

Greenwood, et al.

1 **Article type:** Research article

2 **Short title:** OPS impacts flg22-induced responses

3 **Corresponding author:** Carina Collins, Marian University, Department of Biology, 3200 Cold

4 Spring Road, Indianapolis, IN 46222, USA.

5 Email: ccollins@marian.edu

6

7 **The phloem-resident OCTOPUS protein is a**

8 **novel regulator of flg22-induced responses in *Arabidopsis thaliana***

9

10

11 Kaitlyn N. Greenwood ^{a,1#}, Courtney L. King ^{a,2#}, Isabella Melena ^{a,3#}, Katherine A. Stegemann ^b,

12 Carina A. Collins ^{a,b,4,5}

13

14 ^a Department of Chemistry and Physics, Drury University, Springfield, MO 65802

15 ^b Department of Biology, Marian University, Indianapolis, IN 46222

16

17 ORCIDS: 0000-0003-1081-3518 (K.N.G); 0000-0003-0368-4324 (C.L.K.); 0000-0001-7894-

18 0682 (I.M.); 0000-0002-5584-5890 (K.A.S.); 0000-0001-9150-1924 (C.A.C)

19

20

Greenwood, et al.

21 **Footnotes**

22 # These authors contributed equally to this work.

23 **Author contributions:** CAC supervised the experiments; KNG, CLK, IM, and KAS performed
24 experiments with assistance of CAC; CAC, KNG, CLK, IM, and KAS designed experiments and
25 analyzed the data; CAC, KNG, CLK, IM, and KAS wrote the article; CAC agrees to serve as the
26 author responsible for contact and ensures communication.

27

28 **Funding information:** This research was funded by start-up funds from Drury University (CAC)
29 and Marian University (CAC), Drury University Research Experience in the Natural Sciences
30 Undergraduate Summer Fellowship (KNG and CLK), and American Society of Plant Biologists
31 Summer Undergraduate Research Fellowship (KAS).

32

33 **Present addresses:**

34 KNG: DaVita Dialysis, Overland Park, KS 66210, US

35 CLK: University of Notre Dame, Department of Chemistry and Biochemistry, South Bend, IN
36 46556, USA

37 IM: Washington University in St. Louis, School of Medicine, St. Louis, MO 63130, USA

38 CAC: Marian University, Department of Biology, Indianapolis, IN 46222, USA.

39

40 **Email address of Author for Contact:** Carina Collins, ccollins@marian.edu

41

42

Greenwood, et al.

43 **Abstract**

44 Phloem is a critical tissue that transports photosynthates and extracellular signals in
45 vascular plants. Although a functional phloem is necessary for plant health, it is also an ideal
46 environment for pathogens to access host nutrients to promote pathogenesis. Even though many
47 vascular pathogens induce economically relevant crop damage, very little is known about the
48 mechanism(s) by which phloem cells detect potential pathogens and signal to minimize damage.
49 Our lab searched existing phosphoproteomic databases, mining for proteins that were
50 phosphorylated in response to the defense-elicitor flagellin, or flg22, AND were expressed in
51 vascular cells, and we identified Octopus (OPS). OPS is polarly associated with the plasma
52 membrane (PM) of sieve element cells and promotes their differentiation from procambial
53 precursor cells by inhibiting the function of BIN2 in brassinosteroid-related signaling. The
54 observation that OPS is differentially phosphorylated in response to flg22 led us to examine
55 whether OPS may function in flg22-induced signaling using *Arabidopsis* T-DNA insertion
56 mutants lacking a functional *OPS*. In wild-type (WT) seedlings, flg22 binds to the PM receptor
57 flagellin sensing 2 (FLS2) to initiate three branches of a signaling cascade that culminates in
58 increased expression of distinct marker genes. Ultimately these signaling pathways lead to the
59 restriction of pathogen growth. Two independent alleles of *ops* were treated with 100 μ M flg22
60 and marker genes from all three branches of FLS2 signaling exhibited higher expression than WT.
61 We also found that in the absence of any flg22, *ops* mutants displayed increased flg22 signaling
62 responses. Our results indicate that OPS may function as a negative regulator of flg22-induced
63 signaling events and is one of very few phloem-resident proteins with a documented role in flg22
64 signaling. These results indicate that the phloem may be able to sense and response to the threat
65 of bacterial pathogens in a unique way.

66

67 **Key words:** plant immunity, FLS2, BRI1, phloem, gene expression, *Arabidopsis*

68

Greenwood, et al.

69 **Introduction**

70 The phloem is a critical tissue in vascular plants that transports photosynthetic sugars from
71 source tissues (leaves) to sink tissues (roots, flowers, and fruit); but this carbon-rich nutrient
72 content also makes the phloem an ideal tissue for pathogenic microbes to colonize (1). There are
73 many examples of phloem-limited bacterial pathogens, including the devastating citrus pathogen
74 *Candidatus Liberibacter asiaticus* (CLAs), the causal agent of citrus-greening disease (or
75 ‘Huanglongbing’). CLAs gains access to the phloem of citrus trees after being deposited by the
76 brown planthopper or related insect vectors (2, 3). The phloem is such a rich source of nutrients
77 that some pathogens that do not colonize the phloem directly will stimulate the aberrant
78 development of phloem cells in other tissues to tap its resources (4). Two distinct cell types
79 comprise the phloem transport system in plants: sieve elements and companion cells. Sieve
80 elements are elongated cells whose primary function is to transport sap contents (photosynthetic
81 sugars, RNA, peptides, and other small organic molecules) from source to sink. Adjacent to sieve
82 elements are the companion cells, which are responsible for loading sap contents into sieve
83 elements (5).

