

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

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

Tetiana KALACHOVA<sup>1+\*</sup>, Hana LEONTOVYČOVÁ<sup>1,2,3+</sup>, Oksana IAKOVENKO<sup>1,2,4+</sup>, Romana
POSPÍCHALOVÁ<sup>1</sup>, Petr MARŠÍK<sup>5</sup>, Pavel KLOUČEK<sup>5</sup>, Martin JANDA<sup>1,2,6</sup>, Olga
VALENTOVÁ<sup>2</sup>, Daniela KOCOURKOVÁ<sup>1</sup>, Jan MARTINEC<sup>1</sup>, Lenka BURKETOVÁ<sup>1</sup>, Eric
RUELLAND<sup>6,7</sup>

- <sup>7</sup> <sup>1</sup>Institute of Experimental Botany, The Czech Academy of Sciences, Rozvojová 263, Prague 160
- 8 000, CZECH REPUBLIC
- <sup>9</sup> <sup>2</sup>University of Chemistry and Technology Prague, Department of Biochemistry and Microbiology,
- 10 Prague 166 28, CZECH REPUBLIC
- <sup>3</sup>Department of Biochemistry, Faculty of Science, Charles university, Hlavova 8, 128 43, CZECH
   REPUBLIC
- <sup>4</sup>V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, National Academy of
   Sciences of Ukraine, 1 Murmanska street, 02094 Kyiv, UKRAINE
- <sup>5</sup>Department of Food Quality and Safety, Faculty of Agrobiology, Food and Natural Resources,
- 16 Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, CZECH REPUBLIC
- <sup>6</sup>Ludwig-Maximilians-University of Munich (LMU), Faculty of Biology, Biocenter, Department
- 18 Genetics, Grosshaderner Str. 2-4, D-82152 Martinsried, GERMANY
- <sup>7</sup>CNRS, Institut d'Ecologie et des Sciences de l'Environnement de Paris, UMR 7618, Créteil,
  FRANCE
- <sup>8</sup>Université Paris-Est, UPEC, Institut d'Ecologie et des Sciences de l'Environnement de Paris,
- 22 Créteil, FRANCE

#### <sup>+</sup> equally contributed

## 24 \* corresponding author; <u>kalachova@ueb.cas.cz</u>

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27

## 28 ABSTRACT

29 Actin cytoskeleton is indispensable for plant cell integrity. Besides, increasing evidences illustrate its crucial role in plant responses to environment, including defence against pathogens. Recently, 30 we have demonstrated that pre-treatment with actin disrupting drugs latrunculin B (latB) and 31 cytochalasin E can enhance plant resistance against bacterial and fungal pathogens via activation 32 of salicylic acid (SA) pathway. Here, we show that actin depolymerization in Arabidopsis thaliana 33 seedlings not only triggers SA biosynthesis by ICS1, but also induces callose deposition via callose 34 35 synthase PMR4. This effect is SA-independent since still present in mutants that do not accumulate SA. LatB also triggers the expression of several defence related genes. We could distinguish genes, 36 37 induced in a SA-dependent manner (PR1, WRKY38, ICS1) and those that are SA-independent (PR2, PAD4, BAP1). As actin cytoskeleton is tightly connected with membrane trafficking, we 38 39 assayed the effect of latB on mutant plants invalidated in phosphatidylinositol 4-kinase beta1 and beta2 (PI4K $\beta$ 1 $\beta$ 2). Deficiency in PI4K $\beta$ 1 $\beta$ 2 enhanced latB-triggered actin filaments 40 41 depolymerisation. Yet, it did not lead to a stronger callose deposition or SA biosynthesis in response to latB. Surprisingly, introduction of NahG construct or pad4 mutation in  $pi4k\beta l\beta 2$ 42 43 background had much lower effect on SA induction and SA-dependent gene expression changes than it has in wild type. We can thus conclude that actin disruption itself triggers immune-like 44 responses: there is an induction of SA via PAD4 coupled to ICS1; it leads to the induction of PR1 45 and WRKY38, and this requires a functional PI4K $\beta$ 1 $\beta$ 2 to be properly regulated. However, an 46 alternative, SA-independent, pathway also exists that leads to the enhanced expression of PR2 and 47 48 to callose accumulation.

