

# 1 Inhibition of jasmonate-mediated plant defences by the fungal 2 metabolite higginsianin B

3 Jean-Félix Dallery <sup>1,4</sup>, Marlene Zimmer <sup>2</sup>, Vivek Halder <sup>3,§</sup>, Mohamed Suliman <sup>3,†</sup>, Sandrine Pigné <sup>1</sup>, Géraldine  
4 Le Goff <sup>4</sup>, Despoina D. Gianniou <sup>5</sup>, Ioannis P. Trougkos <sup>5</sup>, Jamal Ouazzani <sup>4</sup>, Debora Gasperini <sup>2</sup>, Richard J.  
5 O'Connell <sup>1</sup>

6 <sup>1</sup> UMR BIOGER, INRA, AgroParisTech, Université Paris-Saclay, Thiverval-Grignon, France

7 <sup>2</sup> Department of Molecular Signal Processing, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany

8 <sup>3</sup> Chemical Biology Laboratory, Max Planck Institute for Plant Breeding Research, Cologne, Germany

9 <sup>4</sup> Centre National de la Recherche Scientifique, Institut de Chimie des Substances Naturelles ICSN, Gif-sur-  
10 Yvette, France

11 <sup>5</sup> Department of Cell Biology and Biophysics, Faculty of Biology, National and Kapodistrian University of  
12 Athens, Greece

13 § Current address: Rijk Zwaan, De Lier, 2678 ZG, Netherlands

14 †Current address: Desert research center, Cairo, Egypt

15 **Corresponding authors:** Richard J. O'Connell, [richard.oconnell@inra.fr](mailto:richard.oconnell@inra.fr) ; Debora Gasperini,  
16 [debora.gasperini@ipb-halle.de](mailto:debora.gasperini@ipb-halle.de)

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## 21 **Highlight**

22 A diterpene secondary metabolite produced by a fungal pathogen suppresses plant jasmonate defense  
23 signalling by preventing the proteasomal degradation of JAZ repressor proteins.

## 24 **Abstract**

25 Infection of *Arabidopsis thaliana* by the ascomycete fungus *Colletotrichum higginsianum* is  
26 characterised by an early symptomless biotrophic phase followed by a destructive necrotrophic  
27 phase. The fungal genome contains 77 secondary metabolism-related biosynthetic gene clusters  
28 (BGCs), and their expression during the infection process is tightly regulated. Deleting *CclA*, a  
29 chromatin regulator involved in repression of some BGCs through H3K4 trimethylation, allowed  
30 overproduction of 3 families of terpenoids and isolation of 12 different molecules. These natural  
31 products were tested in combination with methyl jasmonate (MeJA), an elicitor of jasmonate  
32 responses, for their capacity to alter defence gene induction in *Arabidopsis*. Higginsianin B inhibited  
33 MeJA-triggered expression of the defence reporter *VSP1p:GUS*, suggesting it may block bioactive  
34 JA-Ile synthesis or signalling *in planta*. Using the JA-Ile sensor Jas9-VENUS, we found that  
35 higginsianin B, but not three other structurally-related molecules, suppressed JA-Ile signalling by  
36 preventing degradation of JAZ proteins, the repressors of JA responses. Higginsianin B likely blocks  
37 the 26S proteasome-dependent degradation of JAZ proteins because it inhibited chymotrypsin- and  
38 caspase-like protease activities. The inhibition of target degradation by higginsianin B also extended  
39 to auxin signalling, as higginsianin B treatment reduced IAA-dependent expression of *DR5p:GUS*.  
40 Overall, our data indicate that specific fungal secondary metabolites can act similarly to protein  
41 effectors to subvert plant immune and developmental responses.

42 **Keywords:** *Colletotrichum*; fungal natural product; higginsianin; jasmonate signalling; JAZ protein; plant  
43 chemical biology; plant immunity; proteasome; secondary metabolite

## 44 Introduction

45 The perception of microbial plant aggressors is mediated by the recognition of pathogen-associated  
46 molecular patterns (PAMPs) by plant cell surface receptors, which in turn activates a cascade of  
47 PAMP-triggered immune (PTI) responses (Dodds and Rathjen 2010; Zipfel and Robatzek 2010).  
48 Downstream of PTI activation, these immune responses are regulated by an interconnected network  
49 of phytohormone signalling pathways in which jasmonic acid (JA), ethylene (ET) and salicylic acid  
50 (SA) play a central role (Pieterse *et al.*, 2012). Antagonistic and synergistic interactions between  
51 these pathways provide an additional layer of regulation in which hormone cross-talk allows the  
52 plant to fine-tune its immune responses to particular pathogens (Bigeard *et al.*, 2015, Pieterse *et al.*,  
53 2012). A broad range of microbes target these hormones signalling pathways using secreted protein  
54 or small molecule effectors in order to manipulate or circumvent plant immunity (Plett *et al.* 2014;  
55 Patkar *et al.*, 2015; Gimenez-Ibanez *et al.* 2016; Katsir *et al.* 2008; Groll *et al.* 2008; Stringlis *et al.*,  
56 2018).

57 The ascomycete fungus *Colletotrichum higginsianum* causes anthracnose disease in numerous wild  
58 and cultivated members of the Brassicaceae, including *Arabidopsis thaliana*. The latter interaction  
59 provides a model pathosystem in which both partners are amenable to genetic manipulation and rich  
60 genetic resources are available for the plant host. Infection of *A. thaliana* by *C. higginsianum* is  
61 characterised by an early symptomless biotrophic phase followed by a destructive necrotrophic phase  
62 (O'Connell *et al.*, 2004). As with other hemibiotrophic pathogens, it is assumed that during the  
63 biotrophic phase the fungus manipulates living host cells to evade plant defences, while fungal toxins  
64 and degradative enzymes are secreted in the necrotrophic phase to kill host cells and mobilise  
65 nutrients (Collemare *et al.*, 2019). We previously reported that *C. higginsianum* tightly regulates the  
66 expression of secondary metabolism biosynthetic gene clusters (BGCs) at different stages of the  
67 infection process (Dallery *et al.*, 2017). Remarkably, no fewer than 14 BGCs are specifically induced  
68 early, during penetration and biotrophic colonization, whereas only five are preferentially activated  
69 during necrotrophy. Hence, not including possible biosynthetic intermediates, up to 14 different

70 secondary metabolites are potentially delivered to the first infected host cell, where they may  
71 contribute to establishing a biotrophic interaction with *A. thaliana*. The transient production of these  
72 fungal metabolites exclusively *in planta* presents a major challenge to their structural  
73 characterization and functional analysis. In the past decade, deleting proteins involved in shaping the  
74 chromatin landscape has allowed the isolation of numerous novel metabolites from diverse  
75 axenically grown fungi (e.g. Bok *et al.*, 2009, Fan *et al.*, 2017, Studt *et al.*, 2016, Wu *et al.*, 2016).  
76 Recently, we reported a  $\Delta cclA$  mutant of *C. higginsianum* affected in the trimethylation of histone  
77 proteins at H3K4 residues which overproduces 12 different metabolites belonging to three terpenoid  
78 families, including five new molecules (Dallery *et al.*, 2019a, Dallery *et al.*, 2019b).

79 Despite the huge efforts made in recent years to characterise the natural products produced by plant-  
80 associated microorganisms, to date most studies have only reported on their antimicrobial activity or  
81 phytotoxicity and have neglected their potential activity against components of PTI and hormone  
82 signalling (Collemare *et al.*, 2019). Indeed, only 30 chemical screens relating to plant biology have  
83 been reported in the literature, of which nine tested activity on plant immunity and only one  
84 concerned JA signalling (Meesters *et al.*, 2014, Serrano *et al.*, 2015). Using a forward chemical  
85 genetic screen, we here identify a fungal natural product that suppresses JA-mediated plant defences.  
86 Using different JA-reporter lines in *Arabidopsis*, we show that higginsianin B, a terpenoid metabolite  
87 produced by *C. higginsianum*, can prevent the MeJA-dependent degradation of JAZ repressor  
88 proteins. Three structural analogues of higginsianin B were found to lack this activity, providing  
89 clues to the structure-activity relationship and suggesting candidate functional groups which could  
90 help in identifying target binding sites. We also found that the active metabolite is able to inhibit the  
91 plant developmental signalling pathway mediated by auxin. Finally, we present evidence that  
92 higginsianin B is likely to exert its activity through inhibition of the 26S proteasome. Taken together,  
93 our work highlights the importance of fungal secondary metabolites in manipulating plant hormone  
94 signalling.