84 Current models of immune signaling in response to a pathogen generally assume that all
85 host cells must individually recognize extracellular pathogen-associated molecular patterns
86 (PAMPs). PAMPs are detected by plasma membrane (PM)- localized pattern recognition receptor
87 (PRR) proteins that, after PAMP binding, can initiate intracellular signaling and activate robust
88 defenses (6, 7). The classic example of a PRR is FLAGELLIN SENSING 2 (FLS2), a member of
89 the leucine-rich repeat receptor-like kinase (LRR-RLK) family of receptors. FLS2 binds to the
90 bacterial motor protein flagellin or to a conserved 22-amino acid peptide, flg22, derived from
91 bacterial flagellin, to initiate a cascade of intracellular signaling events that contribute to the host
92 immune response (8). Interestingly, FLS2 signaling events fall into one of three branches, each of
93 which culminates in expression of marker genes (9, 10). For example, the calcium-dependent
94 branch of FLS2 signaling induces expression of *PHOSPHATE-INDUCIBLE 1 (PHI1)*, the
95 mitogen-activated protein kinase (MAPK) pathway activates *FLG22-INDUCED RECEPTOR*
96 *KINASE (FRK1)*, and activation of the salicylic acid (SA) pathway causes increased expression of
97 *PATHOGENESIS-RELATED1 (PRI)* (7, 11, 12). In addition to changes in gene expression, FLS2
98 also initiates the production of extracellular reactive oxygen species (ROS) and the deposition of

Greenwood, et al.

99 callose at the cell wall (7, 11, 12). Combined, these independent signaling events promote the
100 restriction of pathogen growth and promote host immunity (12).

101 Largescale phosphoproteomic screens have been used to detect proteins that are
102 differentially phosphorylated in response to flg22, potentially identifying proteins involved in the
103 regulation of flg22-induced signaling (9, 13, 14). To find potential regulators of flg22-induced
104 signaling in the phloem, we mined existing flg22 phosphoproteomic datasets and identified
105 OCTOPUS (OPS), a protein that is differentially phosphorylated in response to flg22 in
106 *Arabidopsis* suspension cell cultures (15). This finding was interesting because to the extent that
107 its function is known, previous data indicates that OPS has a role in phloem development, not
108 immune signaling (16-20). *Arabidopsis ops* mutant plants, which lack OPS, display decreased
109 phloem pattern complexity, and contain undifferentiated sieve element cells, resulting in gaps in
110 phloem strands of the root (16-18, 21). Further investigations indicated that OPS may regulate
111 phloem developmental processes by promoting signaling events after perception of the hormone
112 brassinolide (BL), a member of the brassinosteroid (BR) class (22). BL is an endogenous steroid
113 hormone detected by the BRassinosteroid Insensitive-1 (BRI1) receptor kinase. When BRI1 binds
114 BL, a series of downstream signaling events occurs, leading to cell elongation and growth. OPS
115 interacts with a member of the GLYCOGEN SYNTHASE KINASE3 (GSK3) family,
116 Brassinosteroid Insensitive-2 (BIN2), at the PM (22). The retention of BIN2 at the PM prevents
117 BIN2 from inhibiting transcriptional changes needed to induce cell growth. Using β -glucuronidase
118 (GUS) and GFP to visualize expression of OPS in *Arabidopsis*, these previous OPS studies found
119 that OPS is present only in the phloem (16). Combined, this evidence indicates that OPS is an ideal
120 candidate for studying flg22-signaling in the phloem.

121 Despite the devastating effects of phloem-dwelling pathogens on crops, it is not yet known
122 in detail how the cells of the phloem detect the presence of bacterial pathogens or how they respond
123 to them (1). Identifying the contributions of the phloem to pathogen detection and response will
124 increase our knowledge of the tissue-specific mechanisms immune signaling. Using loss-of-
125 function T-DNA mutants and gene expression analysis, we identify the sieve element protein
126 OCTOPUS (OPS) as a regulator of flg22-induced signaling events in *Arabidopsis* seedlings. To
127 our knowledge, this is the first example of a protein localized in sieve elements with such a role in
128 flg22 signaling.

129

Greenwood, et al.

130 **Results**

131 *Root length is reduced in ops-3 and ops-4 seedlings*

132 To explore a potential role for OPS in flg22-elicited immune signaling, we obtained
133 previously described T-DNA insertion mutant lines *ops-3* and *ops-4* (20). Using qPCR, we
134 confirmed that both lines exhibit dramatically reduced *OPS* transcript levels (Fig. 1A-B).
135 Consistent with previous studies (16), seedlings from both *ops* T-DNA lines displayed shorter
136 roots with an increased amount of branching (Fig. 1 C-D). Despite the root growth defects, no
137 gross morphological phenotypes, particularly in aerial tissue, were observed in either mutant as
138 compared to Col-0.

139

140 *flg22-induced responses are enhanced in ops-3 and ops-4 mutants*

141 We hypothesized that because OPS was identified as a protein that was differentially
142 phosphorylated after flg22 treatment in a phosphoproteomic screen (15), OPS might have a role in
143 the regulation of flg22-induced signaling events in *Arabidopsis*. We first sought to determine how
144 plants lacking OPS would respond to flg22 using a seedling growth inhibition assay. When Col-0
145 plants are grown in the presence of flg22 for an extended period (2 weeks), the seedlings
146 experience growth stunting as a result of flg22 detection (8, 23). Col-0 and both independent *ops-*
147 *3* and *ops-4* mutant alleles were grown in liquid media supplemented with either 0 or 1 μ M flg22
148 for two weeks before measuring their fresh weight. Growth of the Col-0 seedlings was inhibited
149 by 74% (Fig. 2). Interestingly, both *ops* mutants showed significantly increased percent growth
150 inhibition; 80% and 82%, respectively, when compared to Col-0 (Fig. 2). Importantly, the seedling
151 growth inhibition was similar between *ops-3* and *ops-4*, indicating that this phenotype is indeed
152 the result of loss of *OPS*.

153 Because we observed an increase of flg22-induced growth inhibition in *ops* mutants, which
154 may be indicative of a defect in flg22 perceptions, we sought to investigate whether the lack of
155 OPS led to a more specific flg22 signaling defect. In *Arabidopsis* when FLS2 binds flg22, a
156 network of multiple signaling pathway branches are initiated, and each independent branch results
157 in the expression of a pathway-specific marker gene (9, 10). To test if OPS has a role in one or
158 more of these specific pathways, we measured induction of the flg22-induced and Ca²⁺ pathway-
159 dependent expression of the marker gene *PHII*. In Col-0 seedlings treated with 100 nM flg22,
160 *PHII* expression remains low prior to flg22 exposure (0 minutes), peaks within 30 minutes, and

Greenwood, et al.

