control. When a
237 pUBQ::PMR4-GFP construct was expressed in WT or *pmr4* backgrounds, basal callose level in
238 cotyledons remained low. Yet, when latB was added, callose accumulation was restored (Fig. 1B).
239 Besides, such an induction did not rely on transcriptional changes, as soon as the expression of
240 *PMR4* was not affected (Fig. 1C).

241

242 ***Latrunculin B effects on callose accumulation is SA-independent***

243 The fact that latB treatment causes callose accumulation through PMR4 strongly suggests
244 that other defence-related responses should be studied. We therefore decided to study the role of
245 SA content and signaling pathway in the observed callose deposition. To do so, we first measured
246 SA levels in seedlings after latB treatment. A 20-fold increase of SA was observed in WT
247 seedlings. Notably, *pmr4* plants behaved similarly to WT, suggesting callose to be either
248 downstream, either independent on SA (fig. 1D, Supplementary table ST3). To address this, we
249 analyzed SA induction and callose deposition in plants with altered SA pathway: *NahG* mutant
250 plants, expressing the bacterial salicylate hydroxylase that degrades SA to inactive catechol and
251 therefore unable to accumulate SA; *sid2* plants, deficient in ISOCHORISMATE SYNTHASE 1
252 (ICS1); and *pad4* plants, with a mutation in PHYTOALEXIN DEFICIENT 4 (PAD4) (fig.2,
253 Supplementary table ST3). PAD4 protein has been shown to regulate the synthesis of SA *via* the
254 ICS1 pathway (Cui et al, 2017). As expected, both *NahG* and *pad4* plants had lower basal content
255 of SA, and smaller (approx. 5-fold) increase in SA after latB comparing to WT. *sid2* plants showed
256 no increase in SA, pointing out that the detected SA was produced by ICS1. On the contrary,
257 callose production was not affected any of the studied mutants, confirming SA-independent nature
258 of the process (fig. 2, B,C).



259

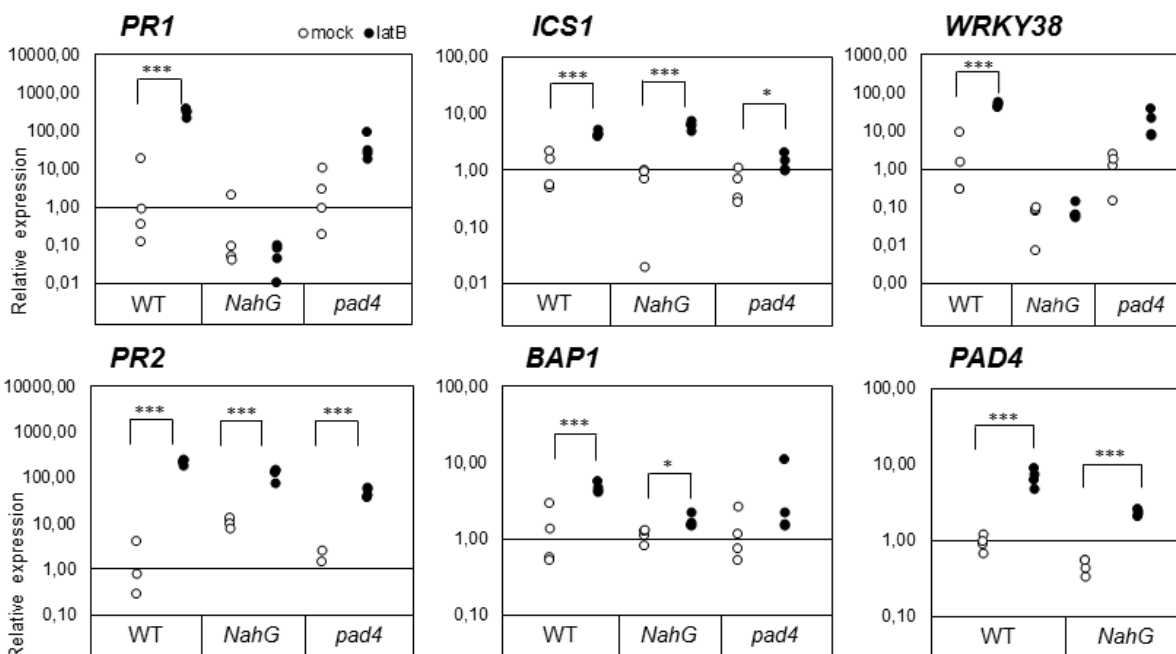
260 **Figure 2. SA-independent induction of callose accumulation by latB treatment.** Ten-day-old
 261 seedlings of WT, *NahG*, *sid2* and *pad4* were treated by 200 nM latB or 0.01 % DMSO as the
 262 control. Seedlings were harvested 24 hr after the treatment A. SA content. *** - samples are
 263 different, $p < 0.001$, two-tailed *t*-test. B. Representative images of aniline blue stained cotyledons,
 264 scale bar 500 μ m. C. Relative area of callose particles. Central line of the boxplot represents the
 265 median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of
 266 distribution and the ends of whiskers are set at 1.5 times the interquartile range. Values outside
 267 this range are shown as outliers. Different letters indicate variants that are significantly different,
 268 one-way ANOVA with Tukey-HSD test, $p < 0.05$. At least 30 independent cotyledons were
 269 analysed for each variant.

270

271 **Latrunculin B affects gene expression in both SA-dependent and -independent manner**

272 We had already shown that latB could induce the expression of some genes related to plant
 273 defence (Leontovyčova et al, 2019; Matoušková et al, 2014). Here we studied whether intracellular
 274 level of SA or an intact SA-signaling pathway is needed for latB to trigger gene expression changes
 275 (figure 3, Supplementary figure S2, Supplementary table ST2). To do so, we analysed the
 276 expression of several defence marker genes in *NahG* and *pad4* mutants. Eleven-day-old *in vitro*
 277 grown plants (whole seedlings) of WT and mutant genotypes were harvested for gene expression
 278 studies 24 hpt by 200nM latB. Concerning the gene response in WT plants, we show here that the
 279 defence-associated *PRI*, *PR2*, *WRKY38*, *ICS1* and *PAD4* were induced by latB (Figure 3). We also
 280 detected an activation of the wounding marker *BAP1*, even though no cell death was yet detected
 281 by trypan blue staining (Supplemental figure S1). On the contrary, no significant changes were
 282 observed in the expression of SA biosynthetic genes other than *ICS1* (*ICS2*, *PAL1*, *PAL2*, *PAL3*).
 283 To be noted, *ICS2* expression seems to be very dependent on the growth conditions, as we
 284 previously detected its induction after latB treatment (Leontovyčova et al, 2019). In addition,
 285 neither jasmonate biosynthesis marker genes (*LOX2*, *AOS1*), nor jasmonate responsive genes
 286 (*VSP2* and *PDF1.2*), nor ethylene marker gene (*ERF1*) were significantly affected by the treatment
 287 (Supplemental figure S2, Supplemental Table ST2).

288



289

290 **Figure 3. SA-independent expression of defence-related genes by latB treatment.** Ten-day-
291 old seedlings of WT, *NahG* and *pad4* were treated by 200 nM latB or 0.01 % DMSO as the control.
292 Seedlings were harvested 24 hr after the treatment. Data from the representative experiment are
293 shown. Values were normalized for mock treated WT, note that data are plotted at the log₁₀ scale.
294 Asterisks indicate values, different from respective controls for each genotype, * - p<0,05; *** -
295 p<0.001, two-tailed Student *t*-test). Reference gene *TIP41*.

296 However, we detected that *PR1* and *WRKY38* inductions by latB were abolished in *NahG*
297 and *pad4* mutants, strongly suggesting these inductions rely on a SA-dependent pathway (fig.3).
298 This also involves that SA is accumulated in response to latB (Fig.2 A). On the contrary, the
299 expression of *PR2* in response to latB appears not to be dependent on SA since it was still present
300 in *NahG* and *pad4* mutants. Similarly, the expression of the *PAD4* by latB was still present in
301 *NahG* plants: it does not rely on SA accumulation. The expression of *ICS1* in response to latB does
302 not appear to be controlled by SA level in the tissues, as seen in *NahG* mutant. Yet, *PAD4* protein
303 regulates *ICS1* expression, at least partly. As for the induction of the expression of *BAP1* by latB,
304 it was diminished in *NahG* and *pad4* mutants (though still present), and so can be considered as
305 partly SA-dependent.