## 50 INTRODUCTION

51 Plants are always confronted to changes in their environment. This can concern physical parameters such as light intensity and/or quality, temperature or water availability, or interactions 52 with other organisms, including bacteria or fungi. Some of the interactions can lead to diseases. 53 Nowadays, plant responses to the environmental challenges, either abiotic or biotic, are an object 54 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 depolymerize at the opposite one (Li et al, 2017). The filaments are indispensable for cell shaping, 60 61 growth and polarity (Andreeva et al, 2010), cytokinesis (Brill et al, 2000), but also for intracellular trafficking of vesicles (Geitmann & Nebenführ, 2015). Yet, the direct mechanisms regulating actin 62 63 dynamics in regards to vesicle trafficking are still waiting to be revealed. A particular role has been proposed for phospholipids, especially phosphoinositides (Ebine & Ueda, 2015). 64 Phosphoinositides are phosphorylated forms of phosphatidylinositol (PI). From the position and 65 number of the phosphate groups on the inositol ring, we can distinguish PI-3-phosphate (PI3P), 66 67 PI-4-phosphate (PI4P) and PI-4,5-bisphosphate (PIP<sub>2</sub>). Phosphoinositides play a key role in vesicle trafficking (Delage et al, 2013) and also interact with cytoskeleton: for instance, F-actin capping 68 protein interaction with actin monomers is regulated by PIP<sub>2</sub> (Pleskot et al, 2012). This makes 69 phosphoinositides potential central players in the regulation of cytoskeleton dynamics versus 70 vesicle trafficking. Phosphatidylinositol-4 kinases (PI4Ks) are the first enzymes committing PI 71 into phosphoinositides phosphorylated at position 4 of inositol. The study of these enzymes and of 72 73 their corresponding mutants can be useful to investigate the regulation of the vesicle trafficking together with cytoskeleton dynamics. In Arabidopsis thaliana, the isoforms AtPI4KB1 and 74 75 AtPI4Kβ2 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 plate (Lin et al, 2019). 77

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

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

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

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

117 We firstly show that actin disruption by latB triggers callose accumulation in A. thaliana seedlings through the callose synthase PMR4. Using mutants altered in SA signaling (pad4), 118 119 biosynthesis (sid2) and accumulation (NahG), we demonstrate that this callose deposition is SAindependent. Furthermore, we found that latB effect on gene expression does not correspond to a 120 121 general transcriptome deregulation, but that specific pathways related to the response to pathogens are the ones triggered by latB. Amongst the gene responses, we were also able to reveal SA-122 123 dependent and SA-independent effects of latB. Interestingly, we show that deficiency in PI4K $\beta$ 1 $\beta$ 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 $\beta$ 1 $\beta$ 2 in SA-independent responses to actin disruption, we have introduced NahG construct as well as sid2 and pad4 126 mutations into  $pi4k\beta l\beta 2$  background. Surprisingly, the latB treatment still strongly induced SA in 127  $pad4pi4k\beta1\beta2$  and  $NahGpi4k\beta1\beta2$  triple mutants, while SA accumulation was prevented in pad4 128 129 and *NahG* mutant plants. To conclude, here we characterize the immunity-like responses to latB, showing that actin depolymerisation acts through both SA-dependent and SA-independent 130 pathways. Moreover, we reveal the existence of an unexpected cross-talk between actin filaments 131 and phosphoinositides in the regulation of those defence-related responses. 132

133

## 134 Materials and methods

135 Plant material

The following *Arabidopsis thaliana* genotypes were used: Columbia-0 (WT); *NahG* (Delaney et
al, 1994); *NahGpi4kβ1β2* (Šašek et al, 2014); pad4.1 (NASC stock N3806); pad4pi4kβ1β2 (this
study); sid2, pi4kβ1β2, sid2pi4kβ1β2 (Kalachova et al, 2019); pmr4; pUBQ::PMR4-GFP in WT
background; pUBQ::PMR4-GFP in pmr4 background (Kulich et al, 2018); Lifeact-GFP
(Cvrčková & Oulehlová, 2017); pUBQ::mCitrine-2xFAPP1 (Simon et al, 2014).

#### 141 *Plant cultivation and treatment*

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

Latrunculin B (latB, Sigma-Aldrich, USA) was dissolved in DMSO at 2 mM stock solution. The 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 (approximatively 300 µg fresh weight) from three wells were immediately frozen in liquid nitrogen. The tissue was homogenized in plastic Eppendorf tubes with 1 g of 1.3 152 153 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<sup>-</sup> Point Mutant reverse transcriptase (Promega Corp., USA) and an anchored oligo dT21 primer 156 157 (Metabion, Germany). Gene expression was quantified by qPCR using a LightCycler 480 SYBR Green I Master kit and LightCycler 480 (Roche, Switzerland). The PCR conditions were 95 °C for 158 159 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 analysis was then conducted. Relative expression was normalized to the housekeeping gene 160 AtTIP41. A list of the analysed genes and primers is available in Supplemental table ST1. 161