## 95 **Methods**

### 96 **Biological materials**

97 The *Colletotrichum higginsianum* wild-type (WT) strain (IMI 349063A) was maintained on Mathur's  
98 medium as previously described (O'Connell *et al.*, 2004). *Arabidopsis thaliana* accession Columbia  
99 (Col-0) was used as the WT line and served as genetic background for the previously described  
100 reporters used in this study: *VSP1p:GUS* (Zheng *et al.*, 2006), *PR1p:GUS* (Shapiro and Zhang 2001),  
101 *CaMV35Sp:JAZ1-GUS* (Thines *et al.*, 2007), *CaMV35Sp:Jas9-VENUS-NLS* (Larrieu *et al.*, 2015),  
102 *JAZ10p:GUSPlus* (Acosta *et al.*, 2013), and *DR5p:GUS* (Ulmasov *et al.*, 1997). Unless otherwise  
103 specified, *Arabidopsis* was grown axenically in half-strength Murashige and Skoog (MS) medium  
104 ( $0.5 \times$  MS,  $0.5 \text{ g}\cdot\text{L}^{-1}$  MES hydrate, pH 5.7). For solid medium, agar was added at 0.7% and 0.85%  
105 for horizontal and vertical growth, respectively.

### 106 **Chemicals**

107 *C. higginsianum* compound fractions were generated by purifying crude culture extracts using flash  
108 chromatography. The pure secondary metabolites used in this study, namely the diterpenoids  
109 higginsianin A, B, C and 13-*epi*-higginsianin C, were isolated and structurally identified as  
110 previously reported (Dallery *et al.*, 2019b). All fractions and pure compounds were dissolved in  
111 dimethyl sulfoxide (DMSO) as stock solutions.

### 112 **Quantitative assay for inhibition of JA and SA responses**

113 Hydroponically grown 12-day-old transgenic *Arabidopsis* seedlings of *VSP1p:GUS* and *PR1p:GUS*  
114 reporters were used to identify compounds interfering with jasmonate-, or salicylic acid-mediated  
115 defences, respectively. Seedlings were treated with compounds for 1 h before inducing reporter gene  
116 expression with MeJA (100  $\mu\text{M}$ ) or SA (200  $\mu\text{M}$ ) dissolved in DMSO. After 24 h, the liquid medium  
117 was removed carefully from the wells with a vacuum pump. Seedlings were incubated with 150  $\mu\text{L}$   
118 lysis buffer containing 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1 % Triton X-100 and 1  
119 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide (4-MUG; 69602, Sigma-Aldrich) at 37°C for 90 min.  
120 The reaction was stopped by adding 50  $\mu\text{L}$  of 1 M  $\text{Na}_2\text{CO}_3$  and 4-MU fluorescence was measured in  
121 a microplate reader (excitation/emission wavelength 365/455 nm). Activity was expressed as relative  
122 light units (RLU). Each treatment was performed on 5 independent seedlings.

### 123 **Histochemical GUS staining**

124 Samples were fixed in 90 % acetone on ice for 1 h, washed in 50 mM  $\text{NaPO}_4$  buffer pH 7.0, vacuum-  
125 infiltrated with GUS substrate solution [50 mM  $\text{NaPO}_4$  buffer, pH 7.0, 0.1 % (v/v) Triton X-100,  
126 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 1mM 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide] and incubated at 37°C for 2h.

127 Staining was stopped with 70 % ethanol and samples were mounted in 70 % glycerol for observation  
128 with a binocular microscope.

### 129 ***In vivo* Jas9-VENUS degradation**

130 Inhibition of JAZ protein degradation upon MeJA treatment was assayed using the *Arabidopsis* JA-  
131 Ile sensor *CaMV35Sp:Jas9-VENUS-NLS* (Larrieu *et al.*, 2015). After seed stratification for 2 days at  
132 4°C, seedlings were grown vertically for 5 days. Growth conditions were 21°C with a photoperiod of  
133 14h light (100  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Seedlings were pre-treated with either mock (DMSO in 0.5× MS) or the  
134 compound under analysis (30  $\mu\text{M}$ ) in a sterile dish for 30 min, then samples were mounted in 60  $\mu\text{L}$   
135 of 30  $\mu\text{M}$  Methyl-Jasmonate (MeJA) in 0.5 × MS on microscope slides and imaged immediately (0  
136 min) and 30 min after MeJA treatment. In this way, expression of the reporter was evaluated in  
137 individual seedling roots (n = 10 for each condition). To ensure that pre-treatments did not cause  
138 reporter degradation, a full sample set was also pre-treated directly on microscopy slides and imaged  
139 at 0 min and after 30 min. VENUS fluorescence in living roots was imaged with a Zeiss LSM 700  
140 confocal laser scanning microscope with 488 nm excitation and 490-555 nm emission wavelength.  
141 All images shown within one experiment were taken with identical settings. Image processing was  
142 done with FIJI (<http://fiji.sc/Fiji>).

### 143 **Monitoring Jas9-VENUS degradation by immunoblot**

144 Five-day-old seedlings were grown horizontally in axenic conditions on a nylon mesh (200  $\mu\text{m}$  pore  
145 size) supported on MS solid medium. Growth conditions were 21°C with a photoperiod of 14h light  
146 (100  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Pre-treatment and treatment of seedlings was performed as described for  
147 microscopy, except that treatments were performed in sterile dishes. E-64, a highly selective cysteine  
148 protease inhibitor (E3132, Sigma-Aldrich) and epoxomicin, a specific proteasome inhibitor (E3652,  
149 Sigma-Aldrich) were used as controls. Seedlings were snap-frozen in liquid nitrogen and kept frozen  
150 for disruption using 3 mm diameter tungsten beads in a Qiagen TissueLyser II operating at 30 Hz, 2  
151 × 1 min. Total proteins from 120 seedlings were extracted with 150  $\mu\text{L}$  of extraction buffer (50 mM  
152 Tris-HCl pH 7.4, 80 mM NaCl, 0.1 % Tween 20, 10 % glycerol, 10 mM dithiothreitol, 2× Protease  
153 inhibitor cocktail [11873580001, Roche], 5 mM PMSF). Prior to protein quantification, debris were  
154 removed by centrifugation at 14,000 rpm, 10 min. Total proteins (40  $\mu\text{g}$ ) were separated using SDS-  
155 PAGE (10 % acrylamide) and then blotted onto nitrocellulose membranes (1620112, Biorad). Jas9-  
156 VENUS and ACTIN were detected using mouse monoclonal antibodies anti-GFP 1:1,000  
157 (11814460001, Roche) or anti-actin 1:2,000 (A0480, Sigma-Aldrich), respectively. The secondary  
158 antibody was an anti-mouse coupled to HRP 1:10,000 (W4021, Promega). Detection was performed  
159 with the Pico Plus system (34580, Thermo Scientific) and X-ray films (47410 19284, Fujifilm).

## 160 **Wounding assays**

161 Horizontally-grown 5-day-old *JAZ10p:GUSPlus* reporter seedlings were pre-treated with either 30  
162  $\mu$ M DMSO (mock) or 30  $\mu$ M higginsianin B in water 30 min prior to mechanical wounding one  
163 cotyledon as described by Acosta *et al.*, (2013). Pre-treatment was performed by applying 0.5  $\mu$ L of  
164 test solutions to both cotyledons of all seedlings. Histochemical GUS staining was performed 2h  
165 after wounding (n = 60 per condition). Alternatively, 1 h after mechanical wounding of one  
166 cotyledon, the shoots and roots were collected separately for qRT-PCR analysis of *JAZ10* expression  
167 as described previously (Acosta *et al.*, 2013). RNA and cDNA were prepared as in Gfeller *et al.*,  
168 (2011). Quantitative RT-PCR was performed as described in Chauvin *et al.*, (2013) using the  
169 primers for *JAZ10* (At5g13220) and *UBC21* (At5g25760) previously reported in Gfeller *et al.*,  
170 (2011).