161 then falls to near base levels for 1-3 hours (Fig. 3A). When *ops-3* and *ops-4* seedlings were treated
162 with 100 nM flg22 for the same time course, the expression pattern of *PHII* was observably
163 different. Even without the addition of 100 nM flg22, both *ops-3* and *ops-4* mutants showed
164 significantly higher *PHII* expression than Col-0 and while *PHII* peaked in both mutants 30
165 minutes after treatment, expression levels were significantly higher than Col-0 (Fig. 3A). After
166 observing that even in the absence of flg22, *ops* mutants had increased *PHII* expression, we
167 decided to examine subsequent marker genes in the absence of flg22.

168 MAPK activation after flg22 elicitation occurs independently of Ca²⁺ induction and leads
169 to expression of *WRKY33* and *FRK1*, so we next measured expression of the marker gene *FRK1*.
170 Similarly to *PHII*, *FRK1* expression was significantly higher in both *ops-3* and *ops-4* seedlings,
171 even in the absence of flg22 (Fig. 3B-C). The final pathway we examined was the SA-dependent
172 pathway of FLS2 signaling by measuring expression of the marker gene *PRI*. Consistent with our
173 previous qPCR results, *PRI* expression was significantly increased in both *ops-3* and *ops-4*
174 mutants. Together, these data suggest that OPS may be a negative regulator of flg22-induced
175 immune responses.

176

177 *Brassinosteroid-induced hypocotyl growth is impaired in ops-3 and ops-4*

178 Previous investigations of OPS function showed that OPS interacts with the kinase
179 BRASSINOSTEROID-INSENSITIVE 2 (BIN2) at the PM, preventing BIN2 from inhibiting
180 transcriptional changes needed to induce cell growth (22). These data indicate that OPS plays a
181 role in promoting BL-induced cellular responses which may explain some of the phloem
182 developmental defects observed in *ops* mutants (16, 24, 25); however, these observations were
183 made by studying *Arabidopsis* plants expressing *OPS* under the control of constitutive promoter
184 which may not accurately reflect *in planta* conditions.

185 To gain further evidence of a role for OPS in BL signaling, we treated Col-0 and *ops*
186 mutants with 1 μ M epibrassinolide and measured hypocotyl elongation as a part of a standard BL-
187 response assay. Col-0 seedlings exhibit elongated hypocotyls when exposed to BL, whereas
188 elongation in both *ops-3* and *ops-4* mutants was comparatively reduced. (Fig. 4A). When
189 *Arabidopsis* seedlings are grown in the dark, their hypocotyls elongate to produce a BL-dependent
190 etiolated phenotype (26). To gain further insights into the role of OPS in BR-mediated signaling,
191 we measured the hypocotyls of Col-0, *ops-3* and *ops-4* seedlings grown in the light and the dark.

Greenwood, et al.

192 Col-0 hypocotyls elongate significantly in the dark, and while the length of hypocotyls of both
193 *ops-3* and *ops-4* seedlings do elongate, they are significantly shorter than those of Col-0 (Fig. 4B).
194 These results provide additional evidence that OPS is required for BR-induced hypocotyl
195 elongation.

196

197

198

199 **Discussion**

200 Phloem-limited pathogens cause devastating crop losses, but our understanding of how
201 vasculature tissue responds to pathogen invasion is incomplete, making it imperative to study the
202 contributions of tissue-specific immune signaling to overall plant defenses. A new study
203 demonstrated for the first time that when *Citrus sinensis* or “Valencia” trees are infected with the
204 phloem-limited pathogen CLas they exhibit increased ROS production and callose deposition,
205 indicative of a PAMP-triggered immune response (27). Moreover, authors showed that both sieve
206 elements and companion cells underwent programmed cell death in response to prolonged CLas
207 infection (27). Here, we have identified a protein exclusively expressed in sieve elements of the
208 phloem that also has a role in flg22-induced immune responses, which we believe may be the first
209 of its kind. These recent advances highlight the likelihood that phloem cells can detect the presence
210 of bacterial pathogens and are active in signaling a response.

211 In this work, we demonstrate that two independent *Arabidopsis* lines harboring T-DNA
212 insertions in the *OPS* gene lack *OPS* expression and display constitutive and increased flg22-
213 induced expression of several immunity-related marker genes. Furthermore, we found that
214 expression of these genes can be detected even in the absence of flg22. This combination of results
215 indicates that OPS functions as a negative regulator of these flg22-induced responses and therefore
216 OPS exhibits a suppressive effect on this pathway (Fig. 5). While the specific mechanism of OPS
217 function in flg22 signaling remains unclear, OPS may suppress activation of *PHI1*, *FRK1*,
218 *WRKY33*, and *PR1* expression by directly inhibiting a signaling event in the flg22 pathway. One
219 possibility is OPS may be directly targeting another member of the GSK3 protein family that is
220 instead known to regulate flg22-induced signaling, such as *Arabidopsis* Protein Kinase α (ASK α)
221 (28). Because genes from three independent branches of FLS2-flg22 signaling show the same
222 increased expression in the *ops* mutant backgrounds, it is likely that if OPS is acting directly to

Greenwood, et al.

223 regulate these signals, it must function early in flg22 signaling. A second possibility remains that
224 OPS has an indirect role in flg22 signaling. OPS is expressed in the sieve elements of the phloem
225 in *Arabidopsis* and previous studies identified that *ops* mutants display in incomplete sieve element
226 differentiation which results in a discontinuous protophloem and metaphloem cell file in the roots
227 (16, 17, 24). Therefore, our observation that *ops* mutants exhibit increased flg22 marker gene
228 expression could be the result of incomplete phloem transport, resulting in the loss of transport of
229 a yet unknown phloem-mobile regulator of flg22 responses.

