

1 **A microbially derived tyrosine sulfated peptide mimics a plant peptide hormone**

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23 **Summary**

24 • The biotrophic pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) produces a sulfated
25 peptide named RaxX, which shares similarity to peptides in the PSY (*p*lant *p*eptide containing
26 sulfated *t*yrosine) family. We hypothesize that RaxX functionally mimics the growth stimulating
27 activity of PSY peptides.

28 • Root length was measured in Arabidopsis and rice treated with synthetic RaxX peptides.
29 We also used comparative genomic analysis and Reactive Oxygen Species (ROS) burst assay to
30 evaluate the activity of RaxX and PSY peptides.

31 • Here we found that a synthetic sulfated RaxX derivative comprising 13 residues
32 (RaxX13-sY), highly conserved between RaxX and PSY, induces root growth in Arabidopsis
33 and rice in a manner similar to that triggered by PSY. We identified residues that are required for
34 activation of immunity mediated by the rice XA21 receptor but that are not essential for root
35 growth induced by PSY. Finally, we showed that a *Xanthomonas* strain lacking *raxX* is impaired
36 in virulence.

37 • These findings suggest that RaxX serves as a molecular mimic of PSY peptides to
38 facilitate *Xoo* infection and that XA21 has evolved the ability to recognize and respond
39 specifically to the microbial form of the peptide.

40
41 Key words: molecular mimicry, PSY1, RaxX, tyrosine sulfated peptide, root growth, XA21,
42 *Xanthomonas oryzae* pv. *oryzae*

43

44 **Introduction**

45 Some plant and animal pathogens employ molecular mimicry to gain evolutionary
46 advantages (Mitchum *et al.*, 2012). Such microbial molecules include those that mimic ligands of
47 host receptors, substrates of host enzymes, or host proteins themselves (Knodler *et al.*, 2001;
48 Nesic *et al.*, 2010). Some plant pathogens produce small molecules that mimic plant hormones
49 required for growth, development and regulation of innate immunity.

50 A well-studied case of hormone mimicry in plants is the production of coronatine by the
51 gram-negative biotrophic bacterium *Pseudomonas syringae* (Weiler *et al.*, 1994). Coronatine
52 structurally and functionally mimics jasmonoyl-L-isoleucine (JA-Ile), a bioactive form of the
53 plant hormone jasmonic acid (JA) (Weiler *et al.*, 1994). JA positively regulates defense against

54 chewing insects and necrotrophic pathogens and negatively regulates defense against biotrophic
55 and hemibiotrophic pathogens. Coronatine produced during *P. syringae* infection mimics JA
56 action, suppressing the host defense response.

57 Plant parasitic nematodes and fungi also produce mimics of endogenous plant hormones.
58 For example, nematodes produce peptides similar to plant CLAVATA3/ESR (CLE) peptides
59 (Chen *et al.*, 2015), which regulate shoot meristem differentiation, root growth, and vascular
60 development. Nematode CLEs are secreted into plant tissues where they induce specific host
61 cells to differentiate into feeding cells that benefit the parasite (Wang *et al.*, 2005; Mitchum *et al.*,
62 2008; Yamaguchi *et al.*, 2016). Another example is C-TERMINALLY ENCODED PEPTIDES
63 (CEPs), a large and diverse family of effector peptides produced by sedentary plant-parasitic
64 nematodes (PPNs). Plant CEPs inhibit root growth and increase the gene expression of a nitrogen
65 transporter in response to nitrogen starvation. It is hypothesized that the parasite produced CEPs
66 promote nitrogen uptake and reduce the size of the feeding site where the PPNs maintain
67 biotrophic interactions (Eves-Van Den Akker *et al.*, 2016). Finally, the root-infecting fungus
68 *Fusarium oxysporum* secretes a functional mimic of plant regulatory peptide RALF (rapid
69 alkalization factor). RALF from *Fusarium oxysporum* induces extracellular alkalization in
70 the host apoplast which favors pathogen multiplication (Murphy & De Smet, 2014; Masachis *et*
71 *al.*, 2016).

72 We have recently shown that the rice receptor XA21 is activated by a sulfated protein,
73 called RaxX, produced by the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). RaxX
74 triggers a robust and effective immune response in rice expressing XA21 (Song *et al.*, 1995;
75 Pruitt *et al.*, 2015). A synthetic 21-amino acid sulfated derivative of RaxX (RaxX21-sY) from
76 *Xoo* strain PXO99 (Fig. 1A) is sufficient to activate XA21-mediated immune responses (Pruitt *et*
77 *al.*, 2015).

78 Sequence analysis revealed that RaxX21 is similar to the peptide hormone PSY (plant
79 peptide containing sulfated tyrosine), which promotes cellular proliferation and expansion in
80 Arabidopsis (Amano *et al.*, 2007) (Pruitt *et al.*, 2015). Arabidopsis PSY1 (AtPSY1) is the best-
81 characterized member of the plant PSY peptide family. AtPSY1 is an 18-amino acid
82 glycopeptide with a single sulfotyrosine residue (Fig. 1A) (Amano *et al.*, 2007) that is secreted,
83 processed from a 75 amino acid precursor and promotes root elongation primarily through
84 regulation of cell size. AtPSY1 is widely expressed in Arabidopsis tissues (Amano *et al.*, 2007).

85 AtPSY1 promotes acidification of the apoplastic space through activation of membrane proton
86 pumps (Fuglsang *et al.*, 2014). This acidification is thought to activate pH-dependent expansins
87 and cell wall remodeling enzymes that loosen the cellulose network (Cosgrove, 2000; Hager, 2003).
88 Concomitant water uptake by the cell leads to cellular expansion. In addition to PSY, plants
89 produce three other classes of tyrosine sulfated peptides: *phytosulfokine* (PSK) (Matsubayashi &
90 Sakagami, 1996), *root meristem growth factor* (RGF) (Matsuzaki *et al.*, 2010) and Casparian
91 strip integrity factor (CIF) (Doblas *et al.*, 2017; Nakayama *et al.*, 2017). PSK, RGF and CIF are
92 also processed, secreted, and play roles in regulation of growth, development and Casparian strip
93 diffusion barrier formation in the root.

94 Here we demonstrate that RaxX peptides derived from diverse *Xanthomonas* species
95 promote root growth, mimicking the growth promoting activities of PSY peptides. We also show
96 that a *Xanthomonas* strain lacking *raxX* is impaired in its ability to infect rice lacking XA21,
97 suggesting that RaxX is a virulence factor. Unlike RaxX, PSY peptides do not activate XA21-
98 mediated immunity. Thus, XA21 is a highly selective immune receptor capable of specifically
99 recognizing the bacterial mimic. Based on these findings we propose a model whereby *Xoo* and
100 other *Xanthomonas* strains produce RaxX to reprogram the host environment by hijacking PSY
101 signaling. XA21 later evolved to recognize and respond to specifically to RaxX.