## 171 ***In vitro* proteasome activity assays**

172 To assess the direct binding-inhibition of proteasomal subunits by higginsianin B, human new born  
173 foreskin (BJs) normal fibroblast cells were lysed by using a lysis buffer containing 0.2 % Nonidet P-  
174 40, 5 mM ATP, 10 % glycerol, 20 mM KCl, 1 mM EDTA, 1mM dithiothreitol and 20 mM Tris, pH  
175 7.6). Protein concentration was determined prior to treatment with increasing concentrations of  
176 higginsianin B or one of two known proteasome inhibitors (bortezomib or epoxomicin).  
177 Chymotrypsin-like (LLVY) and caspase-like (LLE) activities were determined by recording the  
178 hydrolysis of fluorogenic peptides Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC,  
179 respectively (excitation 350 nm; emission 440 nm).

## 180 **Cell-based proteasome activity assays**

181 Measurement of proteasome peptidase activities following cell exposure to the compounds was  
182 performed as described previously (Sklirou *et al.*, 2015). Briefly, cells were plated in 60 mm dishes,  
183 left to adhere overnight and then treated with the test compounds for 24 or 48 h. The cells were then  
184 lysed and proteasome activities were assayed as described above.

## 185 **Auxin treatment**

186 Five-day-old *DR5p:GUS* auxin reporter seedlings were grown vertically as described above. Pre-  
187 treatment with mock (DMSO in 0.5 $\times$  MS) or higginsianin B solution (30  $\mu$ M in 0.5 $\times$  MS) was  
188 performed in sterile dishes for 30 min, followed by 2 h treatment with either mock or  
189 naphthaleneacetic acid (NAA, 5  $\mu$ M in 0.5 $\times$  MS), a synthetic auxin analogue.

## 190 **Statistical Analyses**

191 Statistical analyses were conducted using R software (version 3.4.2) and the packages *Rcmdr*  
192 (version 2.4-4) and *conover.test* (version 1.1.5), all available from The Comprehensive R Archive



193 Network (CRAN). The statistical significance of compound treatments on *VSP1p:GUS* and  
194 *PR1p:GUS* activation was performed using the Kruskal-Wallis test followed by multiple  
195 comparisons using the Conover-Iman test with Benjamini-Hochberg adjustment of *P*-values for false  
196 discovery rate (FDR). All proteasome activity tests were performed at least in duplicate and data  
197 were statistically analysed with an ANOVA single factor test.

## 198 Results

### 199 ***Chemical genetic screens identify an inhibitor of JA signalling***

200 Chemical biology screens using transgenic *Arabidopsis* lines expressing suitable reporter genes are  
201 powerful tools to detect small molecules interfering with components of plant defence and hormone  
202 signalling (Meesters and Kombrink 2014, Serrano *et al.*, 2015). To search for such activities among  
203 *C. higginsianum* metabolites, we generated a small library of partially purified fractions (F1 – F4)  
204 and one pure molecule, namely higginsianin B, isolated from liquid cultures of the *C. higginsianum*  
205  $\Delta cclA$  mutant (Dallery *et al.*, 2019b). These were then screened for potential inhibitory activity  
206 against SA- and JA-induced defence responses using transgenic plants expressing the  $\beta$ -  
207 glucuronidase reporter under the SA-responsive *PATHOGENESIS RELATED 1 (PR1)* promoter or  
208 the JA-responsive *VEGETATIVE STORAGE PROTEIN 1 (VSP1)* promoter, respectively (Shapiro  
209 and Zhang 2001, Zheng *et al.*, 2006). Seedlings grown hydroponically in 96-well plates were first  
210 treated with fungal metabolites before inducing expression of the reporter genes with SA or methyl  
211 jasmonate (MeJA), respectively. The use of 4-methylumbelliferyl- $\beta$ -D-glucuronide (4-MUG) as  
212 GUS substrate allowed the fluorimetric quantification of reporter gene expression in intact plants  
213 (Halder and Kombrink 2015).

214 None of the tested compounds were able to inhibit or enhance the SA-mediated activation of  
215 *PR1p:GUS* (Supplementary Figure S1). Although seedlings pre-treated with fraction F4 and  
216 higginsianin B showed a higher activation of *PR1p:GUS* compared to the DMSO control, these  
217 differences were not significant (adjusted *P*-value = 0.25, Kruskal-Wallis with Conover-Iman test).  
218 In contrast, fractions F3 and F4 both significantly reduced the MeJA-dependent inducibility of



219 *VSP1p:GUS* expression, by 14 % and 66 %, respectively, compared to mock pre-treated controls  
220 (Figure 1A). Purification of compounds from these two fractions identified higginsianin B as the  
221 only active metabolite at a concentration of 30  $\mu$ M. In agreement with this result, comparison of  
222 HPLC chromatograms of fractions F1-F4 showed that higginsianin B was present only in fractions  
223 F3 and F4 (Supplementary Figure S2). Control seedlings that were not treated with MeJA  
224 (uninduced) displayed only basal activation of *VSP1p:GUS* (8% of the level in induced seedlings,  
225 Figure 1A). Using this assay, we also found that higginsianin B reduced *VSP1p:GUS* activation in a  
226 dose-dependent manner between 3 and 100  $\mu$ M, with maximal inhibition of 56% at 100  $\mu$ M (Figure  
227 1B). Given the pronounced inhibitory effect of higginsianin B on the JA pathway, we investigated  
228 this activity further.

### 229 ***Higginsianin B inhibits JAZ1 degradation***

230 To validate the primary screen result, we tested the effect of higginsianin B on a different marker of  
231 the JA pathway, using a transgenic *A. thaliana* line constitutively expressing the JASMONATE ZIM  
232 DOMAIN PROTEIN 1 (JAZ1) fused to GUS (*p35S:JAZ1-GUS*) (Thines *et al.*, 2007). JAZ proteins  
233 repress JA-responsive genes by binding and inhibiting transcriptional activators such as MYC2  
234 (Pauwels and Goossens 2011). The bioactive jasmonate-isoleucine (JA-Ile) conjugate mediates the  
235 binding of JAZ proteins to the F-box protein CORONATINE INSENSITIVE1 (COI1), a member of  
236 the Skp1/Cullin1/F-box protein COI1 (SCF<sup>COI1</sup>) complex (Fonseca *et al.*, 2009). JAZ proteins are  
237 then poly-ubiquitinated prior to degradation by the 26S proteasome (Chini *et al.*, 2007, Thines *et al.*,  
238 2007). We therefore monitored JAZ1-GUS protein degradation in roots pre-treated with test  
239 compounds and then treated with MeJA as described previously (Meesters *et al.*, 2014). While MeJA  
240 treatment triggered JAZ1-GUS degradation in mock pre-treated roots, higginsianin B pre-treatment  
241 prevented the MeJA-induced degradation of JAZ1-GUS protein at concentrations as low as 0.3  $\mu$ M  
242 and similar to the proteasome inhibitor MG132 (Figure 2) which is known to prevent JAZ1-GUS  
243 degradation (Meesters *et al.*, 2014). One possible explanation for this finding is that higginsianin B  
244 may inhibit the proteasome-mediated destruction of JAZ1; alternatively, it may block the conversion

245 of inactive MeJA into active JA-Ile. In *Arabidopsis*, this conversion is a two-step process involving a  
246 methyljasmonate esterase which produces JA from MeJA and a jasmonoyl-L-amino acid synthetase  
247 called JAR1 which converts JA to JA-Ile (Staswick and Tiryaki 2004). When the active JA-Ile was  
248 used as inducer in place of MeJA, higginsianin B was still able to inhibit JAZ1-GUS degradation,  
249 suggesting that the molecule acts downstream of JA-Ile biosynthesis (Figure 2).