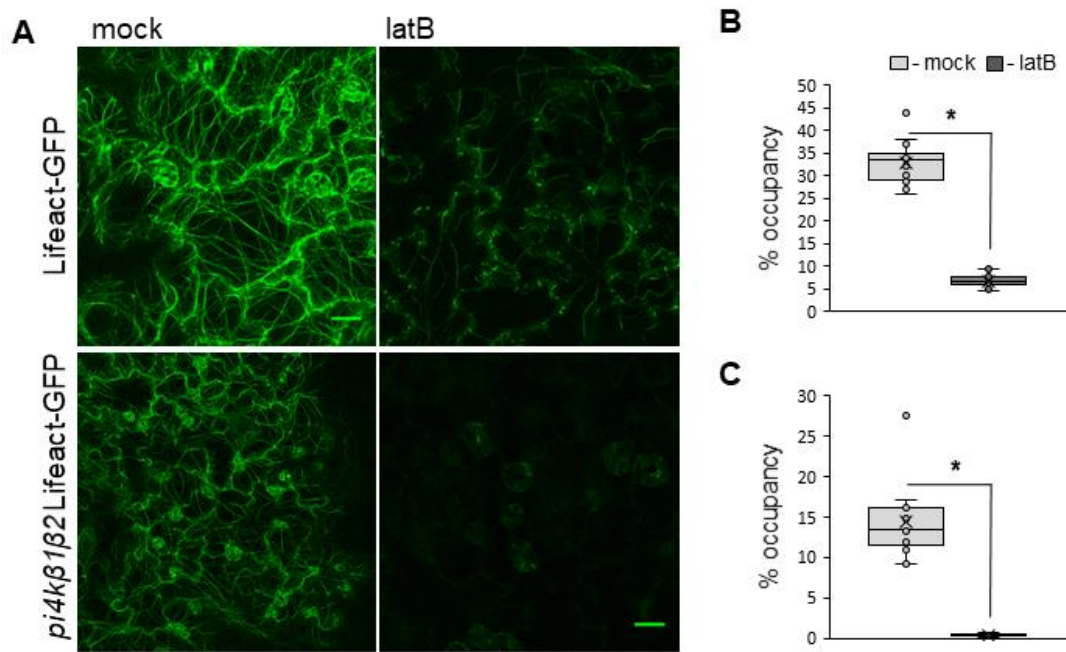
306 We can thus conclude that latB induces several defence-like responses: there is an
307 induction of SA; it leads to the induction of *PR1* and *WRKY38*. However, an alternative, SA-
308 independent, pathway also exists. It controls the expression of *PR2*, *ICS1*, *PAD4* and callose
309 accumulation in response to latB.

310 ***Effects of latB on cytoskeleton are enhanced in pi4kβ1β2 double mutant***

311 Phosphoinositides, that are the phosphorylated forms of phosphatidylinositol (PI), are
312 involved in vesicular trafficking. This process requires correct actin cytoskeleton organization
313 (Pleskot et al, 2014). There is therefore a link between actin cytoskeleton and phosphoinositides.
314 We wanted to investigate whether the effect of latB could be impacted by a modification in
315 phosphoinositide metabolism. To do so, we used the *pi4kβ1β2* double mutant. This mutant is
316 invalidated in the two beta isoforms of phosphatidylinositol-4-kinases. This double mutant was
317 shown to have impaired vesicle trafficking, dwarf phenotype and increased SA level (Kang et al,
318 2011; Šašek et al, 2014).

319 We firstly introduced LifeAct-GFP construct into *pi4kβ1β2* background by crossing. Four
320 independent lines were tested. No major defects in actin filaments organization in epidermal cells
321 were observed while compared to WT (fig.4). After 24h of latB treatment, the density of the

322 filaments in *pi4kβ1β2* plants was 20-times lower than that in non-treated double mutant, while in
 323 the WT background this decrease was 5-fold. Consequently, in *pi4kβ1β2* plants the actin filaments
 324 were almost lost while in WT their occupancy was still 7% (fig 4B). The actin cytoskeleton of
 325 *pi4kβ1β2* mutants thus appear to be more sensitive to latB. Meanwhile, we wanted to evaluate the
 326 impact of latB directly on the PI4P content using genetically encoded biosensor mCitrine-
 327 2xFAPP1 for PI4P (Simon et al., 2014). The sensor mostly labels PI4P localized in plasma
 328 membrane. In our setup, the content of PI4P remained stable in response to latB (fig.S3 A, C).

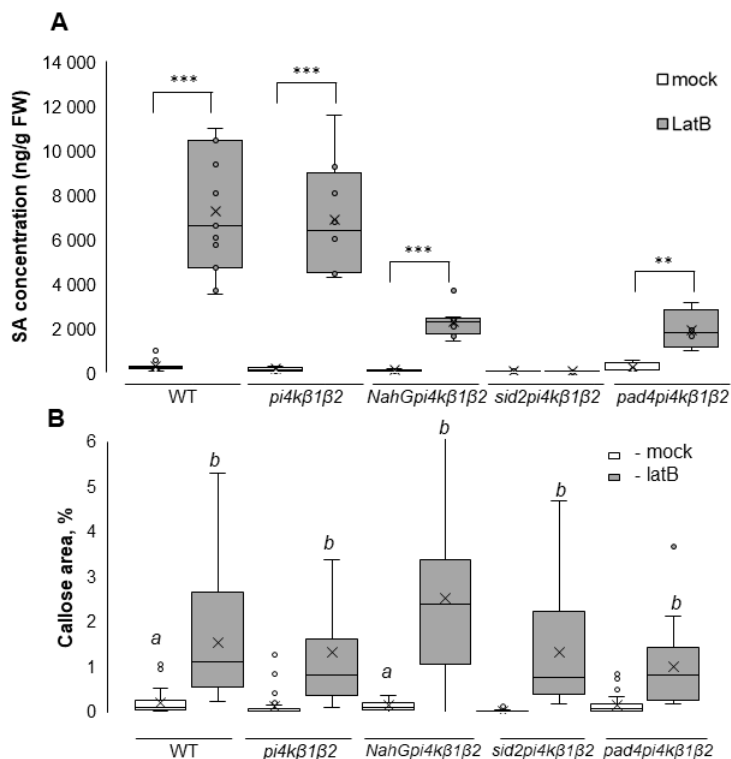


329
 330 **Figure 4. Effect of latB on actin filaments organization of *pi4kβ1β2* mutants.** A. Representative
 331 images of maximum intensity projections of 10µm abaxial epidermis of LifeAct-GFP expressing
 332 WT and *pi4kβ1β2* plants. Confocal microscopy, scale bar = 20µm. Quantification of relative area
 333 occupied with fluorescent filament in WT (B) and *pi4kβ1β2* (C). For each variant at least 15
 334 independent seedlings were imaged, n=12. For quantification, maximum intensity projections of
 335 10µm Z-stacks of abaxial sides of the leaves were used. At least 30 regions of interest (ROI)
 336 measured for each treatment. In the plots, central line represents the median occupancy, cross
 337 represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends
 338 of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as
 339 outliers. Asterisks indicate statistically significant differences with the mock treatment, Student *t*-
 340 test, $p < 0,05$

341

342 *Effects of latB on callose accumulation in pi4kβ1β2 double mutant is still SA-independent*

343 Because it seems there was a positive (enhancing) interaction between latB and PI4Kbeta
 344 mutations on actin filaments, we studied SA levels, callose accumulation and gene expression
 345 responses to latB in *pi4kβ1β2* plants. Indeed, latB triggered SA increase in *pi4kβ1β2* plants (fig.
 346 5A). Surprisingly, introduction of neither *NahG* construct nor *pad4* mutation into *pi4kβ1β2*
 347 background did not fully prevent SA increase as it did in WT background (fig.2A), even though
 348 such an increase was still much lower than in WT or *pi4kβ1β2*. However, *sid2* mutation was
 349 sufficient to block SA production in both WT and *pi4kβ1β2*, confirming that the entire SA pool
 350 induced by latB treatment is synthesized due to ICS1 activity in both backgrounds. As for callose,
 351 its basal levels in 8-, 10- and 14-day-old *pi4kβ1β2* seedlings were similar to that of WT
 352 (Supplemental figure S4). To study the involvement of SA, the callose accumulation was also
 353 tested in triple mutants *NahGpi4kβ1β2* (Šašek et al, 2014), *sid2pi4kβ1β2* (Kalachova et al, 2019)
 354 and *pad4pi4kβ1β2*, obtained by crossing *pi4kβ1β2* to *pad4* (homozygous F3 plants were used).
 355 After latB treatment, accumulation of callose in all studied mutants was similar to that in WT (fig.
 356 5B), suggesting that callose response to latB in *pi4kβ1β2* is still not SA-dependent (fig. 5B). This
 357 is consistent with our findings in WT plants.

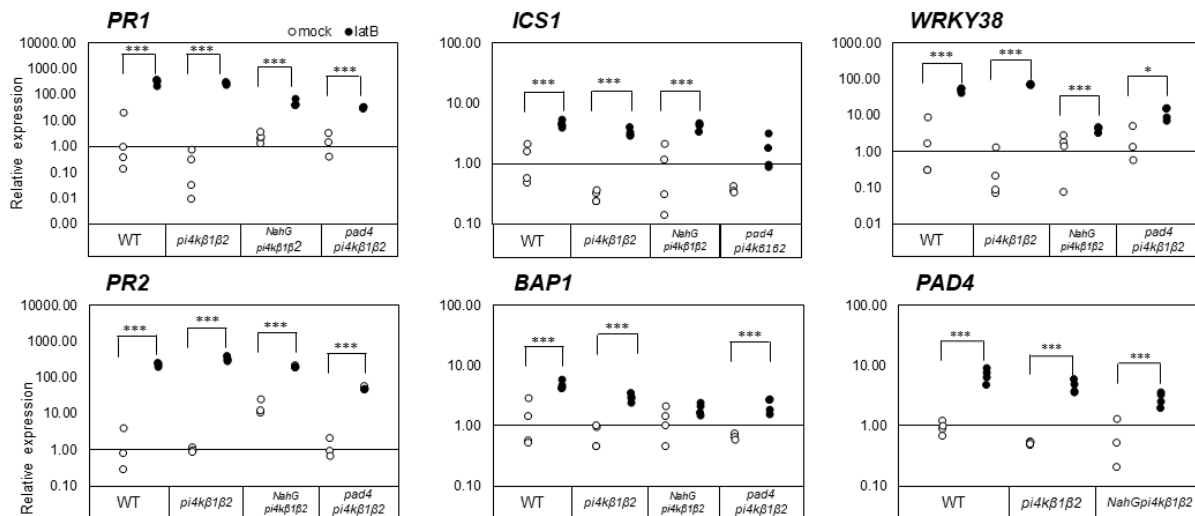


359 **Figure 5. LatB induced SA and callose accumulation in *pi4kβ1β2* mutants.** Ten-days-old
 360 seedlings were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested
 361 24 hr after the treatment. A. SA content. Data from two independent experiments are plotted. B.
 362 Relative area of callose particles in the cotyledons. At least 30 independent cotyledons were
 363 analysed for each variant. Experiment was repeated three times with similar results, the data from
 364 a representative repetition are shown. In boxplots, central line represents the median occupancy,
 365 cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the
 366 ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown
 367 as outliers. Different letters mark significantly different values, $p < 0,05$; Student *t*-test, unequal
 368 variance.