#### 162 *Trypan blue and anilin blue staining*

Seedlings were immersed into the staining solution (10 mL lactic acid (85%, w:w), 10 mL phenol, 10 mL glycerol, 10 mL distilled H<sub>2</sub>O, 40 mg trypan blue) for 2 min (Fernández-Bautista et al, 2016). Seedlings were then washed twice in ethanol:glacial acetic acid (1:3, v:v) until leaves were fully decoloured. Seedlings were rehydrated by incubating in ethanol solutions of decreasing concentrations (70%, 50%, 30%, v:v) for at least 30 min each. They were then stained in 0.01 % aniline blue in 150 mM K<sub>2</sub>HPO<sub>4</sub>, 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  $\mu$ m) was taken from one cotyledon, at 174 least 18 independent cotyledons were analysed per variant.

175

## 176 *Confocal microscopy*

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

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 instrument (MP Biomedicals, CA, United States) with 2 x 500 µl of extraction reagent 190 methanol/water/formic acid (15:4:1, v:v:v) supplemented with stable isotope-labelled 13C-SA 191 192 internal standards, each at 100 ng per sample, to check the recovery during the purification and to validate the quantification. Extract was subjected to solid phase extraction using Oasis MCX 193 cartridges (Waters Co., Milford, MA, United States) and eluted with methanol. The eluate was 194 evaporated to dryness and dissolved in 30 µl of 15% acetonitrile/water (v/v) immediately before 195 the analysis. immediately before the analysis. Quantification was performed on an Ultimate 3000 196

high-performance liquid chromatograph (UHPLC, Dionex; Thermo Fisher Scientific, Waltham, 197 MA, United States) coupled to IMPACT II Q-TOF ultra-high resolution and high mass accuracy 198 mass spectrometer (HRAM-MS; Bruker Daltonik, Bremen, Germany). Separation was carried out 199 200 using column Acclaim RSLC 120 C18 (2.2 µm, 2.1x100 mm; Thermo Fisher Scientific, Waltham, MA, United States) tempered at 30°C and mobile phase consisting of 0.1% formic acid (A) and 201 202 methanol (B) by gradient elution. The gradient started at 1%B and going to 39 % B in 3min, 60 % B in 7 min, 100 % B in 8 min, followed by 3 min of washing by 100% B and 5 min equilibration 203 204 at initial conditions (1%B). Flow rate was changing during separation from 200 µl/min to 300 µl/min in 7 min and then kept at this rate to 16 min. Injection volume was 5 µL. The full-scan data 205 were recorded in negative electrospray ionization (ESI) mode and processed using Data Analysis 206 207 4.3 and TASQ 1.4 software (both Bruker Daltonik, Bremen, Germany). SA and labelled internal 208 standard (<sup>13</sup>C<sub>6</sub>-SA) were monitored as ions of their deprotonated molecules [M-H]- (137.0239 m/z and 143.0440 m/z, respectively). SA content was expressed in ng/g fresh weight (FW). 209

210

#### 211 RESULTS

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

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



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

230*TIP41* was used as the reference gene. D. Salicylic acid content. \*\*\* - variants are significantly231different, two-tailed *t*-test, p<0.001, n=8.

232

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

241

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

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



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

270

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

We had already shown that latB could induce the expression of some genes related to plant 272 defence (Leontovyčova et al. 2019; Matoušková et al. 2014). Here we studied whether intracellular 273 274 level of SA or an intact SA-signaling pathway is needed for latB to trigger gene expression changes (figure 3, Supplementary figure S2, Supplementary table ST2). To do so, we analysed the 275 expression of several defence marker genes in NahG and pad4 mutants. Eleven-day-old in vitro 276 277 grown plants (whole seedlings) of WT and mutant genotypes were harvested for gene expression studies 24 hpt by 200nM latB. Concerning the gene response in WT plants, we show here that the 278 279 defence-associated PR1, PR2, WRKY38, ICS1 and PAD4 were induced by latB (Figure 3). We also detected an activation of the wounding marker BAP1, even though no cell death was yet detected 280 by trypan blue staining (Supplemental figure S1). On the contrary, no significant changes were 281 observed in the expression of SA biosynthetic genes other than ICS1 (ICS2, PAL1, PAL2, PAL3). 282 283 To be noted, ICS2 expression seems to be very dependent on the growth conditions, as we previously detected its induction after latB treatment (Leontovyčova et al, 2019). In addition, 284 285 neither jasmonate biosynthesis marker genes (LOX2, AOS1), nor jasmonate responsive genes (VSP2 and PDF1.2), nor ethylene marker gene (ERF1) were significantly affected by the treatment 286 287 (Supplemental figure S2, Supplemental Table ST2).