### 250 ***Inhibition of JAZ degradation is specific to higginsianin B***

251 To verify if higginsianin B could inhibit JAZ protein degradation *in vivo*, we monitored its effect on  
252 the roots of reporter seedlings constitutively expressing the JA sensor Jas9-VENUS (J9V) consisting  
253 of the JAZ9 degron domain (Jas) fused to the VENUS yellow fluorescent protein and a nuclear  
254 localization signal (Larrieu *et al.*, 2015). Seedling roots were pre-treated with either mock or  
255 compounds under analysis for 30 min, before being treated with MeJA for another 30 min. As  
256 expected, MeJA treatment following mock pre-treatment induced J9V reporter degradation, as  
257 indicated by the low fluorescence intensity visible in root cell nuclei following the 30 min treatment.  
258 (Figure 3A, first row). In contrast, root pre-treatment with higginsianin B (30  $\mu$ M) strongly inhibited  
259 MeJA-induced J9V degradation (Figure 3A, second row). To assess structure-activity relationships,  
260 we also tested three other molecules that are structurally related to higginsianin B, namely  
261 higginsianin A, higginsianin C and 13-*epi*-higginsianin C (Dallery *et al.*, 2019b). However, pre-  
262 treatment with each of these compounds failed to prevent MeJA-induced J9V degradation  
263 (Figure 3A), indicating that the inhibitory effect is specific to higginsianin B. By comparing the  
264 structures of these molecules (Figure 3B), the functional groups most likely to be required for  
265 inhibitory activity are the hydroxyl substituent on the bicyclic core and / or the aliphatic side-chain.

266 To further validate results obtained from live-cell imaging, we monitored J9V reporter degradation  
267 *in planta* by immunoblot assay. *Arabidopsis* seedlings were pre-treated with either mock or  
268 higginsianins for 30 min and subsequently treated with mock or MeJA for 30 min. While MeJA  
269 triggered J9V degradation in mock pre-treated seedlings, pre-treatment with higginsianin B at 30  $\mu$ M

270 prevented J9V degradation (Figure 3C). However, the three other members of this compound family  
271 were again inactive at the same concentration (Supplementary Figure S3). A dose-dependency test  
272 showed that higginsianin B was active at a concentration of 10  $\mu$ M (Figure 3D). As controls in this  
273 assay, E-64, a highly selective cysteine protease inhibitor was used as an inhibitor of non-  
274 proteasomal proteases and epoxomicin as a specific inhibitor of the proteasome. Similar to  
275 higginsianin B, epoxomicin inhibited JAS9-VENUS degradation whereas E-64 was inactive  
276 (Supplementary Figure S3).

### 277 ***Higginsianin B inhibits wound-induced JAZ10 activation in roots***

278 So far, our findings revealed that higginsianin B can inhibit JAZ degradation and JA-induced gene  
279 expression resulting from exogenous MeJA treatment. To test whether the effect of higginsianin B  
280 also extends to suppressing endogenous JA-mediated responses, we assayed JA marker gene  
281 expression following mechanical wounding of seedlings pre-treated with higginsianin B. Mechanical  
282 wounding of seedling cotyledons is a strong elicitor of JA-dependent gene expression in both shoots  
283 and roots, including the activation of the JA-dependent reporter *JAZ10p:GUSPlus (JGP)* (Acosta *et*  
284 *al.*, 2013). Pre-treatment of seedling cotyledons with either mock or higginsianin B did not cause  
285 reporter activation, while mechanical wounding effectively induced *JGP* expression in wounded  
286 shoots in both pre-treatments (Figure 4A). Interestingly, mock pre-treated samples also showed  
287 increased *JGP* expression in their roots, whereas higginsianin B pre-treatment resulted in reduced  
288 wound-induced reporter activation in this organ (Figure 4A). Quantification of *JAZ10* transcripts  
289 further confirmed that higginsianin B pre-treatment reduced wound-induced *JAZ10* accumulation in  
290 both shoots and roots as compared to mock treatments (Figure 4B). Furthermore, higginsianin B pre-  
291 treatment strongly reduced MeJA-induced *JGP* activation in seedling roots (Figure 5A). Taken  
292 together, these results indicate that higginsianin B can suppress endogenous JA-mediated responses.

### 293 ***Higginsianin B affects auxin-mediated signalling***

294 The degradation of JAZ proteins is executed by the 26S proteasome upon poly-ubiquitination by  
295 SCF<sup>COI1</sup> complex (Chini *et al.*, 2007, Thines *et al.*, 2007). Likewise, the 26S proteasome is also  
296 involved in auxin perception by co-receptors, the SCF<sup>TIR1/AFB</sup> ubiquitin ligases and their targets, the  
297 AUX/IAA family of auxin response inhibitors (Gray *et al.*, 2001, Tiwari *et al.*, 2001). If higginsianin  
298 B blocks JAZ degradation by inhibiting proteasome activity, we reasoned that it may also impact  
299 other proteasome-dependent plant responses such as auxin signalling. Treatment of seedling roots  
300 with the synthetic auxin naphthaleneacetic acid (NAA) induces expression of the synthetic auxin  
301 reporter *DR5p:GUS* in the root meristem, including the elongation zone (Liu *et al.*, 2017) (Figure  
302 5B). Although higginsianin B pre-treatment alone had no any visible effect on the *DR5p:GUS*  
303 expression pattern, this pre-treatment not only abolished NAA-mediated reporter induction in the  
304 root elongation zone but also reduced *DR5p:GUS* expression in the quiescent center and root  
305 columella (Figure 5B). This finding supports the hypothesis that higginsianin B could affect other  
306 proteasome-dependent processes, such as the activation of auxin signalling.

### 307 ***The 26S proteasome is a target of higginsianin B***

308 The impact of higginsianin B on JA- and auxin-mediated signalling pathways suggested the  
309 ubiquitin-proteasome system as a possible target. Therefore, to investigate whether higginsianin B  
310 can directly inhibit proteolytic activities of the 26S proteasome *in vitro*, human cell lysates  
311 containing intact proteasomes were treated with increasing concentrations of the molecule and  
312 proteasome activity was measured. Two highly specific proteasome inhibitors, namely bortezomib  
313 and epoxomicin, were used as positive controls. We found that higginsianin B inhibited the  
314 chymotrypsin-like activity of the proteasome in a dose-dependent manner, with a maximal inhibition  
315 of 40% reached at 5  $\mu$ M; both the bortezomib and epoxomicin were more active in this assay  
316 (Figure 6A). Higginsianin B also inhibited the caspase-like proteasomal activity at concentrations of  
317 1 and 5  $\mu$ M, similar to the level of inhibition achieved with epoxomicin and bortezomib (Figure 6B).  
318 To measure the effect of higginsianin B on proteasome activities in cell-based assays, we used  
319 normal human diploid fibroblasts (BJ cells). In cells treated for 24 h or 48 h with higginsianin B the

320 compound reduced both chymotrypsin-like and caspase-like activities in a dose-dependent manner.  
321 The chymotrypsin-like activity was reduced to ~60% at 24 h and ~50% at 48 h relative to the control  
322 (Figure 6C). Caspase-like activity was strongly reduced to 35% of the control at 24 h, but only to  
323 70% of the control at 48 h (Figure 6D). Overall, these results suggest that higginsianin B is a potent  
324 inhibitor of proteasome proteolytic activities.