102

103 **Materials and Methods**

104

105 *Identification of Putative RaxX proteins*

106 Putative PSY orthologs were identified by NCBI Protein BLAST analysis using the
107 default settings for short sequences (Altschul *et al.*, 1990). For *Solanum lycopersicum* BLAST
108 was performed using the Sol Genomics Network with the BLOSUM 62 matrix
109 (<https://solgenomics.net/tools/blast/>). Proteins were identified from a single source for each
110 plant: *Arabidopsis thaliana* Col-0 (refseq_protein, taxid: 3702), *Oryza sativa* Nipponbare
111 (refseq_protein, taxid: 39947), *Triticum aestivum* Chinese Spring (taxid:4565), *Musa acuminata*
112 subsp. *Malaccensis* (refseq_protein, taxid 214687), *S. lycopersicum* cv. Heinz 1706 (ITAG
113 release 2.40). BLAST was initially performed with the 18 amino acid sequence of AtPSY1
114 (DYGDPSANPKHDPGVPPS). Criteria for selection were as follows: (1) Candidates must
115 match the query with an expect-value ≤ 20 for NCBI Protein BLAST analysis (PAM 30 matrix),

116 (2) Candidates must have an invariant Asp-Tyr at the beginning of the query, (3) The full length
117 protein must be between 60 and 200 amino acids with the PSY-like motif in the second half, (4)
118 The protein must be predicted to have a secretion signal by SignalP 4.0 (Petersen *et al.*, 2011).
119 Additional candidates were identified by subsequent iterative BLAST with the 18 amino acid
120 RaxX sequences from candidate RaxX proteins identified in the initial BLAST. The final list is
121 shown in Figure S1. If multiple splicing variants were identified in the search, only one was
122 listed.

123

124 *Sequence analysis and visualization*

125 The sequence alignments in S9 were generated with Geneious software using default
126 parameters (Kearse *et al.*, 2012). Sequence logos (Fig. 1b) were constructed using WebLogo
127 (Schneider & Stephens, 1990; Crooks *et al.*, 2004) with the 13-amino acid RaxX sequences
128 shown in Table S1 and the PSY ortholog sequences in Fig. S1. The bit score for a given residue
129 indicates the conservation at that position, while the size of the individual letters within the stack
130 indicate relative frequency of that amino acid at the position.

131

132 *Arabidopsis growth conditions*

133 All *Arabidopsis thaliana* used in this study were in the Col-0 background. The *AtTPST*
134 mutant, *tpst-1*, (SALK_009847) and homozygous *At1g72300* mutant (SALK_072802C) were
135 obtained from the Arabidopsis Biological Resource Center (ARBC). A homozygous *tpst-1* line was
136 isolated from progeny of the SALK_009847 seeds. The *AtPSKR1/AtPSKR2/At1g72300* triple
137 receptor mutant (Mosher *et al.*, 2013) was obtained from Birgit Kemmerling's laboratory. Plants
138 were grown on the indicated media or on Sungro professional growing mix under continuous light.

139

140 *RaxX and PSY1 peptides*

141 The peptides used in this study are listed in Table S2. All peptides other than RaxX21-Y
142 are tyrosine sulfated as indicated (Y^S). The synthetic AtPSY1 peptide used in these experiments
143 lacks the hydroxy- and L-Ara₃- modifications at the C-terminus. The natural processed, modified
144 state of OsPSY1a is not known. The 18-amino OsPSY1a acid peptide was synthesized based on
145 alignment with AtPSY1. RaxX13-sY was obtained from Peptide 2.0. All other peptides were

146 obtained from Pacific Immunology. One batch of peptides was tested for each sequence. The
147 peptides were resuspended in ddH₂O.

148

149 *Arabidopsis root growth assays*

150 Arabidopsis seeds were treated with 30% bleach for 12 minutes and then washed 4-5
151 times with autoclaved water. Sterilized seeds were incubated in the dark at 4 °C for 3-4 days.
152 Plates were prepared with 0.5× Murashige and Skoog (MS) medium with vitamins (Caisson,
153 MSP09), 1% sucrose, pH 5.7, 0.5% Phytigel (Sigma, P8169). Peptide (or water for mock
154 treatments) was added to the indicated concentration (from a 1 mM stock) just before pouring
155 into a plate. Seeds were placed on the plate (20 seeds per plate), and the lids were secured with
156 Micropore surgical tape (1530-0). Plates were incubated vertically under continuous light (55
157 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 24 °C. Seedlings with delayed germination were marked after 3 days, and were
158 not included in the analysis. Root lengths were measured after 8 days.

159

160 *Arabidopsis live imaging of root growth*

161 Live imaging of roots was performed as described previously with modifications to the
162 media (Duan *et al.*, 2013; Geng *et al.*, 2013). Sterilized *tpst-1* seeds were grown on 1% agar
163 media containing 1× MS nutrients (Caisson, MSP01), 1% sucrose, and 0.5 g l⁻¹ MES, adjusted to
164 pH 5.7 with KOH. After 6 days, seeds were transferred to 0.5% Phytigel (Sigma, P8169) media
165 containing 0.5× MS (Caisson, MSP09, 1% sucrose, and 0.5 g l⁻¹ MES, adjusted to pH 5.7 with
166 KOH) with or without the indicated peptides. Imaging and semiautomated image analysis were
167 performed as described previously (Geng *et al.*, 2013).

168

169 *Rice root growth assays*

170 Seeds of *Oryza sativa* sp. *japonica* cultivars Kitaake (lacking the *Xa21* gene), a
171 transgenic line of Kitaake carrying *Xa21* (XA21-Kitaake), Taipei 309 (TP309), (lacking the
172 *Xa21* gene), or a transgenic line of TP309 carrying *Xa21* driven by its native promoter (XA21-
173 TP309) were dehusked and sterilized with 30% bleach for 30 min. The seeds were washed 4-5
174 times with water and plated to cups with 50 mL 0.5× MS (Caisson MSP09), 1% sucrose (pH 5.7
175 with KOH/ NaOH) containing 0.25% Phytigel. Peptides were added to 100 nM just before
176 pouring into the cups. 20 seedlings were added per cup, and the cups were sealed with clear lids.

177 The seedling roots were measured after 4-6 day incubation in a 28 °C chamber with 13 h/11 h
178 light/dark cycle and a light intensity of 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

179

180 *ROS Assays*

181 Kitaake and XA21-Kitaake rice plants were grown as previously described (Pruitt *et al.*,
182 2015). Briefly, seeds were germinated on water-soaked paper and transplanted in sandy soil in 5.5
183 inch square pots. Plants were grown in tubs filled with fertilizer water in greenhouse. Six weeks
184 after planting the rice was transferred to a growth chamber set to 28 °C/24 °C, 80%/85%
185 humidity, and 14/10 h lighting for the day/night cycle. ROS assays were carried out using leaves
186 of 6-week-old rice plants as described previously (Pruitt *et al.*, 2015). Briefly, leaves were cut
187 longitudinally along the mid vein and then transversely into 1- to 1.5-mm-thick leaf pieces. After
188 overnight incubation floating on sterile water, leaf pieces were transferred into a 96-well white
189 plate (2 pieces per well). Each well contained 100 μl of excitation solution [0.2 mM L-012
190 (Wako) and 50 $\mu\text{g ml}^{-1}$ horseradish peroxidase (Sigma)]. The indicated concentration of peptides
191 was added (or water for mock control), and chemiluminescence was measured for 90 minutes
192 with a TriStar (Berthold) plate reader.