288



Figure 3. SA-independent expression of defence-related genes by latB treatment. Ten-dayold seedlings of WT, *NahG* and *pad4* were treated by 200 nM latB or 0.01 % DMSO as the control.
Seedlings were harvested 24 hr after the treatment. Data from the representative experiment are
shown. Values were normalized for mock treated WT, note that data are plotted at the log<sub>10</sub> scale.
Asterisks indicate values, different from respective controls for each genotype, \* - p<0,05; \*\*\* -</li>
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). This also involves that SA is accumulated in response to latB (Fig.2 A). On the contrary, the 298 299 expression of *PR2* in response to latB appears not to be dependent on SA since it was still present in NahG and pad4 mutants. Similarly, the expression of the PAD4 by latB was still present in 300 301 *NahG* plants: it does not rely on SA accumulation. The expression of *ICS1* in response to latB does not appear to be controlled by SA level in the tissues, as seen in *NahG* mutant. Yet, PAD4 protein 302 303 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.

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

## 310 *Effects of latB on cytoskeleton are enhanced in pi4k* $\beta$ 1 $\beta$ 2 *double mutant*

Phosphoinositides, that are the phosphorylated forms of phosphatidylinositol (PI), are 311 312 involved in vesicular trafficking. This process requires correct actin cytoskeleton organization (Pleskot et al, 2014). There is therefore a link between actin cytoskeleton and phosphoinositides. 313 We wanted to investigate whether the effect of latB could be impacted by a modification in 314 phosphoinositide metabolism. To do so, we used the  $pi4k\beta l\beta 2$  double mutant. This mutant is 315 316 invalidated in the two beta isoforms of phosphatidylinositol-4-kinases. This double mutant was shown to have impaired vesicle trafficking, dwarf phenotype and increased SA level (Kang et al, 317 2011; Šašek et al, 2014). 318

We firstly introduced LifeAct-GFP construct into  $pi4k\beta l\beta 2$  background by crossing. Four independent lines were tested. No major defects in actin filaments organization in epidermal cells were observed while compared to WT (fig.4). After 24h of latB treatment, the density of the filaments in  $pi4k\beta1\beta2$  plants was 20-times lower than that in non-treated double mutant, while in the WT background this decrease was 5-fold. Consequently, in  $pi4k\beta1\beta2$  plants the actin filaments were almost lost while in WT their occupancy was still 7% (fig 4B). The actin cytoskeleton of  $pi4k\beta1\beta2$  mutants thus appear to be more sensitive to latB. Meanwhile, we wanted to evaluate the impact of latB directly on the PI4P content using genetically encoded biosensor mCitrine-2xFAPP1 for PI4P (Simon et al., 2014). The sensor mostly labels PI4P localized in plasma membrane. In our setup, the content of PI4P remained stable in response to latB (fig.S3 A, C).



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Figure 4. Effect of latB on actin filaments organization of  $pi4k\beta 1\beta 2$  mutants. A. Representative 330 images of maximum intensity projections of 10µm abaxial epidermis of LifeAct-GFP expressing 331 WT and  $pi4k\beta l\beta 2$  plants. Confocal microscopy, scale bar = 20µm. Quantification of relative area 332 occupied with fluorescent filament in WT (B) and  $pi4k\beta 1\beta 2$  (C). For each variant at least 15 333 independent seedlings were imaged, n=12. For quantification, maximum intensity projections of 334 10µm Z-stacks of abaxial sides of the leaves were used. At least 30 regions of interest (ROI) 335 336 measured for each treatment. In the plots, central line represents the median occupancy, cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the ends 337 of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown as 338 outliers. Asterisks indicate statistically significant differences with the mock treatment. Student t-339 test, p<0,05 340



Because it seems there was a positive (enhancing) interaction between latB and PI4Kbeta 343 mutations on actin filaments, we studied SA levels, callose accumulation and gene expression 344 responses to latB in  $pi4k\beta1\beta2$  plants. Indeed, latB triggered SA increase in  $pi4k\beta1\beta2$  plants (fig. 345 5A). Surprisingly, introduction of neither NahG construct nor pad4 mutation into  $pi4k\beta l\beta 2$ 346 background did not fully prevent SA increase as it did in WT background (fig.2A), even though 347 such an increase was still much lower than in WT or  $pi4k\beta l\beta 2$ . However, sid2 mutation was 348 sufficient to block SA production in both WT and  $pi4k\beta l\beta 2$ , confirming that the entire SA pool 349 350 induced by latB treatment is synthetized due to ICS1 activity in both backgrounds. As for callose, its basal levels in 8-, 10- and 14-day-old  $pi4k\beta 1\beta 2$  seedlings were similar to that of WT 351 (Supplemental figure S4). To study the involvement of SA, the callose accumulation was also 352 tested in triple mutants NahGpi4k $\beta$ 1 $\beta$ 2 (Šašek et al, 2014), sid2pi4k $\beta$ 1 $\beta$ 2 (Kalachova et al, 2019) 353 354 and  $pad4pi4k\beta1\beta2$ , obtained by crossing  $pi4k\beta1\beta2$  to pad4 (homozygous F3 plants were used). After latB treatment, accumulation of callose in all studied mutants was similar to that in WT (fig. 355 5B), suggesting that callose response to latB in  $pi4k\beta l\beta 2$  is still not SA-dependent (fig. 5B). This 356 is consistent with our findings in WT plants. 357