369

370 **Effects of latrunculin B on defence genes expression in *pi4kβ1β2* double mutant**

371 As for *PR1* and *WRKY38*, after latB treatment, their induction in *pi4kβ1β2* was comparable
 372 to that in WT. Yet, contrary to what was observed in WT background, their induction was also
 373 observed in *NahGpi4kβ1β2* and *pad4pi4kβ1β2* triple mutants (fig. 6). This is in agreement with
 374 the fact that SA is still accumulated in these triple mutants. *BAP1* expression in PI4K deficient
 375 mutants after latB treatment was induced less than in WT and was partly SA-dependent (fig. 6).
 376 Besides, neither induction of SA biosynthetic enzymes other than *ICS1* was detected, nor of JA-,
 377 ABA-, or ethylene markers (Supplemental figure S5, supplemental table ST2).



378

379 **Figure 6. LatB-induced gene expression in *pi4kβ1β2* mutants.** Ten-days-old seedlings of WT,
 380 *pi4kβ1β2*, *NahGpi4kβ1β2* and *pad4pi4kβ1β2* were treated by 200 nM latB or 0.01% DMSO as
 381 mock control. Seedlings were harvested 24 hr after the treatment. Data are normalized to mock-
 382 treated WT, note that data are plotted at the log₁₀ scale. Asterisks indicate values, different from

383 respective controls for each genotype, * - $p < 0,05$; *** - $p < 0.001$, two-tailed Student *t*-test).
384 Reference gene *TIP41*.

385

386 DISCUSSION

387 We show here that latrunculin B induces callose accumulation in seedlings. It is also true for
388 cytochalasin E, another actin filament disruptor (data not shown). Therefore, it is very likely that
389 this effect of latB is due to actin depolymerization. Callose deposition was reported in responses
390 to various stresses, like wounding or pathogen infection (Jacobs et al, 2003). We had previously
391 shown that chemical disruption of actin led to some immunity-related responses such as activation
392 of *PR1* expression (Matouskova et al., 2014). In this regard, callose is particularly important, as
393 another biotic-stress-like response, partly connected with SA. Arabidopsis genome encodes twelve
394 callose synthases (Voigt, 2014). *PMR4* (POWLDERY MILDEW RESISTANT 4, also called
395 GLUCAN SYNTHASE LIKE 5, *GLS5* or *CALS12*) is precisely the one activated during infection
396 (Nishimura et al, 2003). Here we first show that callose accumulation induced by latB fully relies
397 on *PMR4*. It indicates its connection with a biotic stress. Interestingly, using *NahG* and *pad4*
398 mutants, we show that latrunculin B effects on callose accumulation is SA-independent and not
399 downstream of *PAD4*. To be noted, *pmr4* mutant itself was reported to have a constitutively
400 activated SA pathway in adult plants and contribute to most of callose accumulations in studies
401 connected with biotics stres (Nishimura et al., 2003). Nevertheless, SA level in *pmr4* seedlings did
402 not differ from that in WT (Supplementary table ST3). It was also true for *pi4kβ1β2* plants, known
403 as SA and callose accumulators while being grown up to 4-weeks (Kalachova et al, 2019).
404 However, neither SA nor callose levels were increased in non-stimulated seedlings (figure 5). A
405 negative interaction between actin polymerisation, callose and SA levels has been suggested
406 recently: natural polymorphism of genetic disruption of Actin Related Protein Complex 4
407 (*ARPC4*) affected pathogen- and wounding-induced callose deposition, as well as affected
408 expression of *PR1* in response to *Sclerotinia sclerotiorum* (Badet et al, 2019).

409 As latB action on callose appeared as SA-independent, we wanted to check whether the
410 effect of latB on gene expression was also independent on SA. We first better detailed the changes
411 in gene expression triggered by latB. We show here that *PR1*, *PR2*, *WRKY38*, *ICSI* and *PAD4*
412 were induced by latB in WT. It is also the case for a marker of cell death in pathogen and wounding
413 responses, *BAPI* (Yang et al, 2007). *BAPI* was described as wounding marker, but also as a SA-

414 associated gene: it negatively regulates cell death in response to *Pseudomonas syringae* pv *tomato*
415 and *Hyaloperonospora parasitica* acting in the same pathway with *BON1*, *SNCI* and *PAD4* (Yang
416 et al, 2006) *BAP1* expression can be induced by high temperature or ROS formation, however this
417 induction was abolished in mutants with altered SA pathways (*NahG*, *pad4*) (Hedtmann et al,
418 2017; Zhu et al, 2011). In our setup, the expression of *BAP1* was increased independently of the
419 SA content in plants, indicating a new role of this gene in plant stress responses. Yet, no significant
420 changes were observed in the expression of SA biosynthetic genes other than *ICS1* (*ICS2*, *PAL1*,
421 *PAL2*, *PAL3*), nor in the expression of *LOX2* and *AOS*, a JA-biosynthesis marker genes, nor to the
422 jasmonate responsive genes *VSP2* and *PDFI.2*. Genes involved in ethylene biosynthesis (*ACS2*)
423 (Denoux et al, 2008), ethylene response (*ERF1*, *LEA4-1*)(Lorenzo et al, 2003) or to the response
424 of abscisic acid (*ABI1*)(Leung et al, 1997) were not induced by latB. Therefore, it seems that latB
425 action is not “universal”, that it does not lead to a deregulation of the whole transcriptome. Its
426 action seems to be directed mainly towards the SA signalling pathway or toward response to
427 biotrophic pathogens.

428 Clearly, a SA dependent pathway is activated by latB. Expression of *PR1*, a classical
429 representative of defence genes, is usually induced during pathogen attack, but also as a reaction
430 to any SA increase. Besides, *ICS1*, that is responsible for most of the SA accumulation in response
431 to bacteria (Li et al, 2019; Wildermuth et al, 2001; Zheng et al, 2015), was strongly induced.
432 Therefore, the question was the following: did the changes in gene expression, that target SA
433 responsive genes, occur through a SA-dependent pathway. We could show that the expression of
434 *PR1* and *WRKY38* by latB relied on SA accumulation and a functional *PAD4* protein. The fact that
435 *ICS1* expression is controlled by *PAD4* in response to pathogen was established (Cui et al., 2017).
436 Yet, the expression of *PR2* and *ICS1* is independent on SA, as seen in the *NahGpi4kβ1β2*. *PR2* is
437 one of the early defence genes, usually co-expressed with *PR1* and *PR5* and used as a marker of
438 SA pathway (Hamamouch et al, 2011). Nevertheless, here we describe its clear SA-independent
439 induction. It might seem contradictory, unless we take into account that *PR2* protein is β -1,3-
440 glucanase, particularly important for antifungal defences (Ali et al, 2018). *PR2* is crucial for callose
441 degradation and contributes to the SA increase; its overexpression enhances resistance both to
442 fungal and bacterial pathogens (Oide et al, 2013).

443 It thus appears that latB (likely through its action on actin filaments) activates a signaling
444 pathway typical of the response to pathogens. It activates PAD4. Some of the PAD4 action leads
445 to *ICS1* expression, to SA accumulation and to the expression of SA responsive genes. Yet, a part
446 of PAD4 action is not dependent on SA, as seen by *ICS1* expression. LatB also triggers a pathway
447 that does not involve PAD4 nor SA, and that leads to callose accumulation and *PR2* expression.