230 Our results are consistent with reports that FLS2 expression is detected in the vasculature
231 of *Arabidopsis* cotyledons in roots (29, 30), however detection of FLS2 could only be confirmed
232 in the stele, which comprises both sieve elements and companion cells. FLS2 expression in the
233 stele suggests that some cells of the vasculature are capable of detecting and responding to flg22,
234 and while it has yet to be determined whether FLS2 expressed in the vasculature induces flg22
235 signaling events, our results indicate that some element of flg22 signaling does occur in the
236 phloem.

237 In addition to our data demonstrating a role for OPS in flg22 signaling, we found that
238 *Arabidopsis* loss-of-function *ops* mutants have shorter hypocotyls in response to BL treatments
239 and in the dark. This is consistent with other work showing that OPS overexpression lines display
240 increased BR responses (22), and confirms that OPS promotes BR-induced signaling for hypocotyl
241 elongation. Finding that OPS functions in both the BRI1 and FLS2 signaling pathways is
242 particularly interesting because these signaling pathways intersect downstream of initial receptor-
243 ligand perception. Initiation of the BRI1 signaling pathway induces expression of several *WRKY*
244 family transcription factors that inhibit the activation of gene expression of some flg22-induced
245 genes (none that were tested in this work) resulting in a suppression of defense responses (31, 32).
246 That OPS functions as a positive regulator of BL signaling may explain why in *ops* mutants, we
247 observe increased flg22-induced immune responses. While there is much left to understand about
248 the function of OPS, it remains one of the few phloem-localized proteins identified with a role in
249 immunity-related signaling events. Because it remains unknown whether sieve elements or
250 companion cells can directly test and respond to PAMPs, future studies could use OPS as a model
251 to begin answering these questions.

252

Greenwood, et al.

253 **Methods**

254 **Plant Material and Growth Conditions**

255 T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana*) *ops-3* SALK_089722 (20) and *ops-*
256 *4* SALK_042563 (20) were obtained from the Arabidopsis Biological Resource Center at The Ohio
257 State University (<https://abrc.osu.edu/>). Genotypes were confirmed using PCR (primers listed in
258 Supplemental Table 1). Surface-sterilized seeds were sown on 0.5X Murashige and Skoog
259 medium + 1% (w/v) sucrose solidified with 0.6% (w/v) agar as described. After 2 days
260 stratification at 4°C, seedlings were germinated and grown at 22°C with an 10-h-light/14-h-dark
261 photoperiod at 82 mmol m⁻² s⁻¹. Unless otherwise noted, seedlings were grown under these
262 conditions for 10 days before sample analysis.

263

264 **Peptides and Hormones**

265 The peptide flg22 (QRLSTGSRINSAKDDAAGLQIA) was purchased from Genscript and used
266 for elicitation at the indicated concentrations and for the indicated times as described. 24-
267 epibrassinolide (BL) was purchased from MilliporeSigma and used for the indicated times and
268 concentrations described.

269

270 **Root and Hypocotyl Measurements**

271 Root length measurements were performed as described (33). 10-day-old seedlings grown under
272 the conditions described, were traced using Fiji Free-hand tool. For hypocotyl measurements, 4
273 day-old-seedlings were treated with the indicated concentration of BL in liquid media and placed
274 at 22°C for an additional 7 days. Seedlings were removed and hypocotyls were traced using the
275 Fiji Free-hand tool.

276

277 **Flg22 Seedling Growth Inhibition**

278 Seedling growth inhibition was measured as previously described (23). Briefly, four-day-old
279 seedlings were aseptically transferred from MS agar to wells of a 12-well microtiter plate (three
280 seedlings per well) containing 1 ml of liquid MS medium with or without 1 μM flg22. After 14
281 days, seedling fresh weights were recorded.

282

283 **RNA Isolation and RT-qPCR**

Greenwood, et al.

284 RNA isolation, cDNA synthesis, and RT-qPCR were performed as previously described (34).
285 Unless stated otherwise, for each sample, three to five seedlings were elicited with the indicated
286 concentration of flg22 peptide and placed at 22°C for the time indicated. Tissue was flash frozen
287 in liquid nitrogen at the indicated time points. Total RNA was isolated from tissue using Trizol
288 reagent (Sigma-Aldrich) according to the manufacturer's protocol. RT-qPCR was performed on
289 cDNA using a Rotor-Q Real-Time PCR Cycler from Qiagen using gene-specific primers and
290 normalized to the *ACTIN* gene (listed in Supplemental Table 1).