## 325 **Discussion**

326 To date, few chemical genetic screens have been used to systematically search for molecules  
327 interfering with components of plant immunity (Dejonghe and Russinova 2017, Serrano *et al.*, 2015).  
328 The first small molecule found to inhibit JA-mediated responses in a chemical screen was Jarin-1, a  
329 plant-derived alkaloid that was subsequently shown to specifically inhibit the activity of JA-Ile  
330 synthetase JAR1, thereby blocking the conversion of JA into bioactive JA-Ile (Meesters *et al.*, 2014).  
331 Adopting a similar approach combined with the bioassay-guided purification to screen secondary  
332 metabolites produced by the *C. higginsianum*  $\Delta cclA$  mutant, we here identified higginsianin B as a  
333 novel inhibitor of jasmonate-induced plant defence gene expression. We showed that this diterpenoid  
334 can prevent both the wound-induced activation of jasmonate signalling as well as the activation of  
335 this pathway by exogenous MeJA. More precisely, we showed higginsianin B acts downstream of  
336 the enzymatic conversion of MeJA into JA-Ile by inhibiting the degradation of JAZ proteins, the key  
337 repressors of JA signalling in plants. The degradation of JAZ proteins by the ubiquitin-proteasome  
338 system (UPS) is essential for de-repressing plant defence genes regulated by JA signalling (Chini *et*  
339 *al.*, 2007, Thines *et al.*, 2007). We present evidence that higginsianin B directly inhibits two catalytic  
340 activities of the 26S proteasome, suggesting the molecule most likely blocks the activation of JA-  
341 mediated plant defences by inhibiting the proteasomal degradation of JAZ proteins. In agreement  
342 with this proposed mode of action, we show higginsianin B also inhibits another proteasome-  
343 dependent process, namely the activation of auxin signalling (Gray *et al.* 2001).

344 To gain insight into the structural features of higginsianin B that are required for its activity, we  
345 tested the three other known members of this compound family, namely higginsianin A, C and 13-  
346 *epi*-higginsianin C. Remarkably, higginsianin B was the only molecule to show activity in JAZ  
347 degradation assays at the tested concentration of 30  $\mu$ M. The bicyclic core of higginsianin B is  
348 distinguished by harbouring a hydroxyl group and an aliphatic side chain (instead of the 5- or 6-  
349 membered ring present in higginsianin A or higginsianin C and 13-*epi*-higginsianin C, respectively),  
350 suggesting that one or both of these features contribute to the observed inhibitory activity. On the  
351 other hand, a second hydroxyl group located on the pyrone ring in all higginsianins is unlikely to  
352 contribute to this activity, and is therefore a good candidate for tagging higginsianin B with a  
353 fluorescent probe for direct visualization of the active metabolite by live-cell imaging. This group  
354 could also be exploited for the covalent immobilization of higginsianin B onto a solid support to  
355 search for potential protein targets by affinity purification.

356 While many natural proteasome inhibitors have been discovered from actinobacteria, few were  
357 identified from fungi. These include the peptide aldehyde fellutamide B produced by the marine  
358 fungus *Penicillium fellutalum* (Hines *et al.*, 2008) and the TMC-95 family of cyclic peptides from the  
359 soil saprophyte *Apiospora montagnei* (Momose and Watanabe 2017). Proteasome inhibitors are  
360 currently the subject of intense interest as therapeutic agents for the control of cancer and other  
361 diseases (Wang *et al.* 2018; Tsakiri and Trougakos 2015). In this regard it is interesting to note that  
362 higginsianin B was recently shown to have antiproliferative activity against glioma, carcinoma and  
363 melanoma cell lines (Cimmino *et al.*, 2016). As a novel proteasome inhibitor, higginsianin B  
364 therefore merits further investigation as a lead compound for the development of potential  
365 therapeutic applications.

366 Protein turnover by the ubiquitin-proteasome system (UPS) regulates numerous aspects of plant  
367 immunity, from pathogen recognition to downstream defence signalling (Marino *et al.* 2012), and  
368 pathogens have evolved protein and chemical effectors to manipulate the UPS to promote plant

369 colonization (Üstün et al. 2016). For example, *Pseudomonas syringae* pv *syringae* secretes the  
370 nonribosomal peptide syringolin A which binds covalently to catalytic subunits of the 26S  
371 proteasome to inhibit their activity and suppress plant defences (Groll et al. 2008). Two related  
372 bacterial Type 3 (T3) secreted effector proteins, XopJ from *Xanthomonas campestris* pv. *vesicatoria*  
373 and HopZ4 from *P. syringae* pv *lachrymans*, both attenuate SA-mediated defence by inhibiting  
374 proteasome activity through their interaction with RPT6, the ATPase subunit of the 19S regulatory  
375 particle of the 26S proteasome (Üstün et al. 2016). Although we have shown here that higginsianin B  
376 can directly inhibit two catalytic activities of the mammalian proteasome, further studies are now  
377 needed to determine which components of the plant proteasome are the targets of this fungal  
378 metabolite and the nature of their interaction.

379 In the context of JA-mediated defence, the proteasomal degradation of JAZ repressors is targeted by  
380 numerous effectors from both pathogenic and mutualistic microbes. For example, the *P. syringae* T3  
381 effectors HopZ1a and HopX1 both activate JA signalling by targeting JAZ proteins for destruction in  
382 the proteasome (Jiang et al. 2013; Gimenez-Ibanez et al. 2014). In contrast, the symbiotic  
383 ectomycorrhizal fungus *Laccaria bicolor* suppresses JA-mediated defences by secreting the MiSSP7  
384 effector protein, which directly interacts with JAZ proteins to protect them from degradation in the  
385 plant proteasome (Plett et al., 2014). The rice blast fungus *Magnaporthe oryzae* weakens JA-  
386 mediated plant defence by secreting the inactive hydroxylated JA (12OH-JA) and a monooxygenase  
387 enzyme called Abm that hydroxylates JA and depletes levels of endogenous rice JA (Patkar et al.,  
388 2015). However, to our knowledge, higginsianin B is the first example of a small molecule produced  
389 by any plant-associated fungus that suppresses plant jasmonate signalling by blocking the  
390 degradation of JAZ proteins.

391 In conclusion, our findings raise the possibility that higginsianin B could function during infection as  
392 a chemical effector to suppress JA-mediated defences, which are induced at the necrotrophic phase  
393 of *C. higginsianum* infection on *Brassica* and *Arabidopsis* (Narusaka et al. 2004; Narusaka et al.



394 2006). Work is now ongoing to determine at what stage higginsianin B is produced during infection  
395 and to genetically test its contribution to fungal virulence and plant defence suppression.

## 396 **Supporting Information**

397 Supplementary Figure 1: Screening assay for modulation of salicylic acid signalling pathway using  
398 *PR1p:GUS* transgenic line.

399 Supplementary Figure 2: HPLC-ELSD comparison of four fractions of an active crude extract of  
400 *Colletotrichum higginsianum*.

401 Supplementary Figure 3: Pre-treatments with compounds structurally related to higginsianin B do not  
402 influence the MeJA-induced degradation of the JA sensor J9V.

## 403 **Acknowledgments**

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407 interpretation, or writing of the manuscript.

## 408 **Competing Interests**

409 The authors declare that no conflict of interest exists.

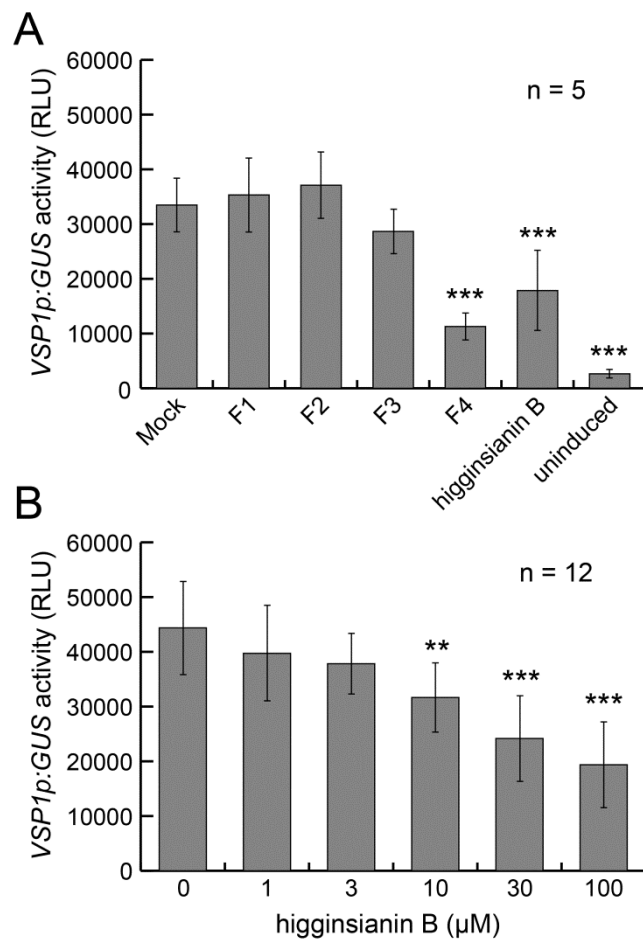
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534 **Figures**