193

194 *Xanthomonas inoculation on rice*

195 TP309 and XA21-TP309 were greenhouse grown as described above for Kitaake. Plants
196 were inoculated 3 days after transfer using the scissors clipping method (Kauffman *et al.*, 1973).
197 PXO99 strains were grown on peptone sucrose agar (PSA) plates at 28 °C with the appropriate
198 antibiotic(s). The bacteria were resuspended in water at a density of 10^6 colony forming units per
199 mL. Water soaked lesions were measured 14 days after inoculation. Bacterial growth analysis *in*
200 *planta* was performed as previously described (Bahar *et al.*, 2014). PXO99 strains used in this
201 study were previously reported (Pruitt *et al.*, 2015). PXO99 Δ *raxX* is a marker free mutant and
202 PXO99 Δ *raxST* is a marker exchange mutant with a spectinomycin resistance gene. The *raxX* and
203 *raxST* sequences including their predicted promoter were cloned into pVSP61 vector (Loper &
204 Lindow, 1994) and transformed into PXO99 strains.

205

206 **Results**

207 *RaxX is similar in sequence to PSY peptides*

208 The region of similarity between RaxX from *Xoo* and AtPSY1 corresponds to amino
209 acids 40-52 of RaxX. RaxX and AtPSY1 share 10 identical residues over this region (Fig. 1a).
210 RaxX is sulfated by the bacterial sulfotransferase RaxST on Y41, which corresponds to the
211 sulfated residue of AtPSY1 (Amano *et al.*, 2007; Pruitt *et al.*, 2015). An aspartate precedes the
212 sulfated tyrosine in both RaxX and AtPSY1. The presence of a nearby acidic residue is a
213 common hallmark of tyrosine sulfation sites (Moore, 2009).

214 We extended our analysis to include PSY orthologs and RaxX peptides from diverse
215 species (Fig. S1-2, Table S1). BLAST search using the 18 amino acid AtPSY1 as a query
216 identified 8 PSY-like proteins in rice (Fig. S1). One of the rice PSY proteins, OsPSY1
217 (Os05g40850), has four nearly identical PSY-like repeats, the first of which (OsPSY1a) is shown
218 in Fig 1. Analysis of Arabidopsis using the same criteria also revealed a total of eight PSY-like
219 proteins including the three that had been previously identified (Fig. S1) (Amano *et al.*, 2007;
220 Matsubayashi, 2014). We also identified PSY-like proteins in tomato, banana and wheat, three
221 diverse and economically important crops (Fig. S1). Alignment of PSY peptides from these
222 different species revealed a highly conserved 13-amino acid region beginning with the aspartate-
223 tyrosine residue pair (Fig. S1). This 13-amino acid sequence corresponds precisely to the region
224 of sequence similarity between RaxX and AtPSY1 (Fig. 1a).

225 Alignment of the RaxX sequences from diverse strains reveals a region of high
226 conservation immediately around the tyrosine, which is sulfated in *Xoo* strain PXO99 (Fig. S2).
227 Sequence logos were constructed for the PSY-like motif using the identified RaxX and PSY
228 sequences (Fig. 1b). These logos further highlight the similarity of 13-amino acid region of
229 RaxX and PSY sequences. Residues that are highly variable in RaxX are also highly variable in
230 PSY. Based on the similarity of RaxX and PSY peptides and the finding that RaxX is also
231 tyrosine sulfated (Pruitt *et al.*, 2015), we hypothesized that RaxX serves as a functional mimic of
232 PSY peptides and that RaxX may have PSY-like activity.

233
234 *RaxX promotes root growth similar to PSY peptides*

235 To test our hypothesis that RaxX is a functional mimic of PSY peptides, we evaluated the
236 effect of RaxX21 treatment on root growth. We first tested the peptides on Arabidopsis seedlings,
237 because PSY signaling has been studied exclusively in this system. RaxX21-sY promoted root
238 growth in a similar manner to that observed for AtPSY1 in Arabidopsis (Fig. 2a, b). After 8 days

239 on media containing 100 nM RaxX21-sY, the average root length of Col-0 seedlings was 61 mm
240 whereas seedlings grown on plates without peptide had an average root length of 54 mm. Similar
241 root growth-promoting effects were observed in experiments using AtPSY1 and OsPSY1a
242 peptides (Fig. 2a, b).

243 We also performed root growth experiments on an Arabidopsis line lacking AtTPST, the
244 tyrosine sulfotransferase responsible for modification of PSY, PSK and RGF peptides (Komori
245 *et al.*, 2009; Matsuzaki *et al.*, 2010). *tpst-1* mutant plants are dwarf and have stunted roots
246 (Komori *et al.*, 2009). Because this mutant lacks endogenous PSY, PSK and RGF signaling,
247 effects of exogenous application of sulfated peptides can be better quantified (Igarashi *et al.*,
248 2012; Mosher *et al.*, 2013). Consistent with earlier reports, we observed that mock treated
249 *tpst-1* mutant seedlings have much shorter roots than Col-0 (Fig. 2a-d). Treatment of *tpst-1*
250 plants with RaxX21-sY or AtPSY1 increases root growth 1.5-2 fold relative to mock treatment
251 (Fig 2c, d).

252 We determined the minimum concentration of RaxX21-sY needed to induce root growth
253 in Arabidopsis. *tpst-1* seeds were grown on plates containing 0.1-250 nM peptide. RaxX21-sY
254 was effective at inducing root growth at concentrations in the low nanomolar range (Fig. S3).
255 This activity is comparable to PSK (Fig. S3). Nonsulfated RaxX21 (RaxX21-Y) also promoted
256 root growth, but was less active than the sulfated version (Fig. 2a-d, S3). AtPSY1 was less active
257 than RaxX21-sY and PSK. We hypothesize that the reduced potency of the synthetic AtPSY1
258 used in this study was due to the lack of glycosylation (See materials and methods).
259 Glycosylation of AtPSY1 was previously shown to be important for full activity (Amano *et al.*,
260 2007).

261 We next used a live root imaging system (Duan *et al.*, 2013; Geng *et al.*, 2013) to assess
262 changes in root growth rate upon exposure to RaxX21-sY. Root growth of *tpst-1* seedlings on
263 plates containing 250 nM RaxX21-sY, AtPSY1 or no peptide (Mock) was monitored over 24 h.
264 Within 4-5 hours, seedlings grown on RaxX21-sY- or AtPSY1-containing plates had an
265 increased root growth rate compared to seedlings on mock plates (Fig. 2e).