Figure 5. LatB induced SA and callose accumulation in  $pi4k\beta1\beta2$  mutants. Ten-days-old 359 seedlings were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 360 24 hr after the treatment. A. SA content. Data from two independent experiments are plotted. B. 361 Relative area of callose particles in the cotyledons. At least 30 independent cotyledons were 362 analysed for each variant. Experiment was repeated three times with similar results, the data from 363 a representative repetition are shown. In boxplots, central line represents the median occupancy, 364 cross represents the mean, bottom and top edges of the box are 25 and 75% of distribution and the 365 ends of whiskers are set at 1.5 times the interquartile range. Values outside this range are shown 366 as outliers. Different letters mark significantly different values, p<0.05; Student *t*-test, unequal 367 variance. 368

369

## **Effects of latrunculin B on defence genes expression in** $pi4k\beta 1\beta 2$ double mutant

As for *PR1* and *WRKY38*, after latB treatment, their induction in  $pi4k\beta1\beta2$  was comparable to that in WT. Yet, contrary to what was observed in WT background, their induction was also observed in *NahGpi4k\beta1\beta2* and *pad4pi4k\beta1\beta2* triple mutants (fig. 6). This is in agreement with the fact that SA is still accumulated in these triple mutants. *BAP1* expression in PI4K deficient mutants after latB treatment was induced less than in WT and was partly SA-dependent (fig. 6). Besides, neither induction of SA biosynthetic enzymes other than *ICS1* was detected, nor of JA-, ABA-, or ethylene markers (Supplemental figure S5, supplemental table ST2).



<sup>378</sup> 

**Figure 6. LatB-induced gene expression in**  $pi4k\beta l\beta 2$  mutants. Ten-days-old seedlings of WT, *pi4k\beta l\beta 2, NahGpi4k\beta l\beta 2* and *pad4pi4k\beta l\beta 2* were treated by 200 nM latB or 0.01\% DMSO as mock control. Seedlings were harvested 24 hr after the treatment. Data are normalized to mocktreated WT, note that data are plotted at the log\_10 scale. Asterisks indicate values, different from

respective controls for each genotype, \* - p<0.05; \*\*\* - p<0.001, two-tailed Student *t*-test). Reference gene *TIP41*.

385

#### 386 DISCUSSION

387 We show here that latrunculin B induces callose accumulation in seedlings. It is also true for cytochalasin E, another actin filament disruptor (data not shown). Therefore, it is very likely that 388 389 this effect of latB is due to actin depolymerization. Callose deposition was reported in responses to various stresses, like wounding or pathogen infection (Jacobs et al, 2003). We had previously 390 391 shown that chemical disruption of actin led to some immunity-related responses such as activation of PR1 expression (Matouskova et al., 2014). In this regard, callose is particularly important, as 392 393 another biotic-stress-like response, partly connected with SA. Arabidopsis genome encodes twelve callose synthases (Voigt, 2014). PMR4 (POWLDERY MILDEW RESISTANT 4, also called 394 395 GLUCAN SYNTHASE LIKE 5, GLS5 or CALS12) is precisely the one activated during infection (Nishimura et al, 2003). Here we first show that callose accumulation induced by latB fully relies 396 397 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 downstream of PAD4. To be noted, *pmr4* mutant itself was reported to have a constitutively 399 activated SA pathway in adult plants and contribute to most of callose accumulations in studies 400 connected with biotics stres (Nishimura et al., 2003). Nevertheless, SA level in pmr4 seedlings did 401 not differ from that in WT (Supplementary table ST3). It was also true for  $pi4k\beta l\beta 2$  plants, known 402 as SA and callose accumulators while being grown up to 4-weeks (Kalachova et al, 2019). 403 404 However, neither SA nor callose levels were increased in non-stimulated seedlings (figure 5). A negative interaction between actin polymerisation, callose and SA levels has been suggested 405 recently: natural polymorphism of genetic disruption of Actin Related Protein Complex 4 406 407 (ARPC4) affected pathogen- and wounding-induced callose deposition, as well as affected expression of *PR1* in response to *Sclerotinia sclerotiorum* (Badet et al, 2019). 408