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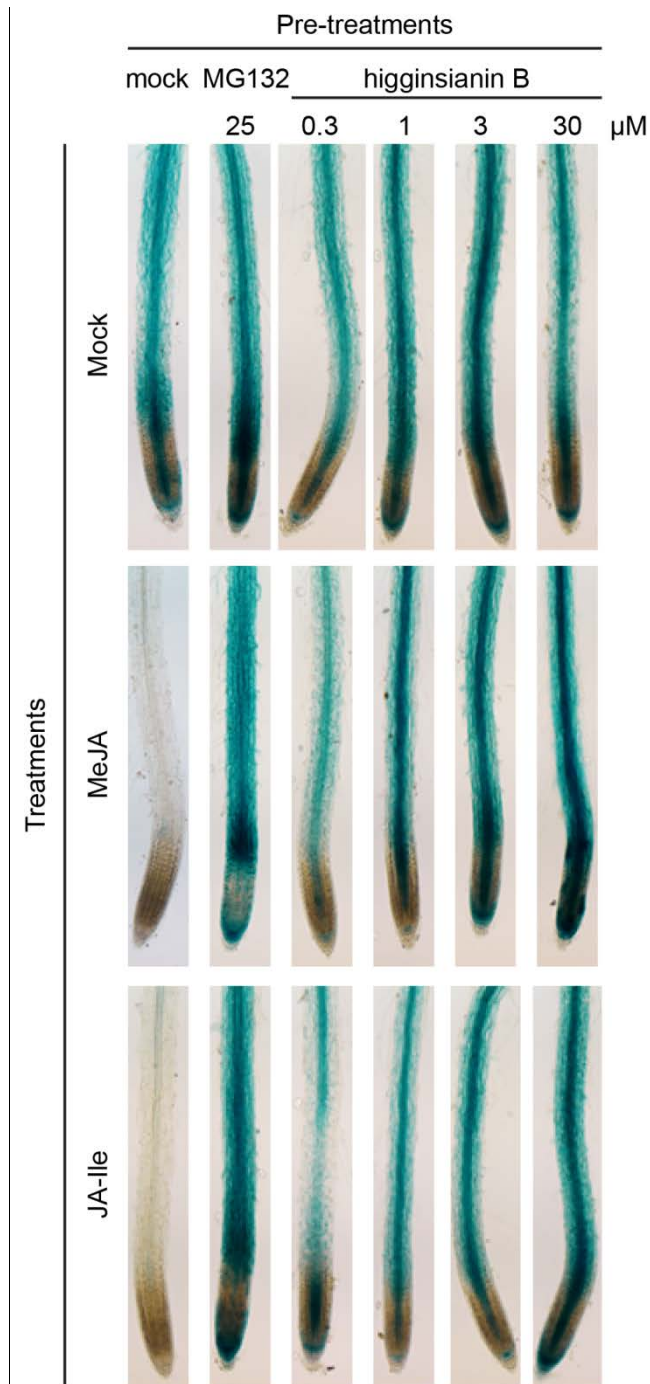
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**Figure 1 – Primary screening identified higginsianin B as a potential inhibitor of JA-mediated plant defence signalling.** (A) *Arabidopsis* seedlings expressing GUS under the *VSP1* promoter, a marker of JA-mediated plant defences, were pre-treated with metabolite fractions or pure compounds for 1h before MeJA treatment (100 µM for 24 h). Bars represent means *VSP1p:GUS* activity of 5 independent seedlings, ± SD from one representative experiment performed twice. (B) Inhibition of *VSP1p:GUS* activity by higginsianin B pre-treatment is dose-dependent. Bars represent means *VSP1p:GUS* activity of 12 independent seedlings, ± SD from one representative experiment performed twice. RLU: Relative Light Unit. \*\*: adjusted *P*-value < 0.01; \*\*\*: adjusted *P*-value < 0.001 (Kruskal-Wallis with Conover-Iman test).