266 Because RaxX21-sY comes from the rice pathogen *Xoo*, we tested whether this peptide
267 also has growth promoting activity in rice seedlings. AtPSY1 and RaxX21-sY treatment
268 significantly enhanced root growth on rice varieties Tapei 309 (Fig. 2f) and Kitaake (Fig. S4).
269 We also tested if the root growth promotion activity is attenuated in the presence of XA21. We

270 found that treatment of RaxX21-sY still induced longer roots in XA21-TP309 plants (Fig. S5).
271 We hypothesize that RaxX21-sY fails to activate XA21 in young seedlings, because XA21-
272 mediated immune response is developmentally controlled in rice (Century *et al.*, 1999).
273 Collectively, these results indicate that RaxX21-sY promotes root growth in a similar manner to
274 PSY and PSK peptides in both Arabidopsis and rice.

275

276 *RaxX induces root growth through the same signaling pathway as PSY1*

277 To determine if RaxX induces root growth using the same signaling pathway as AtPSY1,
278 we grew Arabidopsis seedlings on plates containing both RaxX and AtPSY1 peptides. Roots of
279 Arabidopsis seedlings grown on plates containing 100 nM RaxX21-sY and 100 nM AtPSY1
280 were similar in length to those grown on plates with 100 nM RaxX21-sY alone (Fig. 3). Similar
281 results were observed when seedlings were co-treated with 100 nM RaxX21-sY and 100 nM
282 PSK (Fig. 3). The observation that RaxX, AtPSY1, and PSK do not have additive effects on root
283 growth suggests that these peptides induce root growth via the same pathway. Alternatively, it
284 may be that the 100 nM RaxX21-sY treatment already reached the maximum growth potential
285 (Matsuzaki *et al.*, 2010).

286

287 *At1g72300 is not required for induction of root growth by RaxX or AtPSY1*

288 The leucine-rich repeat receptor kinase encoded by *At1g72300* has been proposed to
289 serve as the AtPSY1 receptor (Amano *et al.*, 2007). We therefore tested if *At1g72300* is required
290 for perception of RaxX21-sY. For these assays we used the *At1g72300* mutant line
291 SALK_072802C. This is the same line used in all published studies of PSY1/At1g72300,
292 (Amano *et al.*, 2007; Mosher & Kemmerling, 2013; Mosher *et al.*, 2013; Fuglsang *et al.*, 2014;
293 Mahmood *et al.*, 2014), and was shown to have the lowest transcript level of available mutants
294 (Fuglsang *et al.*, 2014). We independently validated the mutant genotype (Fig. S6). We found
295 that treatment of the *At1g72300* mutant line with either RaxX21-sY or AtPSY1 increased root
296 growth in a similar manner to that observed for treatment of wild type Col-0 seedlings (Fig. 2a, b,
297 4). We also found that a mutant lacking *At1g72300* and the homologous PSK receptors,
298 *AtPSKR1* and *AtPSKR2*, (*pskr1/pskr2/At1g72300*) also responds to RaxX and AtPSY1
299 treatment (Fig. 4). *pskr1/pskr2/At1g72300* did not respond to synthesized Arabidopsis PSK

300 (AtPSK), whereas PSK promotes root growth of wild-type Col-0 and *At1g72300* (Fig. 4). These
301 results indicate that *At1g72300* is not required for perception of RaxX21-sY or AtPSY1.

302

303 *RaxX21-sY and PSY do not attenuate elf18-induced growth inhibition*

304 Exogenous addition of PSK has previously been shown to attenuate the Arabidopsis
305 immune response to biotrophic pathogens (Igarashi *et al.*, 2012; Mosher & Kemmerling, 2013;
306 Mosher *et al.*, 2013). Although PSK and AtPSY1 share no sequence similarity, they have
307 nevertheless been hypothesized to serve similar roles (Mosher & Kemmerling, 2013; Mosher *et*
308 *al.*, 2013; Matsubayashi, 2014). Thus, we hypothesized that induction of PSY signaling by PSY
309 or RaxX21-sY may also attenuate plant immune responses. To test this hypothesis, we employed
310 a seedling growth inhibition assay. Arabidopsis seedlings were grown in the presence of the
311 bacterial elicitor elf18, which causes activation of immune response and impairs growth. We
312 demonstrated that co-incubation of seedlings with PSK attenuates elf18-mediated growth
313 inhibition as previously reported (Igarashi *et al.*, 2012) (Fig. S7). However, RaxX21-sY and
314 AtPSY1 do not prevent elf18-triggered growth inhibition in Arabidopsis under the conditions
315 tested (Fig. S7). These results indicate that RaxX21-sY and PSY1 do not have the same effects
316 on immune modulation as PSK in Arabidopsis seedlings in response to elf18 treatment.

317

318 *RaxX and PSY peptides differentially activate PSY-like growth promotion and XA21-immune*
319 *responses*

320 Activation of XA21-mediated immunity by RaxX21-sY triggers a number of immune
321 responses including production of reactive oxygen species (ROS), induction of marker gene
322 expression, and production of ethylene (Pruitt *et al.*, 2015). These immune responses are tightly
323 regulated, because aberrant activation of immunity can have negative effects on plant growth and
324 health (Spoel & Dong, 2012; Rodriguez *et al.*, 2015). We therefore hypothesized that XA21
325 would specifically recognize RaxX but not the homologous PSY peptides.

326 We have previously shown that RaxX21-sY treatment induces robust ROS production in
327 rice leaves expressing XA21 (Pruitt *et al.*, 2015). Therefore, to assess XA21-mediated
328 recognition of the sulfated peptides, we measured ROS production in XA21 rice leaves upon
329 treatment with water, RaxX21-sY, AtPSY1, or OsPSY1a (Fig. 5a). Unlike RaxX21-sY, AtPSY1
330 and OsPSY1a failed to induce ROS production in XA21 rice leaves. Robust ROS production was

331 not observed in rice leaves lacking XA21 (Fig. 5b). PSK also failed to activate XA21-mediated
332 immune response (Fig. 5a, b). These results suggest that the XA21 and PSY receptor(s) have
333 different specificities. PSY signaling with respect to primary root growth is activated by both
334 PSY and RaxX (Fig. 2), whereas the XA21-mediated immune response is only activated by
335 RaxX.