291

References

- 292
293
294 1. Y. Jiang, C. X. Zhang, R. Chen, S. Y. He, Challenging battles of plants with phloem-
295 feeding insects and prokaryotic pathogens. *Proc Natl Acad Sci U S A* **116**, 23390-23397
296 (2019).
297 2. E. D. Ammar, J. George, K. Sturgeon, L. L. Stelinski, R. G. Shatters, Asian citrus psyllid
298 adults inoculate huanglongbing bacterium more efficiently than nymphs when this
299 bacterium is acquired by early instar nymphs. *Sci Rep* **10**, 18244 (2020).
300 3. M. Ghanim *et al.*, 'Candidatus Liberibacter asiaticus' Accumulates inside Endoplasmic
301 Reticulum Associated Vacuoles in the Gut Cells of Diaphorina citri. *Sci Rep* **7**, 16945
302 (2017).
303 4. P. Walerowski *et al.*, Clubroot Disease Stimulates Early Steps of Phloem Differentiation
304 and Recruits SWEET Sucrose Transporters within Developing Galls. *Plant Cell* **30**,
305 3058-3073 (2018).
306 5. R. Breia *et al.*, Plant SWEETs: from sugar transport to plant-pathogen interaction and
307 more unexpected physiological roles. *Plant Physiol* **186**, 836-852 (2021).
308 6. D. Couto, C. Zipfel, Regulation of pattern recognition receptor signalling in plants. *Nat*
309 *Rev Immunol* **16**, 537-552 (2016).
310 7. V. Nicaise, M. Roux, C. Zipfel, Recent advances in PAMP-triggered immunity against
311 bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol* **150**,
312 1638-1647 (2009).
313 8. L. Gomez-Gomez, T. Boller, FLS2: an LRR receptor-like kinase involved in the
314 perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* **5**, 1003-1011
315 (2000).
316 9. J. M. Smith *et al.*, Loss of Arabidopsis thaliana Dynamin-Related Protein 2B reveals
317 separation of innate immune signaling pathways. *PLoS Pathog* **10**, e1004578 (2014).
318 10. D. A. Korasick *et al.*, Novel functions of Stomatal Cytokinesis-Defective 1 (SCD1) in
319 innate immune responses against bacteria. *J Biol Chem* **285**, 23342-23350 (2010).
320 11. J. Monaghan, C. Zipfel, Plant pattern recognition receptor complexes at the plasma
321 membrane. *Curr Opin Plant Biol* **15**, 349-357 (2012).
322 12. T. A. DeFalco, C. Zipfel, Molecular mechanisms of early plant pattern-triggered immune
323 signaling. *Mol Cell* **81**, 4346 (2021).
324 13. M. Kalde, T. S. Nuhse, K. Findlay, S. C. Peck, The syntaxin SYP132 contributes to plant
325 resistance against bacteria and secretion of pathogenesis-related protein 1. *Proc Natl*
326 *Acad Sci U S A* **104**, 11850-11855 (2007).
327 14. T. S. Nuhse, A. R. Bottrill, A. M. Jones, S. C. Peck, Quantitative phosphoproteomic
328 analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate
329 immune responses. *Plant J* **51**, 931-940 (2007).
330 15. J. J. Benschop *et al.*, Quantitative phosphoproteomics of early elicitor signaling in
331 Arabidopsis. *Mol Cell Proteomics* **6**, 1198-1214 (2007).
332 16. E. Truernit, H. Bauby, K. Belcram, J. Barthelemy, J. C. Palauqui, OCTOPUS, a polarly
333 localised membrane-associated protein, regulates phloem differentiation entry in
334 Arabidopsis thaliana. *Development* **139**, 1306-1315 (2012).
335 17. M. A. Ruiz Sola *et al.*, OCTOPUS-LIKE 2, a novel player in Arabidopsis root and
336 vascular development, reveals a key role for OCTOPUS family genes in root metaphloem
337 sieve tube differentiation. *New Phytol* **216**, 1191-1204 (2017).

Greenwood, et al.

- 338 18. A. S. Breda, O. Hazak, C. S. Hardtke, Phosphosite charge rather than shootward
339 localization determines OCTOPUS activity in root protophloem. *Proc Natl Acad Sci U S*
340 *A* **114**, E5721-E5730 (2017).
- 341 19. Y. H. Kang, A. Breda, C. S. Hardtke, Brassinosteroid signaling directs formative cell
342 divisions and protophloem differentiation in Arabidopsis root meristems. *Development*
343 **144**, 272-280 (2017).
- 344 20. H. Roschzttardtz *et al.*, The VASCULATURE COMPLEXITY AND CONNECTIVITY
345 gene encodes a plant-specific protein required for embryo provascular development.
346 *Plant Physiol* **166**, 889-902 (2014).
- 347 21. B. Gujas *et al.*, A Reservoir of Pluripotent Phloem Cells Safeguards the Linear
348 Developmental Trajectory of Protophloem Sieve Elements. *Curr Biol* **30**, 755-766 e754
349 (2020).
- 350 22. P. Anne *et al.*, OCTOPUS Negatively Regulates BIN2 to Control Phloem Differentiation
351 in Arabidopsis thaliana. *Curr Biol* **25**, 2584-2590 (2015).
- 352 23. J. C. Anderson *et al.*, Arabidopsis MAP Kinase Phosphatase 1 (AtMKP1) negatively
353 regulates MPK6-mediated PAMP responses and resistance against bacteria. *Plant J* **67**,
354 258-268 (2011).
- 355 24. A. Rodriguez-Villalon *et al.*, Molecular genetic framework for protophloem formation.
356 *Proc Natl Acad Sci U S A* **111**, 11551-11556 (2014).
- 357 25. A. Rodriguez-Villalon, B. Gujas, R. van Wijk, T. Munnik, C. S. Hardtke, Primary root
358 protophloem differentiation requires balanced phosphatidylinositol-4,5-biphosphate
359 levels and systemically affects root branching. *Development* **142**, 1437-1446 (2015).
- 360 26. S. Fujioka *et al.*, The Arabidopsis deetiolated2 mutant is blocked early in brassinosteroid
361 biosynthesis. *Plant Cell* **9**, 1951-1962 (1997).
- 362 27. W. Ma *et al.*, Citrus Huanglongbing is a pathogen-triggered immune disease that can be
363 mitigated with antioxidants and gibberellin. *Nature Communications* **13**, 529 (2022).
- 364 28. H. Stampfl, M. Fritz, S. Dal Santo, C. Jonak, The GSK3/Shaggy-Like Kinase ASKalpha
365 Contributes to Pattern-Triggered Immunity. *Plant Physiol* **171**, 1366-1377 (2016).
- 366 29. M. Beck *et al.*, Expression patterns of flagellin sensing 2 map to bacterial entry sites in
367 plant shoots and roots. *J Exp Bot* **65**, 6487-6498 (2014).
- 368 30. I. Wyrsh, A. Dominguez-Ferreras, N. Geldner, T. Boller, Tissue-specific FLAGELLIN-
369 SENSING 2 (FLS2) expression in roots restores immune responses in Arabidopsis fls2
370 mutants. *New Phytol* **206**, 774-784 (2015).
- 371 31. R. Lozano-Duran *et al.*, The transcriptional regulator BZR1 mediates trade-off between
372 plant innate immunity and growth. *Elife* **2**, e00983 (2013).
- 373 32. Y. Belkhadir, L. Yang, J. Hetzel, J. L. Dangl, J. Chory, The growth-defense pivot: crisis
374 management in plants mediated by LRR-RK surface receptors. *Trends Biochem Sci* **39**,
375 447-456 (2014).
- 376 33. G. Ekanayake *et al.*, DYNAMIN-RELATED PROTEIN DRP1A functions with DRP2B
377 in plant growth, flg22-immune responses, and endocytosis. *Plant Physiol* **185**, 1986-2002
378 (2021).
- 379 34. C. A. Collins *et al.*, EPSIN1 Modulates the Plasma Membrane Abundance of
380 FLAGELLIN SENSING2 for Effective Immune Responses. *Plant Physiol* **182**, 1762-
381 1775 (2020).
- 382