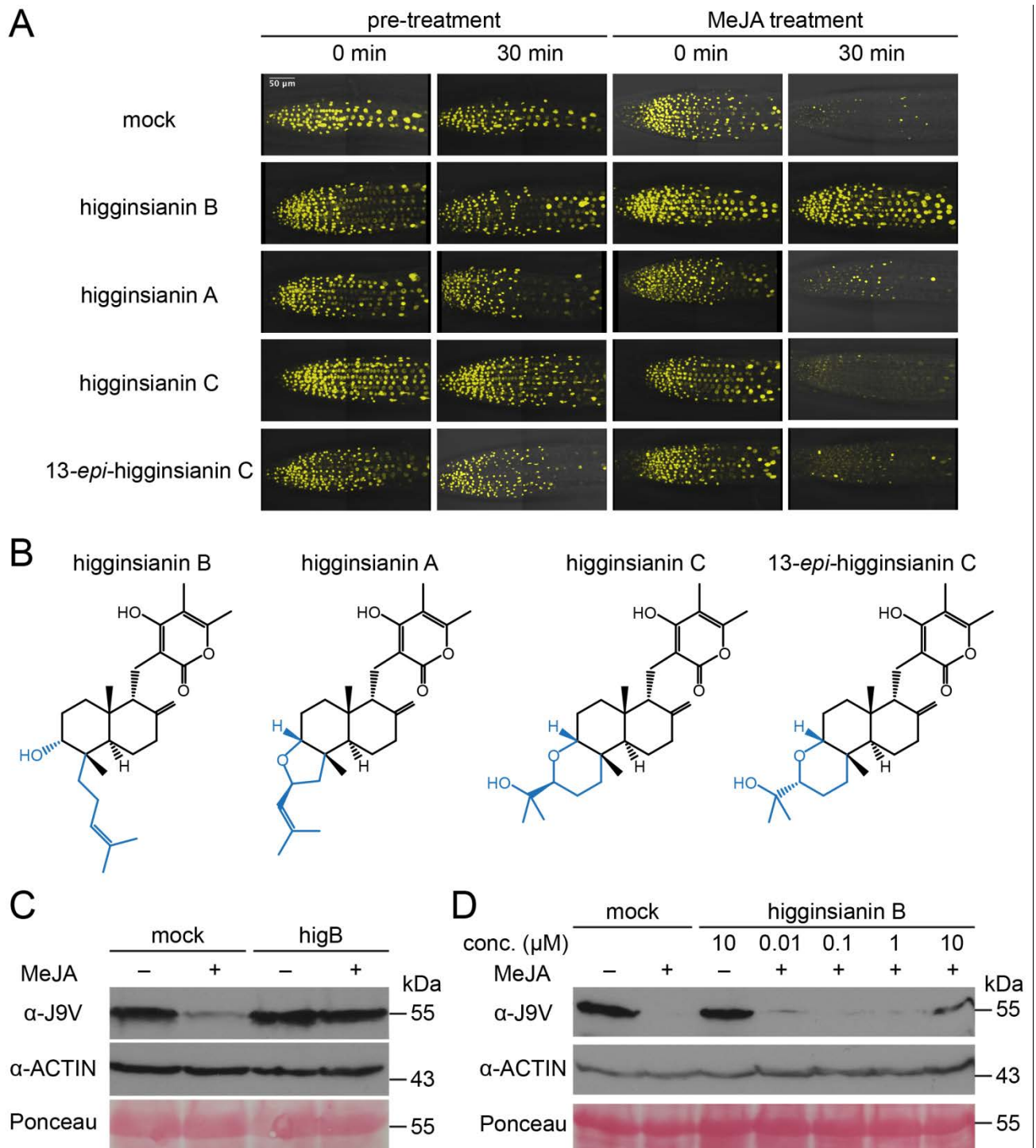


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546 **Figure 2 – Inhibition of JA-mediated degradation of the JAZ1-GUS protein by higginsianin B.** The  
547 constitutively expressed JAZ1-GUS chimeric protein is not degraded by mock pre-treatment (60 min)  
548 followed by mock treatment (30 min), as shown in seedling roots (upper row) whereas MeJA treatment  
549 triggers JAZ1-GUS degradation in mock pre-treated roots (first column, middle row). Pre-treatments with  
550 increasing concentrations of higginsianin B prevent MeJA-mediated degradation of chimeric proteins in a  
551 dose dependent manner. Using 10 μM of JA-Ile as an inducer instead of 10 μM of MeJA gives similar  
552 results indicating that higginsianin B is not inhibiting the conversion of inactive MeJA into the active JA-  
553 Ile (lower row). The proteasome inhibitor MG132 was used as a known inhibitor of JAZ1-GUS  
554 degradation. Each treatment was performed on at least 5 seedlings and one representative image is  
555 presented for each treatment.



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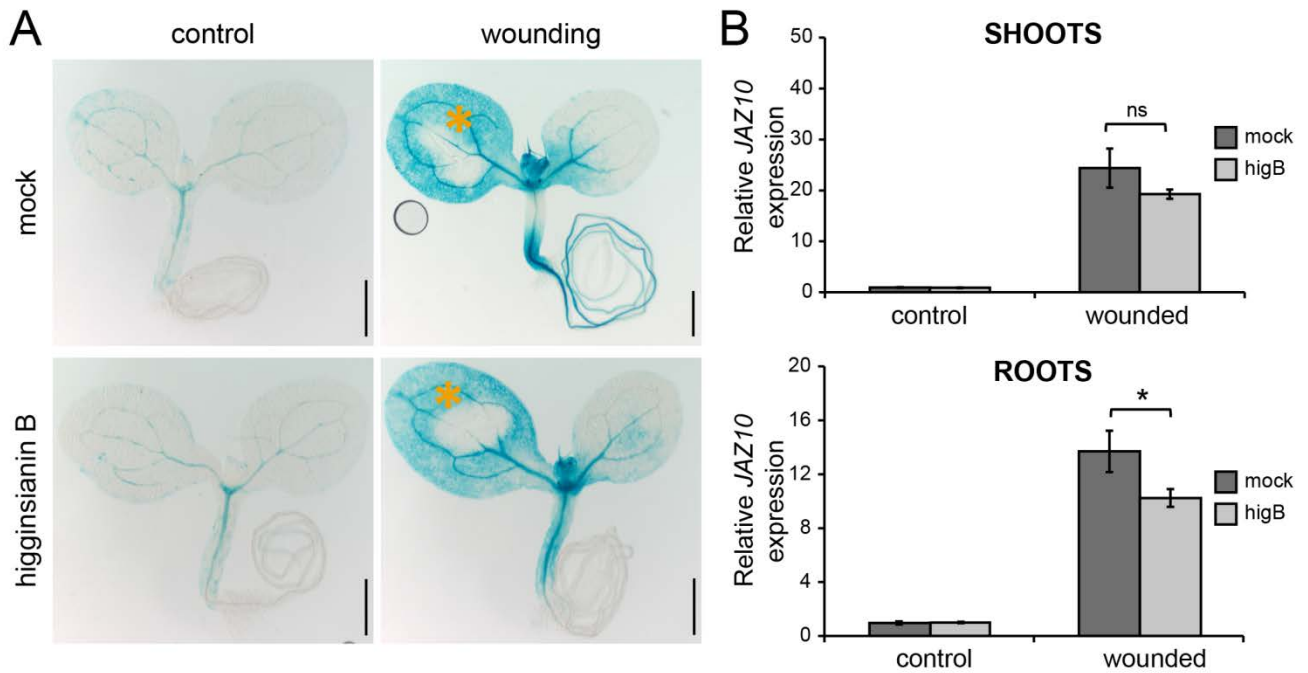
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558 **Figure 3 – Effect of higginsianin B on Jas9-VENUS (J9V) degradation and structure-activity**  
 559 **relationship with other molecules of the higginsianin family.** (A) Primary roots of the JA sensor J9V  
 560 before and after pre-treatment with the indicated compounds (30  $\mu$ M), followed by treatment with  
 561 MeJA (30  $\mu$ M). In the control experiment, mock pre-treatment does not induce reporter degradation, while  
 562 30 min MeJA treatment is sufficient to induce J9V degradation as indicated by the absence of reporter  
 563 fluorescence. Contrariwise, when plants are pre-treated for 30 min with higginsianin B, MeJA treatment is no  
 564 longer able to promote J9V degradation. Other members of the higginsianin family are unable to prevent  
 565 MeJA effect on J9V at the tested concentrations (30  $\mu$ M). (B) Chemical structures of higginsianin B, C, A and