336 To further delineate the region of RaxX required for PSY-like activity and activation of
337 XA21, we synthesized two smaller RaxX peptides based on similarity to AtPSY1. RaxX16-sY
338 begins with the aspartate (D40) at the beginning of the PSY-like motif (Fig. 1a). RaxX13-sY also
339 begins with D40 but is C-terminally truncated relative to RaxX21-sY and RaxX16-sY (Fig. 1a).
340 RaxX13-sY contains the region of highest similarity shared between the RaxX and PSY peptides
341 (Fig. 1, S1). Both the RaxX13-sY and RaxX16-sY peptides are still capable of promoting root
342 growth in Arabidopsis and rice (Fig. 5c, d). We next tested whether these peptides could activate
343 XA21-mediated immunity in the same manner as RaxX21-sY (Pruitt *et al.*, 2015). For this
344 purpose, ROS production was measured in detached XA21 rice leaves treated with water,
345 RaxX13-sY, RaxX16-sY, or RaxX21-sY. RaxX16-sY and RaxX21-sY triggered a ROS response
346 characteristic of the XA21-mediated immune response. In contrast, treatment with RaxX13-sY
347 did not induce ROS production in XA21 rice leaves (Fig. 5a). Thus, RaxX13-sY is able to induce
348 AtPSY1-like growth effects, but fails to activate an XA21-mediated immune response. These
349 experiments reveal that RaxX residues 53-55, which are present in RaxX16 but not RaxX13, are
350 important for activation of XA21 but are not required for root growth promoting activity.

351

352 *RaxX from diverse Xanthomonas species have PSY activity*

353 We next asked whether RaxX from other *Xanthomonas* strains also have PSY-like
354 activity. To address this question, we synthesized 24-amino acid peptides covering the PSY-like
355 region for three different RaxX sequences from *X. oryzae* pv. *oryzicola* strain BSL256 (RaxX24-
356 Xoc-sY), *X. campestris* pv. *musacearum* strain NCPPB4394 (RaxX24-Xcm-sY), and *X.*
357 *euvesicatoria* strain 85-10 (RaxX24-Xe-sY) (Table S2). *Xoc*, *Xcm*, and *Xe* are pathogens of rice,
358 banana, and tomato/pepper, respectively (Table S1). *Xoc* colonizes the mesophyll of rice,
359 whereas *Xoo* colonizes the xylem. All three RaxX sulfated peptides promoted root growth on
360 Arabidopsis seedlings in a manner similar to that of RaxX21-sY derived from *Xoo* strain PXO99
361 (Fig. 6). In other words, the proteins encoded by diverse allelic variants of *raxX* retain PSY like

362 activity. These results demonstrate that the use of RaxX as a mimic of plant PSYs is employed
363 by many *Xanthomonas* species that infect diverse plant species.

364

365 *RaxX facilitates Xoo infection*

366 In some cases, the ability of a pathogen to mimic a host biological process can facilitate
367 pathogen infection (Weiler *et al.*, 1994; Melotto *et al.*, 2006; Mitchum *et al.*, 2012; Chen *et al.*,
368 2015). We therefore tested if RaxX contributes to the virulence of *Xoo* in plants lacking XA21.
369 We did not observe an effect of RaxX on disease lesion development in TP309 rice leaves using
370 standard scissor clipping inoculation (a high inoculum concentration of 10^8 colony forming units
371 per mL) (da Silva *et al.*, 2004; Pruitt *et al.*, 2015). Inoculating with a low inoculum concentration
372 is known to reveal subtle virulence differences between strains (Starkey & Rahme, 2009). Thus,
373 we challenged TP309 leaves with PXO99 strains at a density of 10^6 colony forming units per mL.
374 Under this condition, the PXO99 Δ *raxX* strain, but not the complemented strain
375 (PXO99 Δ *raxX*(*praxX*)), formed shorter lesions compared with wild-type PXO99 (Fig. 7a). We
376 also tested if RaxST-mediated sulfation is required for the virulence activity of RaxX. A PXO99
377 strain lacking RaxST (PXO99 Δ *raxST*) also formed shorter lesion than PXO99 on TP309 rice
378 leaves in low inoculum concentration experiments (Fig. 7a). PXO99 Δ *raxST* (*praxST*) regained
379 the ability to form long lesions similar to the wild-type strain (Fig. 7a). PXO99 wild-type,
380 PXO99 Δ *raxX*(*praxX*) and PXO99 Δ *raxST*(*praxST*) form short lesions on XA21-TP309 at a lower
381 inoculum concentration suggesting activation of the XA21 immune response (Fig. 7b). As
382 expected, PXO99 Δ *raxX* and PXO99 Δ *raxST* evade XA21-mediated immune response and form
383 longer lesions (Fig. 7b). The bacterial populations of PXO99 Δ *raxX* and PXO99 Δ *raxST* were
384 less than those of strains PXO99, PXO99 Δ *raxX*(*praxX*), and PXO99 Δ *raxST* (*praxST*) 12 days
385 after inoculation (Fig. S8). These results suggest that RaxX is a virulence factor that facilitates
386 *Xoo* infection and that RaxST-mediated sulfation is also required for this virulence activity.

387

388 **Discussion**

389 In a classical evolutionary arms race, both the pathogen and host develop and deploy an
390 arsenal of strategies to infect or resist their partner. For example, many pathogens secrete an
391 array of molecular factors designed to manipulate host biology and suppress the immune
392 response. In turn, plants have developed a set of immune receptors that recognize these

393 molecules or their activities and launch mechanisms to destroy the pathogen, which the pathogen
394 then tries to counter.

395 The findings reported here and in previous studies, suggest a model where *Xoo* produces,
396 sulfates, and secretes a peptide that mimics PSY peptides (da Silva *et al.*, 2004; Pruitt *et al.*,
397 2015) (Fig. 8). Plants evolved the receptor XA21 to specifically recognize the bacterial mimic,
398 allowing it to launch a defense response in the presence of the pathogen but not in the presence
399 of the highly similar PSY peptide hormones, which are predicted to be necessary for normal
400 growth and development.

401 The hypothesis that RaxX is a mimic of PSY is well supported by the high level of
402 sequence similarity (Fig. 1), the tyrosine sulfation status of RaxX and PSY peptides (Amano *et*
403 *al.*, 2007; Pruitt *et al.*, 2015), and the similar growth promoting activities of both peptides (Fig. 2,
404 S3-5). Significantly, both RaxX and PSY1 require tyrosine sulfation for full activity. Tyrosine
405 sulfation is an important posttranslational modification that mediates protein-protein interactions.
406 Plants and animals employ tyrosine-sulfated proteins, to regulate growth, development,
407 immunity and other biological processes. Tyrosine sulfated proteins in animal cells have roles in
408 coagulation, leukocyte adhesion, HIV entry, and chemokine signaling (Farzan *et al.*, 1999;
409 Moore, 2009; Stone *et al.*, 2009).

410 Due to the similar sequence and functional mimicry in root growth promotion we
411 hypothesize that PSY1 and RaxX target a common cognate plant receptor. The leucine-rich
412 repeat receptor kinase At1g72300 was originally hypothesized to serve as the receptor for
413 AtPSY1 based on the observation that the root length was not increased by exogenous AtPSY1
414 treatment in an At1g72300 mutant (Amano *et al.*, 2007). However, the At1g72300 mutant line
415 still partially responds to AtPSY1 treatment in proton efflux experiments (Fuglsang *et al.*, 2014),
416 and transcriptomics analysis reveals that many AtPSY1-regulated genes are regulated
417 independently of At1g72300 (Mahmood *et al.*, 2014). We found that RaxX and AtPSY1 still
418 promote root growth in the absence of At1g72300. Collectively, these findings indicate that
419 At1g72300 is not the receptor for PSY peptides or that it is not the only receptor. Additional
420 work is required to understand how PSY and RaxX are perceived in plants.