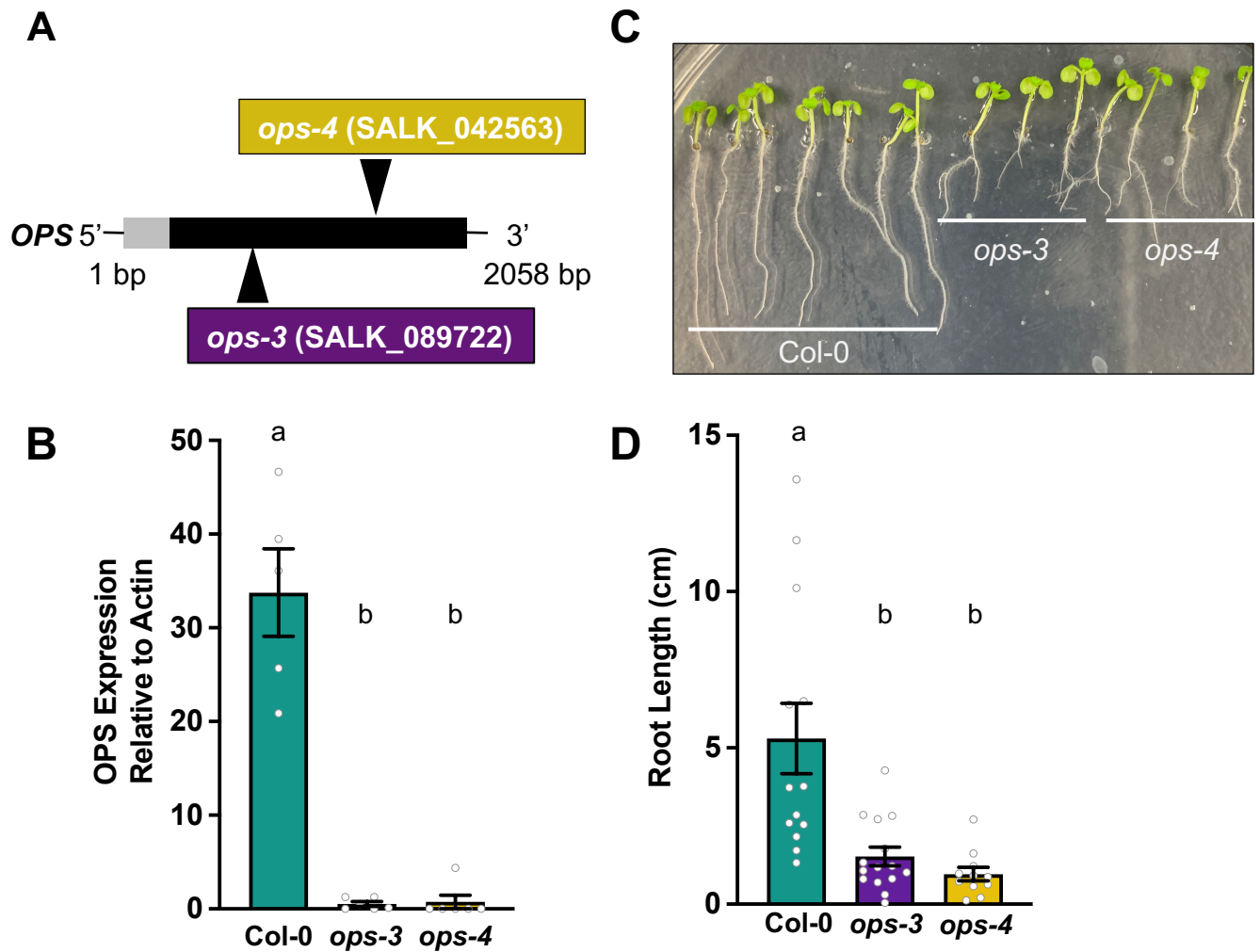


Figure 1. Characterization of two independent *OPS* T-DNA insertion lines

A. Location of T-DNA insertions in *OPS* and mutants used in this study. **B.** Expression of *OPS* mRNA in Col0 and *ops* mutants measured using qPCR. *OPS* expression is shown relative to *Actin*, $n=6$. **C.** Comparison of root length between Col-0 WT, *ops-3* and *ops-4* mutants from vertically grown, day 7 old seedlings. Shown is a representative image. **D.** Analysis of root length in Col-0 WT, *ops-3*, and *ops-4* mutants. Measurements were made from images (as in C) using ImageJ, $n = 20$. Letters above all bars represent statistical differences as measured by a one-way ANOVA with Tukey multiple comparison test where $P < 0.05$. All error bars represent \pm SE.

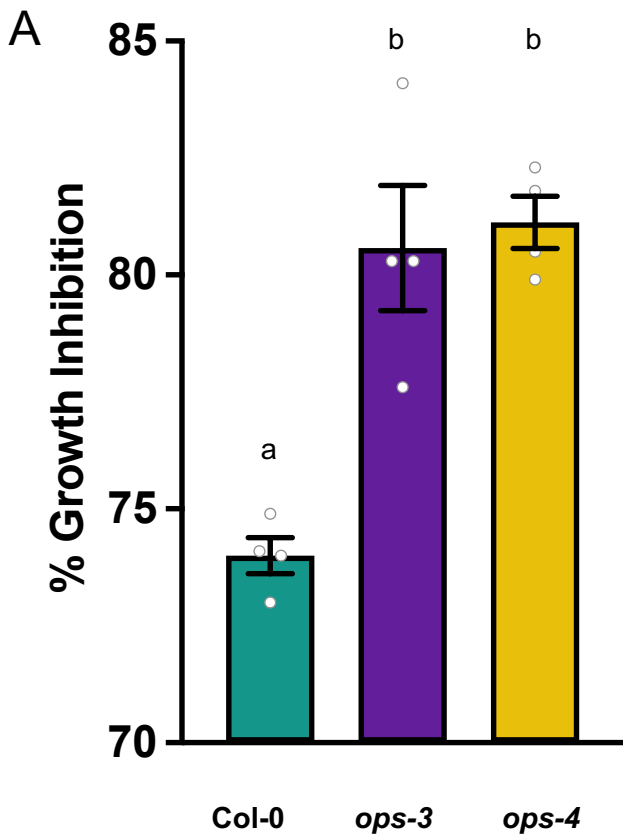


Figure 2. Lack of *OPS* increases flg22-induced growth inhibition

A. Four-day old seedlings were grown in the presence of 0 or 1 μ M flg22 for two weeks before measuring their fresh weights and calculating the percent growth inhibition between growth conditions. Shown are percent change calculations pooled from 4 independent experiments, $n = 40-60$ seedlings per genotype per experiment. ANOVA with Tukey multiple comparison test with a $P < 0.05$. All error bars are \pm SE