As latB action on callose appeared as SA-independent, we wanted to check whether the effect of latB on gene expression was also independent on SA. We first better detailed the changes in gene expression triggered by latB. We show here that *PR1*, *PR2*, *WRKY38*, *ICS1* and *PAD4* were induced by latB in WT. It is also the case for a marker of cell death in pathogen and wounding responses, *BAP1* (Yang et al, 2007). *BAP1* was described as wounding marker, but also as a SA-

associated gene: it negatively regulates cell death in response to *Pseudomonas syringae* pv tomato 414 and Hyaloperonospora parasitica acting in the same pathway with BON1, SNC1 and PAD4 (Yang 415 et al, 2006) BAP1 expression can be induced by high temperature or ROS formation, however this 416 417 induction was abolished in mutants with altered SA pathways (NahG, pad4) (Hedtmann et al, 2017; Zhu et al, 2011). In our setup, the expression of BAP1 was increased independently of the 418 419 SA content in plants, indicating a new role of this gene in plant stress responses. Yet, no significant changes were observed in the expression of SA biosynthetic genes other than ICS1 (ICS2, PAL1, 420 421 PAL2, PAL3), nor in the expression of LOX2 and AOS, a JA-biosynthesis marker genes, nor to the jasmonate responsive genes VSP2 and PDF1.2. Genes involved in ethylene biosynthesis (ACS2) 422 (Denoux et al, 2008), ethylene response (ERF1, LEA4-1)(Lorenzo et al, 2003) or to the response 423 of abscisic acid (ABI1)(Leung et al, 1997) were not induced by latB. Therefore, it seems that latB 424 425 action is not "universal", that it does not lead to a deregulation of the whole transcriptome. Its action seems to be directed mainly towards the SA signalling pathway or toward response to 426 427 biotrophic pathogens.

Clearly, a SA dependent pathway is activated by latB. Expression of *PR1*, a classical 428 representative of defence genes, is usually induced during pathogen attack, but also as a reaction 429 to any SA increase. Besides, *ICS1*, that is responsible for most of the SA accumulation in response 430 431 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 responsive genes, occur through a SA-dependent pathway. We could show that the expression of 433 *PR1* and *WRKY38* by latB relied on SA accumulation and a functional PAD4 protein. The fact that 434 ICS1 expression is controlled by PAD4 in response to pathogen was established (Cui et al., 2017). 435 Yet, the expression of *PR2* and *ICS1* is independent on SA, as seen in the *NahGpi4k* $\beta$ 1 $\beta$ 2. *PR2* is 436 one of the early defence genes, usually co-expressed with PR1 and PR5 and used as a marker of 437 SA pathway (Hamamouch et al, 2011). Nevertheless, here we describe its clear SA-independent 438 induction. It might seem contradictory, unless we take into account that PR2 protein is  $\beta$ -1,3-439 440 glucanase, particularly important for antifungal defences (Ali et al, 2018). PR2 is crucial for callose degradation and contributes to the SA increase; its overexpression enhances resistance both to 441 fungal and bacterial pathogens (Oide et al, 2013). 442

It thus appears that latB (likely through its action on actin filaments) activates a signaling pathway typical of the response to pathogens. It activates PAD4. Some of the PAD4 action leads to *ICS1* expression, to SA accumulation and to the expression of SA responsive genes. Yet, a part of PAD4 action is not dependent on SA, as seen by *ICS1* expression. LatB also triggers a pathway 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 filaments are involved in the control of vesicle trafficking. Phosphoinositides are also involved in 449 450 vesicular trafficking. There is indeed an interaction/interplay between actin filaments and phosphoinositides (Pleskot et al, 2014). F-actin capping protein interaction with actin monomers 451 452 is regulated by phosphatidic acid and phosphatidylinositol-4,5-bisphosphate, a phosphoinositide (Pleskot et al, 2012). In pollen tube, cytoskeletal disruption by latB or jasplakinolide not only 453 454 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 $\beta$ 1 and PI4K $\beta$ 2 were 456 shown to be recruited by RabA4b. Due to this interaction between actin and phosphoinositides in relation to vesicle trafficking, we were interested to investigate whether the action of latB could 457 458 be impacted by alteration in phosphoinositide metabolism. To address this,  $pi4k\beta l\beta 2$  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\beta1 and PI4K\beta2 were shown to be recruited by RabA4b. PI4Kbeta proteins participate in cytokinesis in Arabidopsis roots by 461 targeting actin filaments to the cell plate (Lin et al, 2019). Nevertheless, it is important to consider 462 the fact that  $pi4k\beta 1\beta 2$  plants exhibit constitutive activation of SA-related genes and high basal 463 levels of callose. Yet it is true for mature 4 week-old soil-grown plants (Antignani et al, 2015; 464 Kalachova et al, 2019; Šašek et al, 2014). In younger plants, no PR1 overexpression was detected 465 (Sasek et al., 2014). In the present study we used 10-day-old seedlings grown in vitro. In these 466 conditions, neither SA accumulation (fig.5 A, Supplementary table ST3), nor constitutively high 467 expression of *PR1* and *ICS1* was observed (fig. 6). 468