448 The action of latB is likely to occur through actin filament depolymerization. Actin
449 filaments are involved in the control of vesicle trafficking. Phosphoinositides are also involved in
450 vesicular trafficking. There is indeed an interaction/interplay between actin filaments and
451 phosphoinositides (Pleskot et al, 2014). F-actin capping protein interaction with actin monomers
452 is regulated by phosphatidic acid and phosphatidylinositol-4,5-bisphosphate, a phosphoinositide
453 (Pleskot et al, 2012). In pollen tube, cytoskeletal disruption by latB or jasplakinolide not only
454 affects actin and arrested tube growth, but also causes relocalization and even dissociation of
455 RabA4a and RabA4b-tagged vesicles (Zhang et al, 2010). Interestingly, PI4K β 1 and PI4K β 2 were
456 shown to be recruited by RabA4b. Due to this interaction between actin and phosphoinositides in
457 relation to vesicle trafficking, we were interested to investigate whether the action of latB could
458 be impacted by alteration in phosphoinositide metabolism. To address this, *pi4k β 1 β 2* mutant was
459 a tool of choice. As already mentioned, PI4Ks are the first enzymes that commit PI toward the
460 synthesis of phosphoinositides, and the beta isoforms PI4K β 1 and PI4K β 2 were shown to be
461 recruited by RabA4b. PI4Kbeta proteins participate in cytokinesis in Arabidopsis roots by
462 targeting actin filaments to the cell plate (Lin et al, 2019). Nevertheless, it is important to consider
463 the fact that *pi4k β 1 β 2* plants exhibit constitutive activation of SA-related genes and high basal
464 levels of callose. Yet it is true for mature 4 week-old soil-grown plants (Antignani et al, 2015;
465 Kalachova et al, 2019; Šašek et al, 2014). In younger plants, no *PR1* overexpression was detected
466 (Sasek et al., 2014). In the present study we used 10-day-old seedlings grown *in vitro*. In these
467 conditions, neither SA accumulation (fig.5 A, Supplementary table ST3), nor constitutively high
468 expression of *PR1* and *ICS1* was observed (fig. 6).

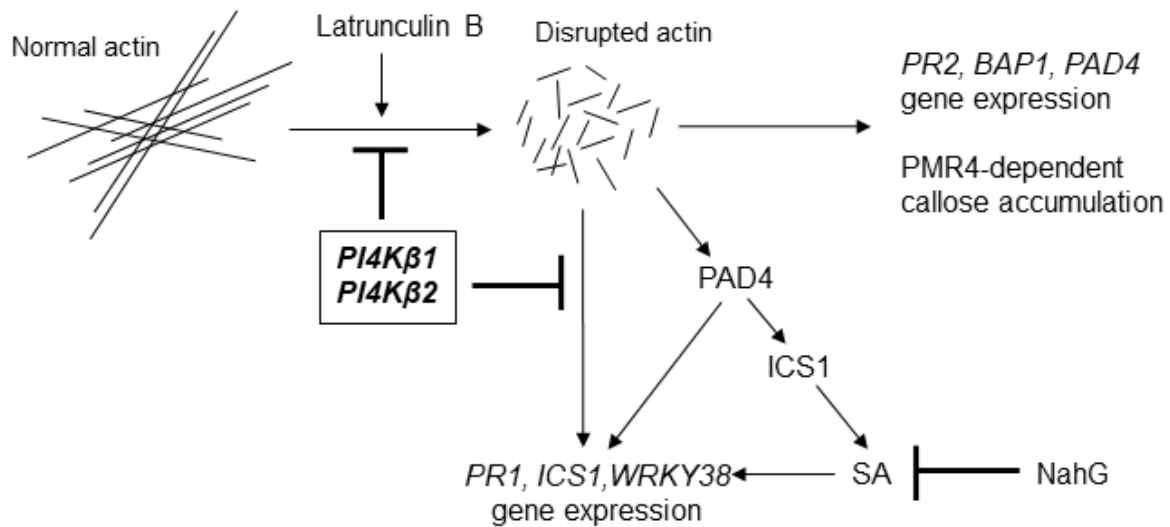
469 The actin cytoskeleton of *pi4k β 1 β 2* mutants appear to be more sensitive to latB (fig.4).
470 This is consistent with the above mentioned references which show F-actin capping protein
471 interaction with actin monomers is regulated by phosphatidic acid and phosphatidylinositol-4,5-
472 bisphosphate, a phosphoinositide (Pleskot et al, 2012). In response to latB, *pi4k β 1 β 2* seedlings

473 accumulated callose, in a SA- and PAD4- independent way, as observed in WT plants. Yet, when
474 we assay the SA increase in response to latB, a process not observed in WT is then detected: the
475 SA induction in *pi4kβ1β2* background still occurs, despite the mutation in PAD4 or expression of
476 NahG. The *pi4kβ1β2* double mutation allows to reveal a signalling pathway, triggered here by
477 latB, that leads to SA biosynthesis and *PR1* expression independently on PAD4. Yet, the *PR1*
478 expression in response to latB was not only observed in *NahGpi4kβ1β2* and *pad4pi4kβ1β2* triple
479 mutants, but also in *sid2pi4kβ1β2* (data not shown). Therefore, our data on the existence of such a
480 SA independent pathway are strong. Is this pathway usually actively inhibited by functional
481 PI4Ks? Is it linked to the effect of *pi4kβ1β2* double mutation on trafficking? Or is it due to a role
482 of phosphoinositide on signalling? An important contribution may come from immune-related
483 MAPKs, whose transient or sustained activation works as a switch between pattern-triggered
484 immunity and effector triggered immunity (Tsuda et al, 2013). Indeed, *Tsuda et al.* described
485 expression of *PR1* in *sid2* background, induced by constitutive activation of MPK3 and MPK6.
486 Another enzyme of the cascade, MPK4, was shown to interact with PI4Kβ1 during cell plate
487 formation in cytokinesis (Lin et al, 2019). More research is necessary to understand and
488 characterize this pathway.

489 To summarize, we here report the defence-like consequences of actin cytoskeleton
490 disruption by latB (fig. 7). Two kinds of signalling pathways were activated by latB treatment: a
491 first pathway is independent on SA; it leads to the expression of genes such as *PR2*, but also to
492 callose accumulation through PMR4. The second pathway involves SA synthesis via PAD4 and
493 ICS1; it leads to the expression of genes such as *PR1* and *WRKY38*. The double mutation in
494 PI4Kβ1β2 makes the effect of latB on filaments more pronounced. Interestingly, the SA increase
495 and *PR1* expression in response to latB in the *pi4kβ1β2* background is also triggered in an
496 unconventional, PAD4-independent pathway. Either this pathway is actively inhibited in WT
497 background by PI4Kβ1β2 (and thus not seen), or it is activated only when the PI4Kβ1β2 are
498 missing.

499

500



501

502 **Figure 7. Graphical summary of the latrunculin B effects in seedlings.** The disruption of actin
 503 polymers by latB activates two kinds of signalling pathways. A first pathway is independent on
 504 SA; it leads to the expression of genes such as *PR2*, but also to callose accumulation. The second
 505 pathway involves SA synthesis via *PAD4* and *ICS1*; it leads to the expression of genes such as
 506 *PR1* and *WRKY38*. The double mutation in *PI4K* beta1 and beta2 makes the effect of latB on
 507 filaments more pronounced. Interestingly, the *PR1* expression in response to latB is no longer SA-
 508 dependent in the *pi4kβ1β2* background: a SA independent pathway leading to *PR1* expression is
 509 thus revealed.

510 Still several open questions remain: what makes actin depolymerization trigger these pathways?
 511 Is it the disrupted actin *per se* that act? Or are these pathways activated as a response to a process
 512 altered due to lack of good filaments? Such a process could be a modified vesicle trafficking due
 513 the missing actin filaments. In this regard, it would be interesting to monitor the processing of
 514 receptors of MAMPs in seedlings submitted to artificial cytoskeleton depolymerization agents.

515 We believe this work sheds light on the connection between cytoskeleton dynamics, vesicular
 516 trafficking and immunity at the cellular and subcellular level and thus will contribute to better
 517 understanding of plant responses to changing environment.

518

519

520

521 FUNDING INFORMATION

522 This work was supported by Czech Science Foundation [GAČR grant no. 17-05151S]. TK
523 benefited from the Program of Postdoctoral Fellowships of the Czech Academy of Sciences
524 [PPPLZ grant no. TK 919220]. OI received the Visegrad scholarship (2018–2019) [grant no.
525 51810647]. HL benefited from Charles University student grant [GAUK no. 992416]. The work
526 was also supported by the Ministry of Education, Youth and Sports of CR from European Regional
527 Development Fund-Project "Centre for Experimental Plant Biology" [grant no.
528 CZ.02.1.01/0.0/0.0/16_019/0000738]. IEB imaging facility is supported by OPVK
529 CZ.2.16/3.1.00/21519.

530

531 ACKNOWLEDGEMENTS

532 We are very grateful to Dr. Ivan Kulich for providing pUBQ::PMR4-GFP expressing lines and Dr.
533 Yvon Jaillais for mCitrine-2xFAPP1 expressing line. We would like to thank Dr. Kateřina
534 Malínská for training and the precious advices with microscopy.