421 The precise role of RaxX in *Xoo* biology is not known. Because bacteria have been
422 demonstrated to employ bio-mimics to hijack the plants' endogenous systems and reprogram the
423 host environment to facilitate pathogen infection (Weiler *et al.*, 1994; Melotto *et al.*, 2006;

424 Mitchum *et al.*, 2012; Chen *et al.*, 2015), we hypothesize that *Xoo* may use RaxX in a similar
425 manner. Here we show that RaxX is required for the full virulence of *Xoo* to infect rice leaves
426 (Fig. 7). *Xoo* is a biotrophic pathogen and thus requires living host tissues, which ensures
427 prolonged supply of carbon and other nutrients necessary for bacterial survival. The ability of
428 *Xoo* to promote the host growth would thus benefit a biotroph (Nino-Liu *et al.*, 2006; Fatima &
429 Senthil-Kumar, 2015).

430 Xanthomonads enter through hydathodes, natural openings in the leaf, or wounds and
431 multiply in the xylem or mesophyll tissues. To date, growth promoting activities for RaxX or
432 PSY1 have only been demonstrated on roots. We used induction of root growth as an indicator of
433 PSY-like activity in this study because this is a robust well-characterized effect of AtPSY1. It is
434 known, however, that *AtPSY1* is widely expressed in various plant tissues (Amano *et al.*, 2007).
435 *Arabidopsis* seedlings overexpressing *AtPSY1* not only have longer roots, but also larger
436 cotyledons (Amano *et al.*, 2007). Recently, a PSY-like peptide in soybean is shown to be
437 translocated from the roots to the xylem (Okamoto *et al.*, 2015). These findings suggest that PSY
438 peptides likely have important unidentified roles outside of the roots.

439 The growth promoting properties of RaxX are reminiscent of the hypertrophy in tomato
440 and pepper leaves induced by the *Xe* effector AvrBs3. AvrBS3 enhances transcription of host
441 genes including auxin-induced and expansin-like genes that contribute to host cell enlargement
442 (Marois *et al.*, 2002). This phenotype is thought to facilitate dissemination because the bacteria
443 are able to multiply in the enlarged cells and escape from the infected site to other plants (Marois
444 *et al.*, 2002; Kay *et al.*, 2007). The AvrBs3 example suggests a possible role for RaxX in
445 bacterial maintenance, persistence or transmission.

446 In this paper we demonstrate that XA21 can be activated by RaxX16 but not by RaxX13,
447 indicating that the C-terminal end of the RaxX16 sequence (RaxX amino acids 53-55) is required
448 for XA21 recognition. This result may explain why PSY1 cannot activate XA21: PSY1 has C-
449 terminal residues which differ from RaxX16. Residues within the RaxX13 region are also
450 important for recognition by XA21. In a previous study, we identified three residues (44, 46, and
451 48) of RaxX from *Xoo* that are involved in XA21 activation (Pruitt *et al.*, 2015). Mutation of
452 RaxX P44 and P48 completely abolishes the immunogenic activity of RaxX on XA21-rice.
453 Mutation of A46 has a partial effect. Interestingly, these residues are not required for root growth

454 promoting activity. For example, RaxX24-Xoc contains amino acid differences at positions 44,
455 46 and 48, but is still capable of inducing root growth in Arabidopsis (Fig. 6, S2, Table S1).

456 Comparison of the RaxX-Xoo and RaxX-Xoc sequences with rice PSY sequences
457 suggests the possibility that RaxX from the *Xanthomonas* strains have evolved to mimic different
458 PSY peptides. The three residues from RaxX-Xoo (strain PXO99) which are required for
459 recognition by XA21 are identical to those in OsPSY1a (Fig. S9). In contrast, the amino acids of
460 RaxX-Xoc (strain BSL256) are similar to those in OsPSY2. If these two peptides have evolved to
461 mimic different PSY peptides, it would indicate that there are multiple PSY receptors in rice,
462 which differentially recognize diverse PSY peptides. Multiple receptors have been reported for
463 RGF peptides. It is not yet clear if the RGF receptors have different affinities for specific RGF
464 peptides (Shinohara *et al.*, 2016). Using multiple receptors and multiple ligands with different
465 affinities would allow for a more complex and tunable signaling network.

466 To further investigate the possibility that RaxX may have evolved to mimic specific host
467 PSY peptides, we compared the sequences of RaxX13 and PSY from various species (Fig. 1B,
468 S10). We did not observe a correlation between the sequences of RaxX from the pathogen and
469 PSYs from a compatible host (Fig. S10). However, alignment of the 13-amino acid region did
470 highlight variation at positions 5, 7, and 9. These residues correspond to RaxX amino acids 44,
471 46, and 48, which are important for XA21 recognition. Notably, the variation is not random. For
472 example, the most common amino acids in position 5 of the sequences analyzed are serine and
473 proline in both RaxX and PSY (Fig. 1B, S10). The amino acids in this position could affect the
474 ability of the peptides to activate specific PSY receptor(s), as they do for XA21. Alternatively,
475 the PSY receptor(s) may simply be able to accommodate serine or proline at this position.
476 Further research, including the characterization of the PSY receptor(s), will help address
477 questions of specificity and lead to a greater understanding of PSY signaling.

478 The study of microbial mimicry of host molecules provides insight into both host and
479 pathogen biology, and can lead to novel strategies for disease prevention (Gardner *et al.*, 2015).
480 Recent studies of the JA receptor have provided new insight into selective recognition of
481 endogenous hormones. The endogenous JA receptor is sensitive to both JA-Ile and the mimic
482 coronatine. By making a structure-guided point mutation of a single amino acid, Zhang *et al.*
483 generated a modified JA receptor which has strongly reduced sensitivity to coronatine while
484 retaining endogenous JA-Ile recognition (Zhang *et al.*, 2015). Arabidopsis with the modified JA

485 receptor displayed enhanced resistance to coronatine producing *Pseudomonas* strains, and have a
486 normal phenotype in the absence of infection (Zhang *et al.*, 2015). The Zhang *et al.* study
487 demonstrates how understanding of bacterial mimicry of host factors can be used to engineer
488 plants with enhanced resistance to bacterial pathogens. The findings presented in this work
489 provide another striking example of co-evolution between the host and pathogen and provide a
490 framework for future work directed at understanding how XA21 and the PSY receptor(s)
491 differentially recognize RaxX and endogenous PSY peptides.