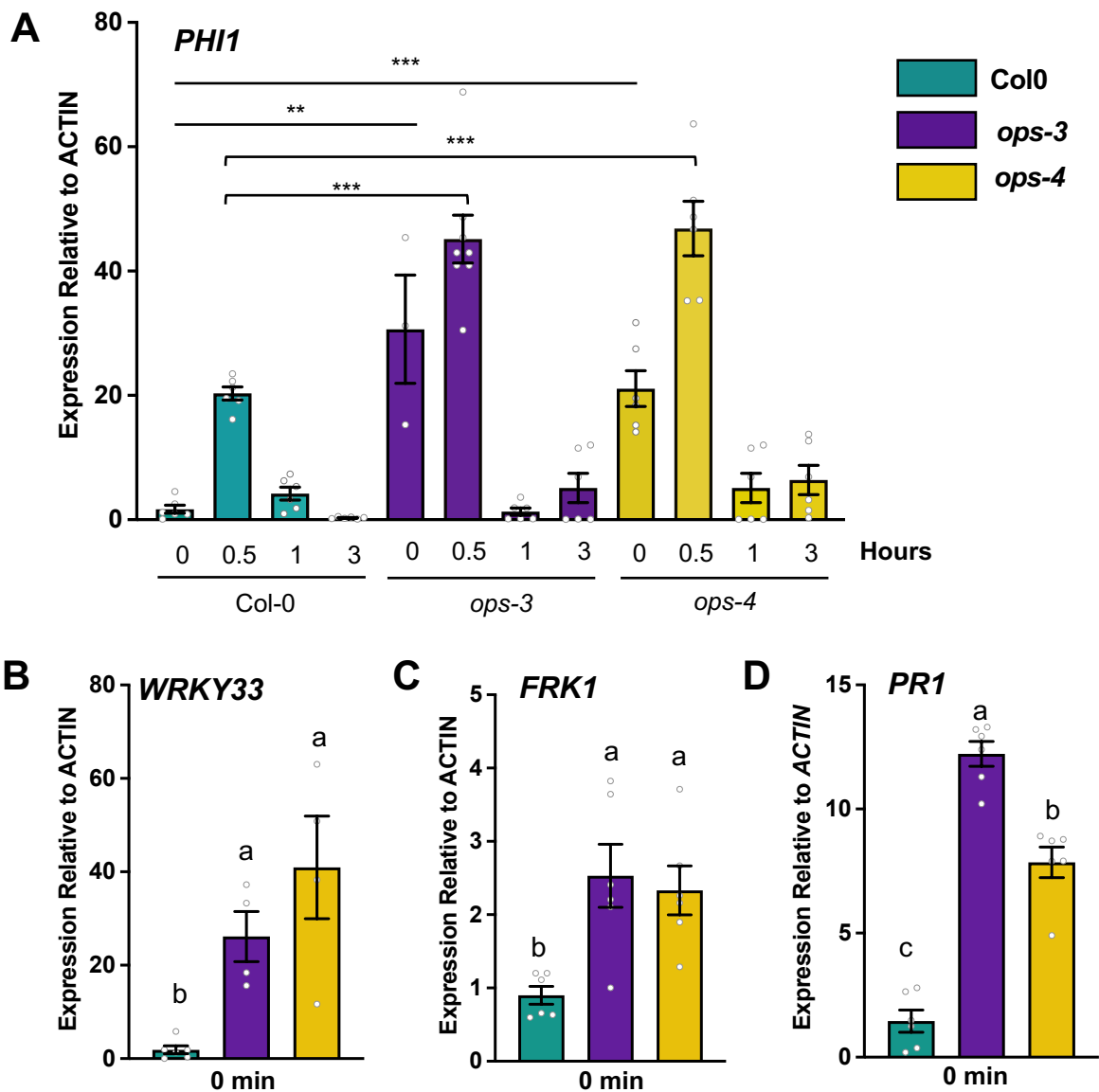


Figure 3. OPS functions as a negative regulator of flg22-induced immune signaling
A. Ten-day old old seedlings (Col-0 WT, *ops-3*, and *ops-4*) were treated with 100 nM flg22 and qPCR was used to measure expression of marker gene *PHI1* over a 3-hour time-course. Shown are means of pooled seedling samples (n = 4). Asterisks indicate significant differences compared with Col-0 0 min (lines) and Col-0 30 min (brackets) using a two-tailed Student's *t*-test ** $P > 0.01$, *** $P > 0.0001$. **B-D.** qPCR analysis of untreated, ten-day old seedlings (Col-0 WT, *ops-3*, and *ops-4*). Expression of the marker genes *WRKY33* (**B**), *FRK1* (**C**), and *PR1* (**D**) was examined. Letters represent statistical differences as measured by one-way ANOVA with Tukey multiple comparison at $P < 0.01$. All error bars are \pm SE. Experiments were repeated at least three times with similar results.