535

536 AUTHOR CONTRIBUTION

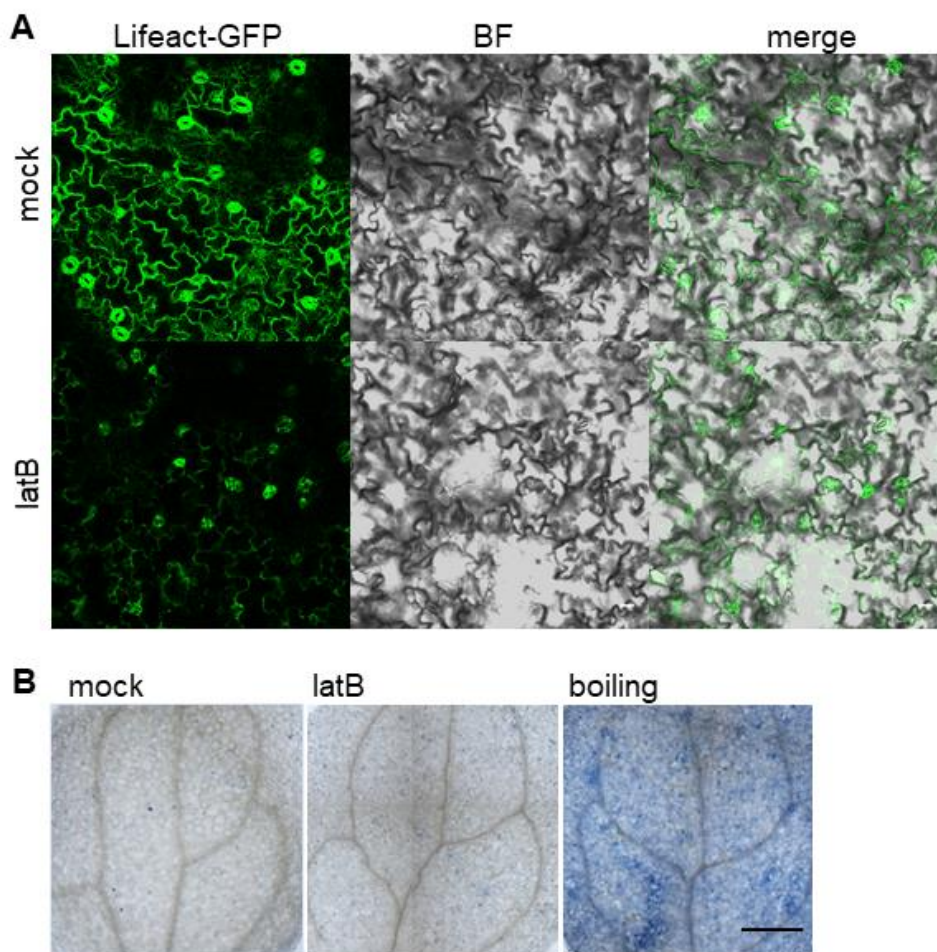
537 TK, MJ and ER - conceptualization; TK, OI, HL, PM, PK and RP - data curation; TK, HL, OI -
538 formal analysis; TK, HL, OI, DK, PM - methodology; LB, OV, JM - funding acquisition,
539 resources, project administration; TK, HL – writing, original draft; TK, MJ, LB, ER - review &
540 editing. All authors commented and approved the manuscript. Authors declare no conflict of
541 interests.

542

543

544 SUPPLEMENTARY DATA:

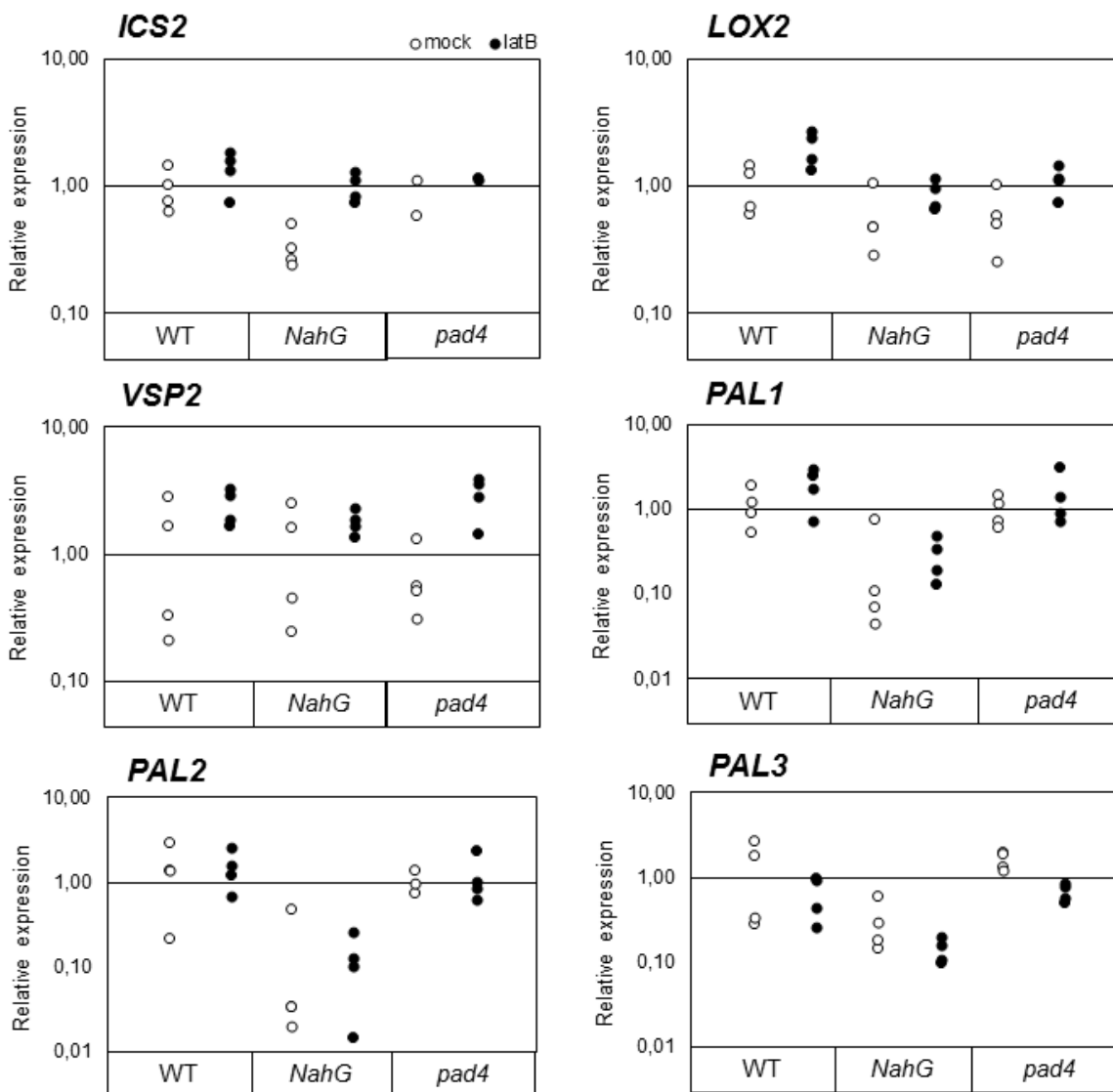
545 **Supplemental figure S1. LatB treatment did not cause cell death in seedlings.** A. Actin
546 depolymerization after latB treatment. Representative confocal images of abaxial epidermis of
547 10-days-old *A.thaliana* seedlings expressing Lifeact-GFP treated with for 24h with 0.05% DMSO
548 (mock) of 200nM latB. Confocal microscopy, scale bar = 20 μ m. B. Trypan blue staining of
549 seedlings. Boiling for 5min was used as a positive control for cell death; color camera, scale bar =
550 500 μ m.



551

552

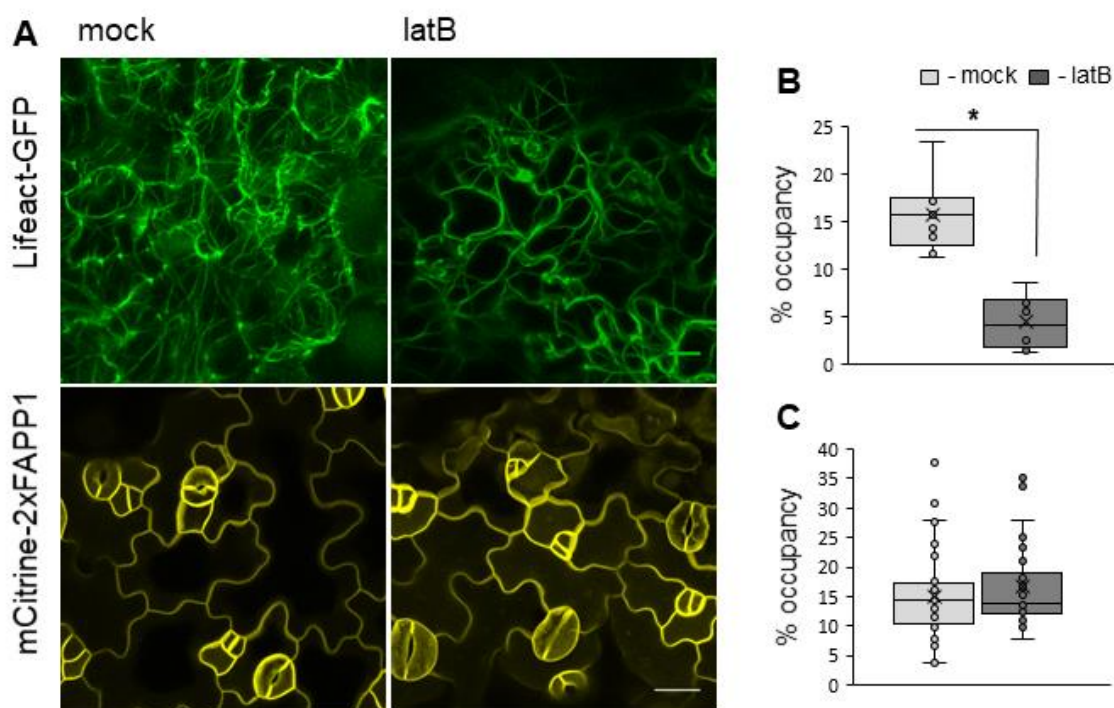
553 **Supplementary figure S2. Relative expression of SA biosynthesis and JA marker genes in**
 554 ***NahG* and *pad4* mutants.** Ten-days-old seedlings of WT, *NahG* and *pad4* were treated by 200
 555 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 24 hr after the treatment.
 556 Data are normalized to mock-treated WT, note that data are plotted at the log₁₀ scale. Asterisks
 557 indicate values, different from respective controls for each genotype, * - p<0,05; *** - p<0.001,
 558 two-tailed Student *t*-test). Reference gene *TIP41*.