492
493

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500

501 **Author contributions**

502 R.N.P., A.J., P.C.R. and W.Z. designed the research, R.N.P., A.J., W.Z. and W.F. performed
503 experiments, J.R.D. provided resources, R.N.P, A.J. and V.S. analyzed data, R.N.P., A.J. and
504 P.C.R. wrote the manuscript, and B.S. and W.Z. helped to revise the manuscript.

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654

Figure 1. Sequence similarity of RaxX and plant PSYs. a) The mature 18 amino acid AtPSY1 (amino acids 48-65 of the AtPSY1 precursor protein) and a synthetic PSY-like repeat from OsPSY1 (amino acids 64-81 of the AtPSY1 precursor protein) were aligned with the sequences of three synthetic RaxX peptides from *Xoo* strain PXO99. The numbers adjacent to the sequence indicate the amino acid positions of the terminal peptide residues within the predicted precursor protein. Endogenous AtPSY1 has 3 postranslationally modified residues, which are shown at the top of alignment: a sulfotyrosine and two hydroxyprolines. The first hydroxyproline is further modified by chain of three L-arabinose residues (L-Ara₃). Residues in the black box are identical in all three sequences. The grey boxes indicate a conserved residue in two sequences among AtPSY1, OsPSY1a and RaxX. The sulfated tyrosine is marked in yellow box. b) Sequence logos depicting the amino acid composition in the conserved 13-amino acid region of RaxX and PSY proteins. The logos were generated from 34 PSY orthologs (Fig. S1) and 17 non-redundant RaxX13 sequences (Table S1).

Figure 2. Sulfated RaxX21 promotes root growth in *Arabidopsis* and rice. Root lengths of *Arabidopsis* a) Col-0 or c) *tpst-1* seedlings grown on 0.5× MS vertical plates with or without 100 nM of the indicated peptides. Bars indicate the average seedling root length measured after 8 days (n ≥ 18). b and d) 8-day old Col-0 and *tpst-1* seedlings grown as in 2a and c, respectively. e) Growth rate of six day old *tpst-1* seedlings following transfer to 0.5× MS plates containing 250 nM RaxX21-sY, 250 nM AtPSY1, or lacking peptide (Mock) (n ≥ 7). Growth was monitored by continual imaging over 20 h. f) Root lengths of 6-day old rice seedlings (Tapei 309) grown on 0.5× MS with or without 100 nM of the indicated peptides (n ≥ 37). Error bars indicate standard error. Statistical analysis was performed using the Tukey-Kramer honestly significant difference test for mean comparison using the JMP software. Different letters represent significant differences within each plant genotype (p ≤ 0.05).

Figure 3. RaxX, AtPSY1, and PSK do not have additive effects on root growth in *Arabidopsis*. *tpst-1* seedlings were grown on 0.5× MS vertical plates with or without 100 nM of each of the indicated peptides. Bars indicate the average seedling root length measured 8 days after plating seeds (n ≥ 18). Error bars indicate standard error. Statistical analysis was performed

using the Tukey-Kramer honestly significant difference test for mean comparison using the JMP software. Different letters represent significant differences within each plant genotype ($p \leq 0.05$). Experiments were performed at least two times with similar results.

Figure 4. The Arabidopsis gene *At1g72300* is not required for RaxX- and PSY-induced root growth. Arabidopsis Col-0, *At1g72300* or *AtPSKR1/AtPSKR2/At1g72300* triple receptor mutant seeds were grown on 0.5× MS plates with or without 100 nM of the indicated peptides. Root lengths were measured 8 days after placing seeds on plates. Error bars indicate standard error ($n \geq 22$). Statistical analysis was performed using the Tukey-Kramer honestly significant difference test for mean comparison using the JMP software. Different letters represent significant differences within each plant genotype ($p \leq 0.05$). The experiment was performed at least three times with similar results.

Figure 5. Differential activities of PSY and RaxX peptides in growth promotion and activation of XA21-mediated immunity. ROS production in leaves of a) XA21 rice (XA21-Kitaake) and b) wild type rice (Kitaake) treated with H₂O (Mock) or 500 nM of the indicated peptide. Bars represent average ROS production over 90 min following addition of peptide ($n = 6$). RLU stands for relative light units. c) TP309 seeds were grown on 0.5× MS media for with or without 100 nM of the indicated peptides. Root lengths were measured 5 days after placing seeds on plates ($n \geq 25$). d) Arabidopsis *tpst-1* seeds were grown on 0.5× MS vertical plates with or without 100 nM of the indicated peptides. Root lengths were measured 8 days after placing seeds on plates ($n \geq 16$). Error bars indicate standard error. Statistical analysis was performed using the Tukey-Kramer honestly significant difference test for mean comparison using the JMP software. Different letters represent significant differences within each plant genotype ($p \leq 0.05$). Experiments were performed at least two times with similar results.

Figure 6. RaxX peptides derived from RaxX encoded by *Xoc*, *Xe*, and *Xcm* promote root growth in Arabidopsis seedlings. *tpst-1* seedlings were grown on 0.5× MS vertical plates with or without 100 nM of the indicated peptides. Bars indicate the average seedling root length measured after 8 days ($n \geq 18$). Error bars indicate standard error. Statistical analysis was performed using the Tukey-Kramer honestly significant difference test for mean comparison

using the JMP software. Different letters represent significant differences within each plant genotype ($p \leq 0.05$). Experiments were performed at least two times with similar results.

Figure 7. The *Xoo raxX* mutant is impaired in virulence on rice. TP309 (A) and XA21-TP309 (B) were inoculated by clipping with scissors dipped in the indicated *Xoo* suspensions at a density of 10^6 colony forming units (CFU) per mL. Bars indicate the mean lesion length \pm standard error (SE) measured 14 days after inoculation ($n \geq 24$). The ‘*’ indicates statistically significant difference from PXO99 within each plant genotype using Dunnett’s test ($\alpha=0.01$). Experiments were performed at least five times with similar results.

Figure 8. Proposed model of RaxX production and activation of PSY and XA21 signaling. PSY is produced and detected by plant cells to regulate growth. RaxX is produced in *Xoo*, sulfated by RaxST, and secreted by a type I secretion system composed of RaxA, RaxB, and RaxC. Secreted sulfated RaxX induces signaling through the endogenous PSY receptor(s). The wild rice *O. longistaminata* subsequently evolved the immune receptor XA21 which is activated by RaxX, but not endogenous PSY peptides.

Supplemental Information

Table S1. RaxX13 sequences from diverse Xanthomonas sources.

Table S2. Synthetic peptides used in this study.

Figure S1. Putative PSY-like proteins from Arabidopsis (At), rice (Os), banana (Ma), tomato (Sl), and wheat (Ta).

Figure S2. Comparison of the RaxX sequences from diverse bacterial strains.

Figure S3. Dose dependent activity of RaxX21-Y, RaxX21-sY, AtPSY1, and PSK on root growth of Arabidopsis *tpst-1* seedlings.

Figure S4. Sulfated RaxX21 promotes root growth in Kitaake rice.

Figure S5. Sulfated RaxX21 promotes root growth in XA21 rice.