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

accumulated callose, in a SA- and PAD4- independent way, as observed in WT plants. Yet, when 473 we assay the SA increase in response to latB, a process not observed in WT is then detected: the 474 SA induction in  $pi4k\beta1\beta2$  background still occurs, despite the mutation in PAD4 or expression of 475 476 NahG. The  $pi4k\beta l\beta 2$  double mutation allows to reveal a signalling pathway, triggered here by latB, that leads to SA biosynthesis and PR1 expression independently on PAD4. Yet, the PR1 477 expression in response to latB was not only observed in NahGpi4k $\beta 1\beta 2$  and pad4pi4k $\beta 1\beta 2$  triple 478 mutants, but also in  $sid2pi4k\beta1\beta2$  (data not shown). Therefore, our data on the existence of such a 479 480 SA independent pathway are strong. Is this pathway usually actively inhibited by functional PI4Ks? Is it linked to the effect of  $pi4k\beta l\beta 2$  double mutation on trafficking? Or is it due to a role 481 of phosphoinositide on signalling? An important contribution may come from immune-related 482 MAPKs, whose transient or sustained activation works as a switch between pattern-triggered 483 484 immunity and effector triggered immunity (Tsuda et al, 2013). Indeed, Tsuda et al. described expression of *PR1* in *sid2* background, induced by constitutive activation of MPK3 and MPK6. 485 486 Another enzyme of the cascade, MPK4, was shown to interact with PI4K $\beta$ 1 during cell plate formation in cytokinesis (Lin et al, 2019). More research is necessary to understand and 487 488 characterize this pathway.

To summarize, we here report the defence-like consequences of actin cytoskeleton 489 490 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 callose accumulation through PMR4. The second pathway involves SA synthesis via PAD4 and 492 ICS1; it leads to the expression of genes such as PR1 and WRKY38. The double mutation in 493 PI4KB1B2 makes the effect of latB on filaments more pronounced. Interestingly, the SA increase 494 and *PR1* expression in response to latB in the  $pi4k\beta l\beta 2$  background is also triggered in an 495 unconventional, PAD4-independent pathway. Either this pathway is actively inhibited in WT 496 497 background by PI4KB1B2 (and thus not seen), or it is activated only when the PI4KB1B2 are 498 missing.

499



## 501

Figure 7. Graphical summary of the latrunculin B effects in seedlings. The disruption of actin 502 polymers by latB activates two kinds of signalling pathways. A first pathway is independent on 503 SA; it leads to the expression of genes such as PR2, but also to callose accumulation. The second 504 pathway involves SA synthesis via PAD4 and ICS1; it leads to the expression of genes such as 505 506 *PR1* and *WRKY38*. The double mutation in PI4K beta1 and beta2 makes the effect of latB on filaments more pronounced. Interestingly, the *PR1* expression in response to latB is no longer SA-507 dependent in the  $pi4k\beta l\beta 2$  background: a SA independent pathway leading to PR1 expression is 508 thus revealed. 509

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

altered due to lack of good filaments? Such a process could be a modified vesicle trafficking due

the missing actin filaments. In this regard, it would be interesting to monitor the processing of

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

trafficking and immunity at the cellular and subcellular level and thus will contribute to better

- 517 understanding of plant responses to changing environment.
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- 520

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535

## 536 AUTHOR CONTRIBUTION

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

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## 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 =  $20\mu$ 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  $500\mu$ m.





551

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 log<sub>10</sub> 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*.



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



**Supplemental figure S4.** Representative images of basal callose accumulation in WT and  $pi4k\beta l\beta 2$  seedlings. Anilin blue staining, epifluorescent microscopy, scale bar 500µm.



Supplementary figure S5. Relative expression of SA biosynthesis and JA marker genes in pi4k\beta1\beta2 mutants. Ten-days-old seedlings of WT, pi4k\beta1\beta2, NahGpi4k\beta1\beta2 and pad4pi4k\beta1\beta2 were treated by 200 nM latB or 0.01% DMSO as mock control. Seedlings were harvested 24 h after the treatment. Data are normalized to mock-treated WT, note that data are plotted at the log<sub>10</sub> scale. Asterisks indicate values, different from respective controls for each genotype, \* - p<0,05; \*\*\* - p<0.001, two-tailed Student *t*-test). Reference gene *TIP41*. 