559

560

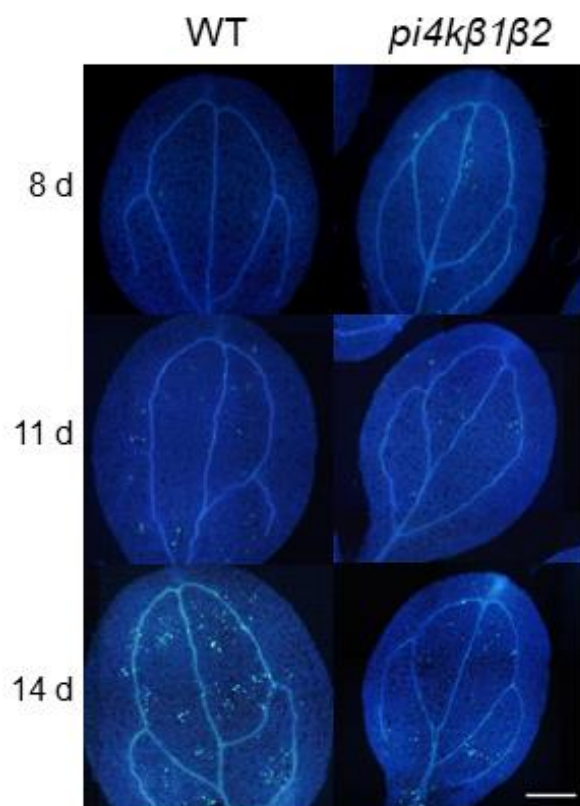
561 **Supplemental figure S3. LatB decrease actin filaments abundance while PI4P level remains**
562 **stable.** Ten-days-old seedlings were imaged 24h after replacing cultivation media with 0.05%
563 DMSO (Mock) of 200nM latrunculin B (latB). For each variant at least 10 individual seedlings
564 were observed. A. Representative images of the cotyledons of Lifeact-GFP and mCitrine-
565 2xFAPP1 expressing plants after latB treatment; confocal microscopy, scale bar 20 μ m.
566 Quantification of the actin filaments by lifeact-GFP signal intensity (B); PI4P levels by mCitrine-
567 2xFAPP1 fluorescent signal intensity (C). For quantification, maximum intensity projections of
568 10 μ m Z-stacks of abaxial sides of the leaves were used. At least 30 regions of interest (ROI)
569 measured for each treatment. In the plots, central line represents the median occupancy, cross
570 represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends
571 of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as
572 outliers. Asterisks indicate statistically significant differences with the mock treatment, Student *t*-
573 test, $p < 0,05$



574

575

576 **Supplemental figure S4.** Representative images of basal callose accumulation in WT and
577 *pi4kβ1β2* seedlings. Anilin blue staining, epifluorescent microscopy, scale bar 500μm.



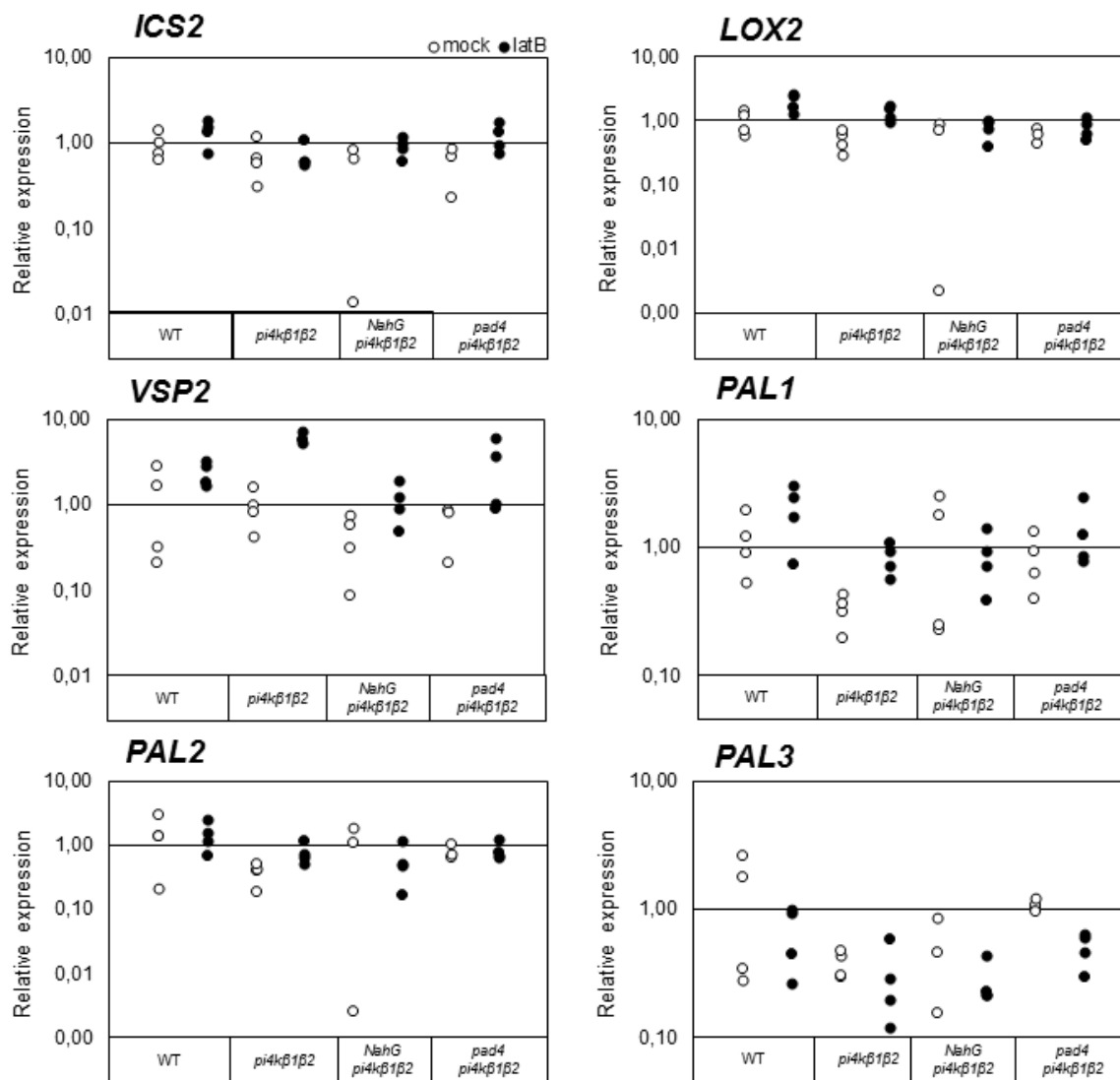
578

579

580 **Supplementary figure S5. Relative expression of SA biosynthesis and JA marker genes in**
 581 ***pi4kβ1β2* mutants.** Ten-days-old seedlings of WT, *pi4kβ1β2*, *NahGpi4kβ1β2* and *pad4pi4kβ1β2*
 582 were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 24 h
 583 after the treatment. Data are normalized to mock-treated WT, note that data are plotted at the log₁₀
 584 scale. Asterisks indicate values, different from respective controls for each genotype, * - p<0,05;
 585 *** - p<0.001, two-tailed Student *t*-test). Reference gene *TIP41*.