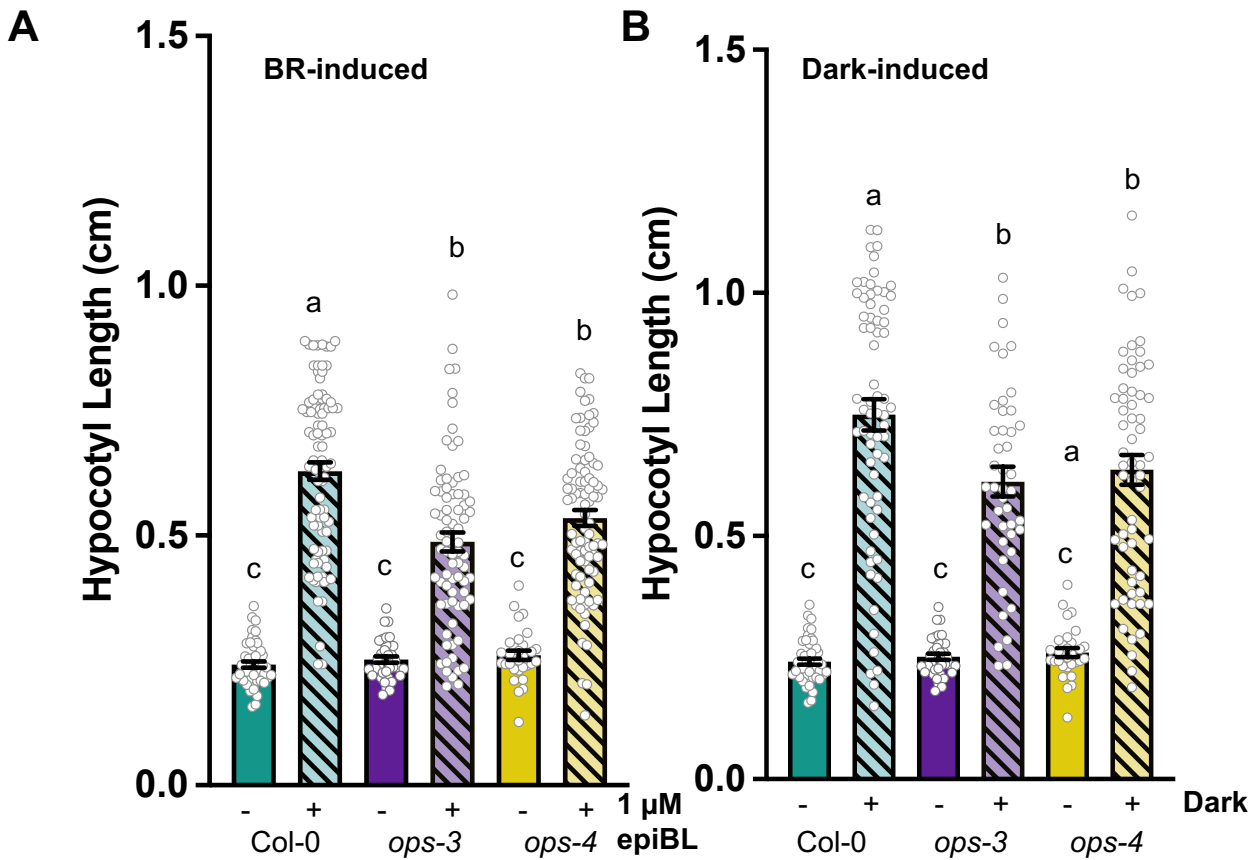
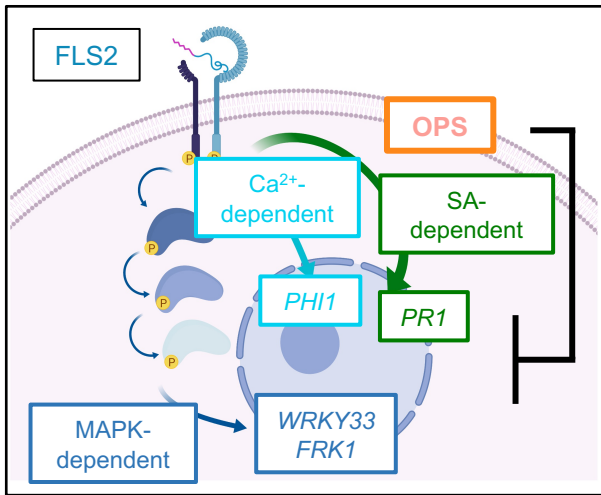


Figure 4. OPS is necessary for hypocotyl elongation

A. Hypocotyl length measurements taken of ten-day old seedlings (Col-0, *ops-3*, and *ops-4*) treated with 0 (-) or 1 (+) μ M epibrassinolide. Values shown are pooled from three independent experiments, $n = 60-90$. **B.** Length of hypocotyls of ten-day old seedlings grown in the light (-) or the dark (+) for Col-0 WT, *ops-3*, and *ops-4*. Measurements are pooled from three independent experiments, $n = 30-60$. Letters represent statistical differences as measured by two-way ANOVA with Tukey multiple comparison test at $P < 0.05$. Error bars are \pm SE and all experiments were repeated at least three times with similar results.

Col-0



ops

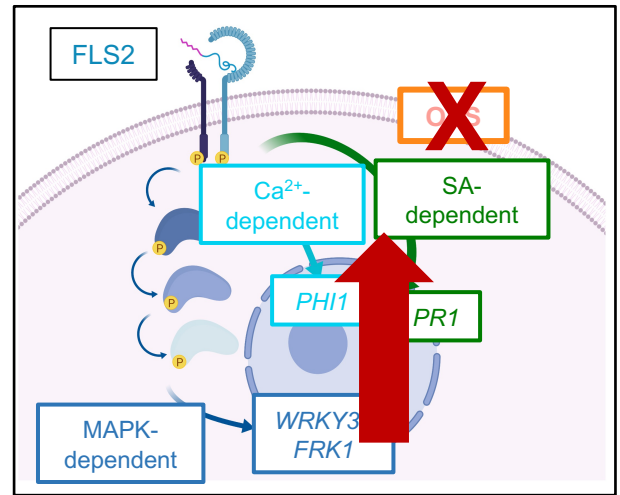


Figure 5. Summary of OPS flg22 signaling phenotype

Loss of the protein OPS (*ops*; right panel) results in increased expression of marker genes from three independent branches of the FLS2 signaling network (red arrow). This indicates that OPS plays a role in suppressing flg22-induced signaling (left panel).