Gene	Accession number	Forward primer	Reverse primer				
TIP41	AT4G34270	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA				
<b>PR1</b>	AT2G14610	AGTTGTTTGGAGAAAGTCAG	GTTCACATAATTCCCACGA				
PR2	AT3G57260	TATAGC CAC TGA CAC CAC	GCCAAGAAACCTATCACTG				
ICS1	At1g74710	GCAAGAATCATGTTCCTACC	AATTATCCTGCTGTTACGAG				
ICS2	AT1G18870	TGTCTTCAAAGTCTCCTCTG	CTTCCTCCAAACTCATCAAAC				
WRKY38	At5g22570	GCCCCTCCAAGAAAAGAAAG	CCTCCAAAGATACCCGTCGT				
LOX2	AT3G45140	ATCCCACCTCACTCATTACT	ATCCAACACGAACAATCTCT				
VSP2	AT5G24770	CCAAACAGTACCAATACGAC	CTTCTCTGTTCCGTATCCAT				
PAL1	AT2G37040	GTGTCGCACTTCAGAAGGAA	GGCTTGTTTCTTTCGTGCTT				
PAL2	AT3G53260	GTGCTACTTCTCACCGGAGA	TATTCCGGCGTTCAAAAATC				
PAL3	AT5G04230 CAACCAAACGCAACAGCA		CTCCAGGTGGCTCCCTTTTA				
PAD4	AT3G52430 GGTTCTGTTCGTCTGATGTT		GTTCCTCGGTGTTTTGAGTT				
BAP1	At3g61190 ATGCCCATCAATGGTAATG		TCCCACACTTATCACCAAA				
PDF1.2a	AT5G44420 ACGCACCGGCAATGGTGGAA		TGCATGATCCATGTTTGGCT				
PDF1.2c	AT5G44430 CTGCTACCATCATCACCTTC		CCGCAAACGCCTGACCATGT				
AOS	AT5G42650 GGTCATCAAGTTCATAACCG		TTTCTCAATCGCTCCCAT				
ACS2	At1g01480 GTTAAGCTCAATGTGTCTCC		AAGCAAATCCTAAACCATCC				
ERF1	At3g23240 ATCAAATCCGTAAGCTCAAG		CCAAACCCTAATACCGTTTC				
LEA4-1	I AT1G32560 AGCAAAAGCTGATGAGAAG		TAGGTCTGAGGAGGCACTGA				
ABI1	AT4G26080 ATGTCGAGATCCATTGGCGAT		TTCCTTTCTCCGCTCATCCG				
PMR4	AT4G03550	ATGTACACATGTAAATGGCG	GAGGCTGGGTCTCGAGGAGTT				
593							

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

	WT		NahG		pad4		pi4k 6162		NahGpi4k8182		pad4pi4k6162	
	mock	LatB	mock	LatB	mock	LatB	mock	LatB	mock	LatB	mock	LatB
PdF1.2a	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 <u>+</u> 0.18	4.79 ± 0.92	3.49 <u>+</u> 0.54	13.29 <u>+</u> 2.34	4.67 <u>+</u> 0.67	4.91 ± 0.96
PdF1.2c	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
ACS2	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
AOS	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
ERF1	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
LEA4-1	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 <u>+</u> 0.12	0.97 ± 0.07	0.95 <u>+</u> 0.22	1.25 ± 0.29	3.45 <u>+</u> 2.07	0.92 <u>+</u> 0.13
ABI1	0.98 ± 0.04	1.18 ± 0.03	0.89 ± 0.05	1.13 ± 0.06	$0.00 \pm 0.00$	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 $\mu$ M latB for 24h. DMSO was used as mock treatment. SA content was measured by LC-MS. Means ± SE from 8 values generated in two independent experiments are shown.

SA content (ng/ g FW)							
	D	0	LatB				
WT	307,70	±	66,59	7247,39	±	793,85	
pmr4	402,10	±	45,02	10179,89	±	756,35	
NahG	64,11	±	6,30	451,38	±	51,03	
sid2	54,30	±	6,84	54,84	±	3,32	
pad4	102,97	±	20,15	788,84	±	158,57	
pi4k 6162	159,56	±	26,04	6879,74	±	864,17	
NahGpi4k8182	105,50	±	18,14	2286,92	±	222,89	
sid2pi4k8182	60,94	±	9,76	62,75	±	10,80	
pad4pi4k6162	237,77	±	92,15	1925,06	±	385,01	

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