586

587



588

589

590

591 **Supplemental table ST1.** Primers used in this study

592

Gene	Accession number	Forward primer	Reverse primer
<i>TIP41</i>	AT4G34270	GTGAAAACCTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTTCGCA
<i>PR1</i>	AT2G14610	AGTTGTTTGGAGAAAGTCAG	GTTACATAATTCCCACGA
<i>PR2</i>	AT3G57260	TATAGC CAC TGA CAC CAC	GCCAAGAAACCTATCACTG
<i>ICS1</i>	At1g74710	GCAAGAATCATGTTCTCTACC	AATTATCCTGCTGTTACGAG
<i>ICS2</i>	AT1G18870	TGTCTTCAAAGTCTCTCTG	CTTCTCCAAACTCATCAAAC
<i>WRKY38</i>	At5g22570	GCCCCCTCCAAGAAAAGAAAG	CCTCCAAAGATACCCGTCGT
<i>LOX2</i>	AT3G45140	ATCCACCTCACTCATTACT	ATCCAACACGAACAATCTCT
<i>VSP2</i>	AT5G24770	CCAAACAGTACCAATACGAC	CTTCTCTGTTCCGTATCCAT
<i>PAL1</i>	AT2G37040	GTGTCGCACTTCAGAAGGAA	GGCTTGTTTCTTTTCGTGCTT
<i>PAL2</i>	AT3G53260	GTGCTACTTCTCACCGGAGA	TATCCGCGCTTCAAAAATC
<i>PAL3</i>	AT5G04230	CAACCAAACGCAACAGCA	CTCCAGGTGGCTCCCTTTTA
<i>PAD4</i>	AT3G52430	GGTTCTGTTCGTCTGATGTT	GTTCTCGGTGTTTTGAGTT
<i>BAP1</i>	At3g61190	ATGCCCATCAATGGTAATG	TCCCACACTTATCACCAA
<i>PDF1.2a</i>	AT5G44420	ACGCACCGGCAATGGTGGAA	TGCATGATCCATGTTTGGCT
<i>PDF1.2c</i>	AT5G44430	CTGCTACCATCATCACCTTC	CCGCAAACGCCTGACCATGT
<i>AOS</i>	AT5G42650	GGTCATCAAGTTCATAACCG	TTTCTCAATCGCTCCCAT
<i>ACS2</i>	At1g01480	GTAAAGCTCAATGTGTCTCC	AAGCAAATCCTAAACCATCC
<i>ERF1</i>	At3g23240	ATCAAATCCGTAAGCTCAAG	CCAAACCCTAATACCGTTTC
<i>LEA4-1</i>	AT1G32560	AGCAAAAGCTGATGAGAAGGCA	TAGGTCTGAGGAGGCACTGA
<i>ABI1</i>	AT4G26080	ATGTGCGAGATCCATTGGCGAT	TTCCTTTCTCCGCTCATCCG
<i>PMR4</i>	AT4G03550	ATGTACACATGTAAATGGCG	GAGGCTGGGTCTCGAGGAGTT

593

594

Supplemental table ST2. Effect of latB on defence gene expression.

	WT		<i>NahG</i>		<i>pad4</i>		<i>pi4k 6162</i>		<i>NahGpi4k6162</i>		<i>pad4pi4k6162</i>	
	mock	LatB	mock	LatB	mock	LatB	mock	LatB	mock	LatB	mock	LatB
<i>PdF1.2a</i>	1.03 ± 0.29	3.48 ± 0.64	2.22 ± 0.78	2.25 ± 0.32	1.84 ± 0.21	4.50 ± 0.70	1.72 ± 0.18	4.79 ± 0.92	3.49 ± 0.54	13.29 ± 2.34	4.67 ± 0.67	4.91 ± 0.96
<i>PdF1.2c</i>	1.17 ± 0.43	4.64 ± 1.05	1.82 ± 0.66	2.78 ± 0.37	2.15 ± 0.31	6.00 ± 1.05	1.73 ± 0.20	6.93 ± 1.55	2.34 ± 0.34	16.99 ± 5.05	4.15 ± 0.69	5.75 ± 1.25
<i>ACS2</i>	3.83 ± 2.15	2.20 ± 0.16	3.07 ± 0.33	4.97 ± 1.12	8.81 ± 1.30	42.83 ± 7.80	0.47 ± 0.18	2.13 ± 0.21	5.05 ± 0.87	10.43 ± 1.26	1.40 ± 0.12	10.30 ± 3.72
<i>AOS</i>	1.14 ± 0.39	6.07 ± 0.87	0.48 ± 0.15	1.27 ± 0.44	1.26 ± 0.67	2.05 ± 1.24	0.62 ± 0.14	2.15 ± 0.28	1.60 ± 0.76	5.08 ± 1.14	2.69 ± 0.92	5.82 ± 1.63
<i>ERF1</i>	2.05 ± 0.80	8.57 ± 0.55	1.09 ± 0.12	5.82 ± 0.55	2.86 ± 0.33	13.32 ± 1.90	0.82 ± 0.27	5.99 ± 0.68	2.51 ± 0.80	13.83 ± 0.92	3.13 ± 0.23	11.10 ± 1.91
<i>LEA4-1</i>	1.14 ± 0.32	1.35 ± 0.23	1.21 ± 0.24	0.87 ± 0.24	0.82 ± 0.15	1.29 ± 0.19	0.93 ± 0.12	0.97 ± 0.07	0.95 ± 0.22	1.25 ± 0.29	3.45 ± 2.07	0.92 ± 0.13
<i>ABI1</i>	0.98 ± 0.04	1.18 ± 0.03	0.89 ± 0.05	1.13 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.56 ± 0.02	0.79 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Supplemental table ST3. Effect of latB on salicylic acid content in seedlings. Seedlings were grown *in vitro* for 10 days and treated with 1 μ M latB for 24h. DMSO was used as mock treatment. SA content was measured by LC-MS. Means \pm SE from 8 values generated in two independent experiments are shown.

SA content (ng/ g FW)				
	DMSO		LatB	
WT	307,70	\pm 66,59	7247,39	\pm 793,85
<i>pmr4</i>	402,10	\pm 45,02	10179,89	\pm 756,35
<i>NahG</i>	64,11	\pm 6,30	451,38	\pm 51,03
<i>sid2</i>	54,30	\pm 6,84	54,84	\pm 3,32
<i>pad4</i>	102,97	\pm 20,15	788,84	\pm 158,57
<i>pi4k 6162</i>	159,56	\pm 26,04	6879,74	\pm 864,17
<i>NahGpi4k6162</i>	105,50	\pm 18,14	2286,92	\pm 222,89
<i>sid2pi4k6162</i>	60,94	\pm 9,76	62,75	\pm 10,80
<i>pad4pi4k6162</i>	237,77	\pm 92,15	1925,06	\pm 385,01

REFERENCES

1. Ali S, Ganai BA, Kamili AN, Bhat AA, Mir ZA, Bhat JA, Tyagi A, Islam ST, Mushtaq M, Yadav P, Rawat S, Grover A (2018) Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiol Res* **212-213**: 29-37
2. Andreeva Z, Barton D, Armour W, Li M, Liao L-F, McKellar H, Pethybridge K, Marc J (2010) Inhibition of phospholipase C disrupts cytoskeletal organization and gravitropic growth in *Arabidopsis* roots. *Planta* **232**: 1263-1279
3. Antignani V, Klocko AL, Bak G, Chandrasekaran SD, Dunivin T, Nielsen E (2015) Recruitment of PLANT U-BOX13 and the PI4K β 1/ β 2 Phosphatidylinositol-4 Kinases by the Small GTPase RabA4B Plays Important Roles during Salicylic Acid-Mediated Plant Defense Signaling in *Arabidopsis*. *The Plant Cell* **27**: 243-261
4. Badet T, Léger O, Barascud M, Voisin D, Sadon P, Vincent R, Le Ru A, Balagué C, Roby D, Raffaele S (2019) Expression polymorphism at the ARPC4 locus links the actin cytoskeleton with quantitative disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *New Phytol* **222**: 480-496
5. Baluška F, Jasik J, Edelman HG, Salajová T, Volkmann D (2001) Latrunculin B-Induced Plant Dwarfism: Plant Cell Elongation Is F-Actin-Dependent. *Dev Biol* **231**: 113-124
6. Bigeard J, Colcombet J, Hirt H (2015) Signaling Mechanisms in Pattern-Triggered Immunity (PTI). *Mol Plant* **8**: 521-539
7. Brill JA, Hime GR, Scharer-Schuksz M, Fuller MT (2000) A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. *Development* **127**: 3855-3864
8. Chen X-Y, Kim J-Y (2009) Callose synthesis in higher plants. *Plant Signaling Behav* **4**: 489-492
9. Chen X-Y, Liu L, Lee E, Han X, Rim Y, Chu H, Kim S-W, Sack F, Kim J-Y (2009) The *Arabidopsis* callose synthase gene *GSL8* is required for cytokinesis and cell patterning. *Plant Physiol* **150**: 105-113
10. Cui H, Gobbato E, Kracher B, Qiu J, Bautor J, Parker JE (2017) A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in *Arabidopsis* immunity. *New Phytol* **213**: 1802-1817
11. Cui W, Lee J-Y (2016) *Arabidopsis* callose synthases *CalS1/8* regulate plasmodesmal permeability during stress. *Nature Plants* **2**: 16034

12. Cvrčková F, Oulehlová D (2017) A new kymogram-based method reveals unexpected effects of marker protein expression and spatial anisotropy of cytoskeletal dynamics in plant cell cortex. *Plant Methods* **13**: 19
13. Delage E, Puyaubert J, Zachowski A, Ruelland E (2013) Signal transduction pathways involving phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate: Convergences and divergences among eukaryotic kingdoms. *Prog Lipid Res* **52**: 1-14
14. Delaney T, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. *Science* **266**: 1247 - 1250
15. Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, De Lorenzo G, Ferrari S, Ausubel FM, Dewdney J (2008) Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. *Mol Plant* **1**: 423-445
16. Drobak BK, Franklin-Tong VE, Staiger CJ (2004) The Role of the Actin Cytoskeleton in Plant Cell Signaling. *The New Phytologist* **163**: 13-30
17. Ebine K, Ueda T (2015) Roles of membrane trafficking in plant cell wall dynamics. *Front Plant Sci* **6**
18. Ellinger D, Glöckner A, Koch J, Naumann M, Stürtz V, Schütt K, Manisseri C, Somerville SC, Voigt CA (2014) Interaction of the Arabidopsis GTPase RabA4c with Its Effector PMR4 Results in Complete Penetration Resistance to Powdery Mildew. *The Plant Cell* **26**: 3185-3200
19. Fernández-Bautista N, Domínguez-Núñez JA, Moreno MMC, Berrocal-Lobo M (2016) Plant Tissue Trypan Blue Staining During Phytopathogen Infection. *Bio-protocol* **6**: e2078
20. Geitmann A, Nebenführ A (2015) Navigating the plant cell: intracellular transport logistics in the green kingdom. *Mol Biol Cell* **26**: 3373-3378
21. Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. *The Plant cell* **11**: 2349-2363
22. Hamamouch N, Li C, Seo PJ, Park C-M, Davis EL (2011) Expression of Arabidopsis pathogenesis-related genes during nematode infection. *Mol Plant Pathol* **12**: 355-364
23. Hardham AR (2007) Cell biology of plant–oomycete interactions. *Cell Microbiol* **9**: 31-39