566 13-*epi*-higginsianin C. **(C, D)** Immunoblot analysis of MeJA-induced degradation of J9V (assayed with  $\alpha$ -  
567 GFP). Each lane was loaded with 40  $\mu$ g of total protein extracts from 60 seedlings. ACTIN (assayed with  $\alpha$ -  
568 actin) and Ponceau S represent loading controls. Protein molecular mass is shown on the right. **(C)**  
569 Higginsianin B pre-treatment (30  $\mu$ M) reduced MeJA-induced Jas9-VENUS degradation. **(D)** Inhibition of  
570 MeJA-induced J9V degradation by higginsianin B is dose dependent.  
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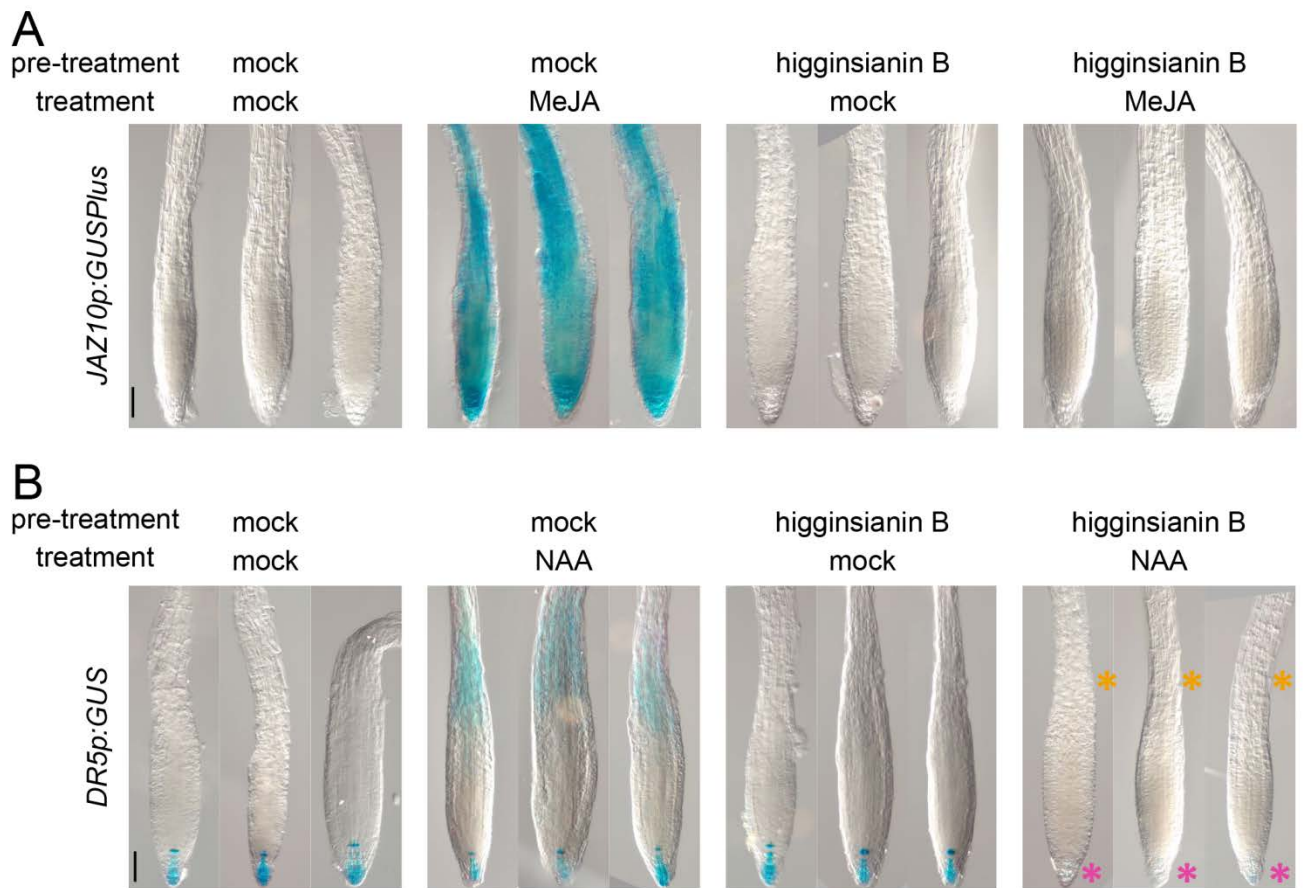
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**Figure 4 – Effect of higginsianin B on wound-induced *JAZ10p:GUSPlus* activation.** (A) Horizontally grown 5-day old *JAZ10p:GUSPlus* reporter seedlings were pre-treated with mock (30  $\mu$ M DMSO) or 30  $\mu$ M higginsianin B by applying 0.5  $\mu$ L of the pre-treatment solution to their cotyledons for 30 min, after which one cotyledon was mechanically wounded as indicated by orange asterisks. GUS staining was performed 2 h after wounding. Bars = 0.5 mm. (B) Quantitative RT-PCR (qRT-PCR) of *JAZ10* expression following 30min pre-treatments with mock or higginsianin B (higB) combined with mechanical wounding. Shoots and roots were collected independently 1 h after wounding aerial organs. *JAZ10* transcript levels were normalised to those of *UBC21* and displayed relative to the expression of mock controls. Bars represent the means of three biological replicates ( $\pm$ SD), each containing a pool of organs from ~60 seedlings. ns, not significant ( $P$ -value = 0.08, t-test); \*:  $P$ -value < 0.05 (t-test).

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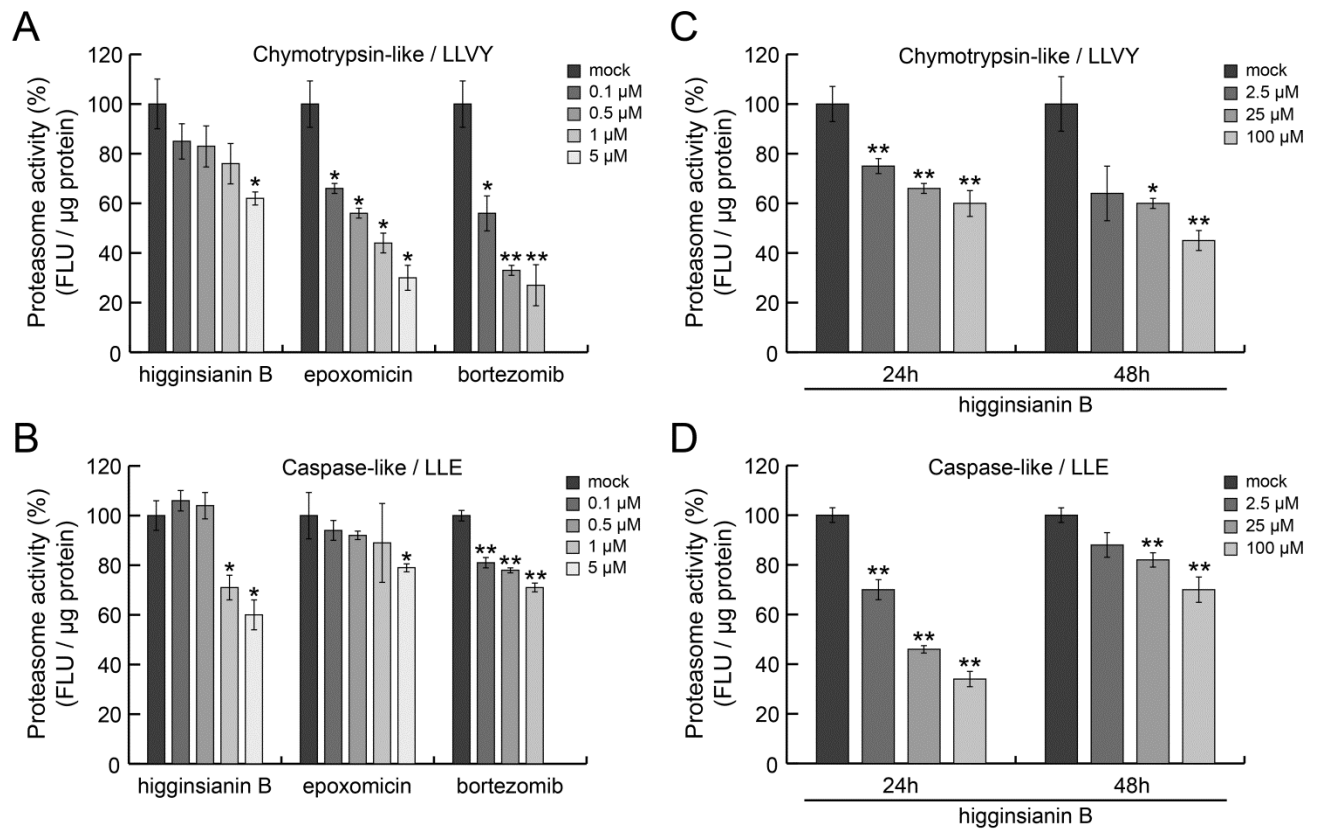
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**Figure 5** – Higginsianin B negatively impacts JA- and IAA-triggered gene expression. **(A)** Higginsianin B pre-treatment abolishes the MeJA-mediated induction of *JAZ10p:GUSPlus* in *Arabidopsis* roots. **(B)** Similarly, higginsianin B also inhibits naphthaleneacetic acid (NAA)-mediated induction of the auxin reporter *DR5p:GUS*. Note the absence of *DR5p:GUS* staining in the elongation zone of higginsianin B pre-treated / NAA treated roots (orange asterisks), as well as reporter absence from the meristem (pink asterisks). Pre-treatments: 30 min (DMSO or 30  $\mu$ M higginsianin B); Treatments: 2 h. Bars = 50  $\mu$ m.



593

594 **Figure 6** – Histograms of inhibition of 26S proteasome activities. (A, C) Chymotrypsin-like activity. (B, D)  
 595 Caspase-like activity. (A, B) *In vitro* direct inhibition of chymotrypsin-like (panel A) and caspase-like (panel  
 596 B) activities in a dose-dependent manner by higginsianin B and two known proteasome inhibitors, i.e.  
 597 epoxomicin and bortezomib. (C, D) Cell-based assays showing dose-dependent inhibition of chymotrypsin-  
 598 like (panel C) and caspase-like (panel D) proteasomal activities in BJ cells exposed for 24 h and 48 h to  
 599 higginsianin B. Data points correspond to the mean of the independent experiments and error bars denote  
 600 standard deviation (SD). FLU, Fluorescence unit. \*: *P*-value < 0.05; \*\*: *P*-value < 0.01 (ANOVA test).