24. Hedtmann C, Guo W, Reifschneider E, Heiber I, Hiltcher H, van Buer J, Barsch A, Niehaus K, Rowan B, Lortzing T, Steppuhn A, Baier M (2017) The Plant Immunity Regulating F-Box Protein CPR1 Supports Plastid Function in Absence of Pathogens. *Front Plant Sci* **8**
25. Henty-Ridilla JL, Shimono M, Li J, Chang JH, Day B, Staiger CJ (2013) The Plant Actin Cytoskeleton Responds to Signals from Microbe-Associated Molecular Patterns. *PLOS Pathogens* **9**: e1003290
26. Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis Callose Synthase, GSL5, Is Required for Wound and Papillary Callose Formation. *The Plant Cell* **15**: 2503-2513
27. Kalachova T, Janda M, Šašek V, Ortmannová J, Nováková P, Dobrev IP, Kravets V, Guivarc'h A, Moura D, Burketová L, Valentová O, Ruelland E (2019) Identification of salicylic acid-independent responses in an Arabidopsis phosphatidylinositol 4-kinase beta double mutant. *Annals of Botany*
28. Kang B-H, Nielsen E, Preuss ML, Mastronarde D, Staehelin LA (2011) Electron Tomography of RabA4b- and PI-4K β 1-Labeled Trans Golgi Network Compartments in Arabidopsis. *Traffic* **12**: 313-329
29. Keppler BD, Song J, Nyman J, Voigt CA, Bent AF (2018) 3-Aminobenzamide Blocks MAMP-Induced Callose Deposition Independently of Its Poly(ADPribose)ylation Inhibiting Activity. *Front Plant Sci* **9**: 1907-1907
30. Kulich I, Vojtíková Z, Sabol P, Ortmannová J, Neděla V, Tihlaříková E, Žárský V (2018) Exocyst Subunit EXO70H4 Has a Specific Role in Callose Synthase Secretion and Silica Accumulation. *Plant Physiol* **176**: 2040-2051
31. Leontovyčova H, Kalachova T, Trda L, Lamparova L, Pospichalova R, Dobrev P, Malinska K, Burketova L, Valentova O, Janda M (2019) Actin depolymerization is able to increase plant resistance against pathogens via activation of salicylic acid signalling pathway. *Scientific Reports* **in press**
32. Leung J, Merlot S, Giraudat J (1997) The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *The Plant cell* **9**: 759-771
33. Li J, Cao L, Staiger CJ (2017) Capping Protein Modulates Actin Remodeling in Response to Reactive Oxygen Species during Plant Innate Immunity. *Plant Physiol* **173**: 1125-1136

34. Li N, Han X, Feng D, Yuan D, Huang L-J (2019) Signaling Crosstalk between Salicylic Acid and Ethylene/Jasmonate in Plant Defense: Do We Understand What They Are Whispering? *International Journal of Molecular Sciences* **20**: 671
35. Lin F, Krishnamoorthy P, Schubert V, Hause G, Heilmann M, Heilmann I (2019) A dual role for cell plate-associated PI4K β in endocytosis and phragmoplast dynamics during plant somatic cytokinesis. *The EMBO Journal* **38**: e100303
36. Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant cell* **15**: 165-178
37. Lu Y-J, Day B (2017) Quantitative Evaluation of Plant Actin Cytoskeletal Organization During Immune Signaling. *Methods in molecular biology (Clifton, NJ)* **1578**: 207-221
38. Matoušková J, Janda M, Fišer R, Šašek V, Kocourková D, Burketová L, Dušková J, Martinec J, Valentová O (2014) Changes in actin dynamics are involved in salicylic acid signaling pathway. *Plant Sci* **223**: 36-44
39. Morton WM, Ayscough KR, McLaughlin PJ (2000) Latrunculin alters the actin-monomer subunit interface to prevent polymerization. *Nat Cell Biol* **2**: 376
40. Moscatelli A, Idilli AI, Rodighiero S, Caccianiga M (2012) Inhibition of actin polymerisation by low concentration Latrunculin B affects endocytosis and alters exocytosis in shank and tip of tobacco pollen tubes. *Plant Biol* **14**: 770-782
41. Nishimura MT, Stein M, Hou B-H, Vogel JP, Edwards H, Somerville SC (2003) Loss of a Callose Synthase Results in Salicylic Acid-Dependent Disease Resistance. *Science* **301**: 969
42. Oide S, Bejai S, Staal J, Guan N, Kaliff M, Dixelius C (2013) A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *New Phytol* **200**: 1187-1199
43. Pleskot R, Pejchar P, Staiger CJ, Potocký M (2014) When fat is not bad: the regulation of actin dynamics by phospholipid signaling molecules. *Front Plant Sci* **5**: 5-5
44. Pleskot R, Pejchar P, Žárský V, Staiger CJ, Potocký M (2012) Structural Insights into the Inhibition of Actin-Capping Protein by Interactions with Phosphatidic Acid and Phosphatidylinositol (4,5)-Bisphosphate. *PLOS Computational Biology* **8**: e1002765

45. Šašek V, Janda M, Delage E, Puyaubert J, Guivarc'h A, López Maseda E, Dobrev PI, Caius J, Bóka K, Valentová O, Burketová L, Zachowski A, Ruelland E (2014) Constitutive salicylic acid accumulation in pi4kIIIβ1β2 Arabidopsis plants stunts rosette but not root growth. *New Phytol* **203**: 805-816
46. Sassmann S, Rodrigues C, Milne SW, Nenninger A, Allwood E, Littlejohn GR, Talbot NJ, Soeller C, Davies B, Hussey PJ, Deeks MJ (2018) An Immune-Responsive Cytoskeletal-Plasma Membrane Feedback Loop in Plants. *Current biology : CB* **28**: 2136-2144.e2137
47. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. *Nat Meth* **9**: 676
48. Schneider R, Hanak T, Persson S, Voigt CA (2016) Cellulose and callose synthesis and organization in focus, what's new? *Curr Opin Plant Biol* **34**: 9-16
49. Shimono M, Lu Y-J, Porter K, Kvitko BH, Henty-Ridilla J, Creason A, He SY, Chang JH, Staiger CJ, Day B (2016) The Pseudomonas syringae Type III Effector HopG1 Induces Actin Remodeling to Promote Symptom Development and Susceptibility during Infection. *Plant Physiol* **171**: 2239-2255
50. Simon MLA, Platre MP, Assil S, van Wijk R, Chen WY, Chory J, Dreux M, Munnik T, Jaillais Y (2014) A multi-colour/multi-affinity marker set to visualize phosphoinositide dynamics in Arabidopsis. *Plant J* **77**: 322-337
51. Thiele K, Wanner G, Kindzierski V, Jürgens G, Mayer U, Pachel F, Assaad FF (2009) The timely deposition of callose is essential for cytokinesis in Arabidopsis. *Plant J* **58**: 13-26
52. Tsuda K, Mine A, Bethke G, Igarashi D, Botanga CJ, Tsuda Y, Glazebrook J, Sato M, Katagiri F (2013) Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in Arabidopsis thaliana. *PLoS genetics* **9**: e1004015-e1004015
53. Voigt CA (2014) Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Front Plant Sci* **5**: 168
54. Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**: 562-565
55. Yang H, Li Y, Hua J (2006) The C2 domain protein BAP1 negatively regulates defense responses in Arabidopsis. *Plant J* **48**: 238-248

56. Yang H, Yang S, Li Y, Hua J (2007) The Arabidopsis BAP1 and BAP2 genes are general inhibitors of programmed cell death. *Plant Physiol* **145**: 135-146
57. Závěská Drábková L, Honys D (2017) Evolutionary history of callose synthases in terrestrial plants with emphasis on proteins involved in male gametophyte development. *PLoS one* **12**: e0187331-e0187331
58. Zhang Y, He J, Lee D, McCormick S (2010) Interdependence of Endomembrane Trafficking and Actin Dynamics during Polarized Growth of Arabidopsis Pollen Tubes. *Plant Physiol* **152**: 2200-2210
59. Zheng X-y, Zhou M, Yoo H, Pruneda-Paz JL, Spivey NW, Kay SA, Dong X (2015) Spatial and temporal regulation of biosynthesis of the plant immune signal salicylic acid. *PNAS* **112**: 9166
60. Zhu Y, Yang H, Mang H-G, Hua J (2011) Induction of BAP1 by a moderate decrease in temperature is mediated by ICE1 in Arabidopsis. *Plant Physiol* **155**: 580-588