Figure S6. Validation of the *At1g72300* mutants.

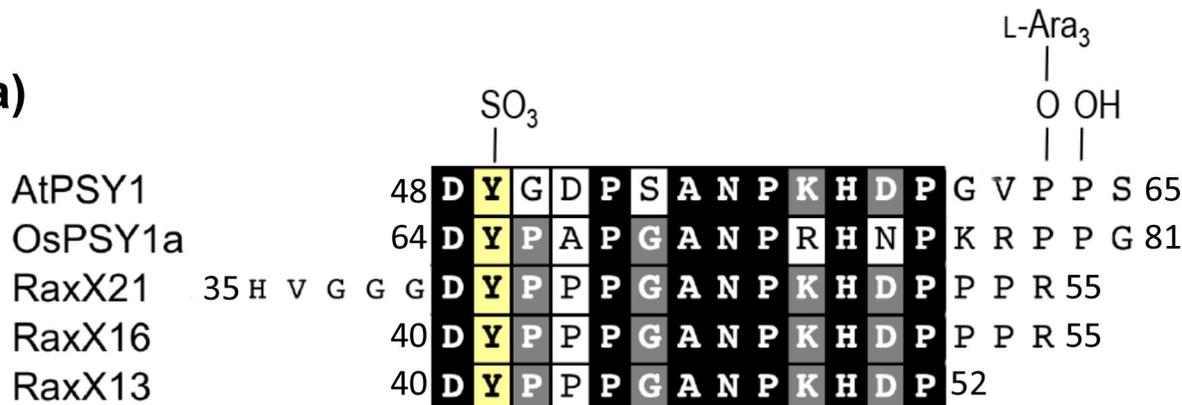
Figure S7. Addition of PSK partially blocks elf18-triggered growth inhibition in Arabidopsis seedlings, whereas RaxX21-sY and AtPSY1 do not.

Figure S8. PXO99 strain lacking RaxX is impaired in virulence.

Figure S9. Sequence similarity of RaxX from *Xoo* and *Xoc* with selected rice PSYs.

Figure S10. Comparison of RaxX and PSY peptides from various species.

(a)



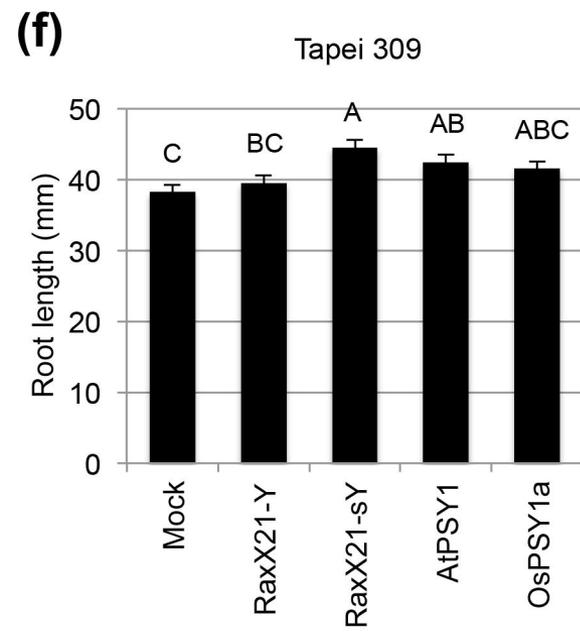
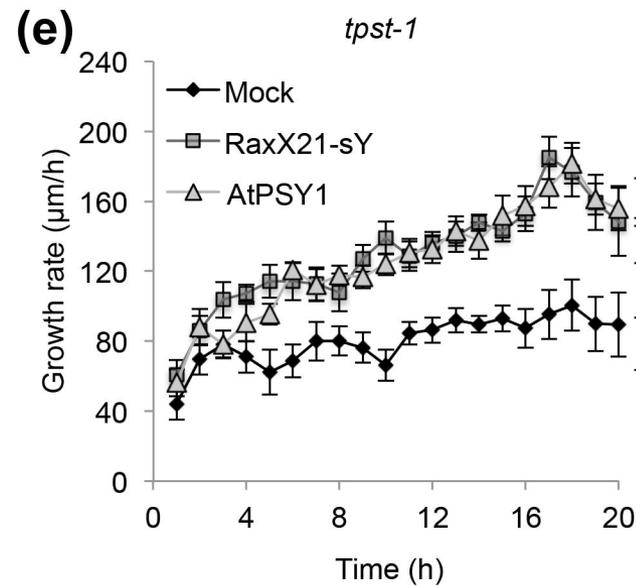
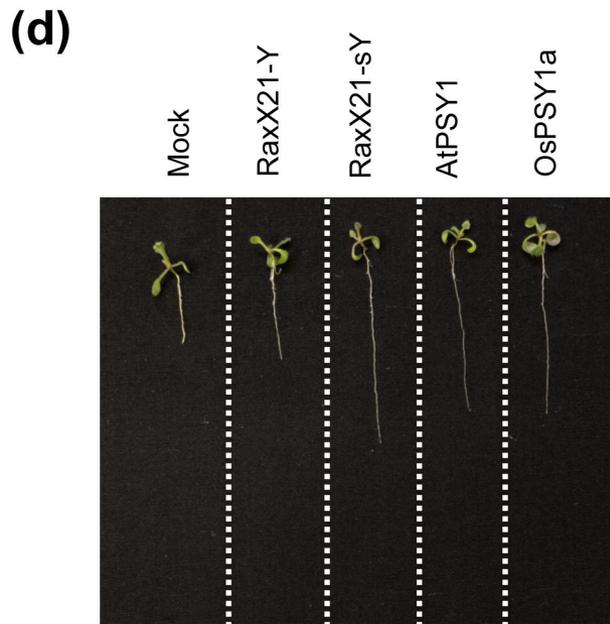
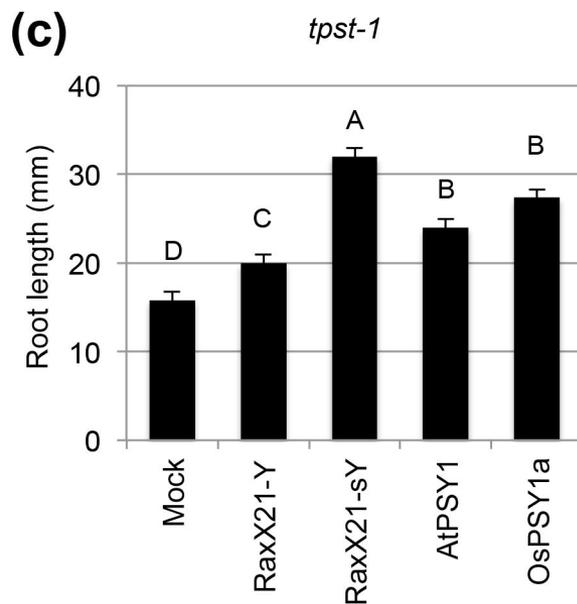
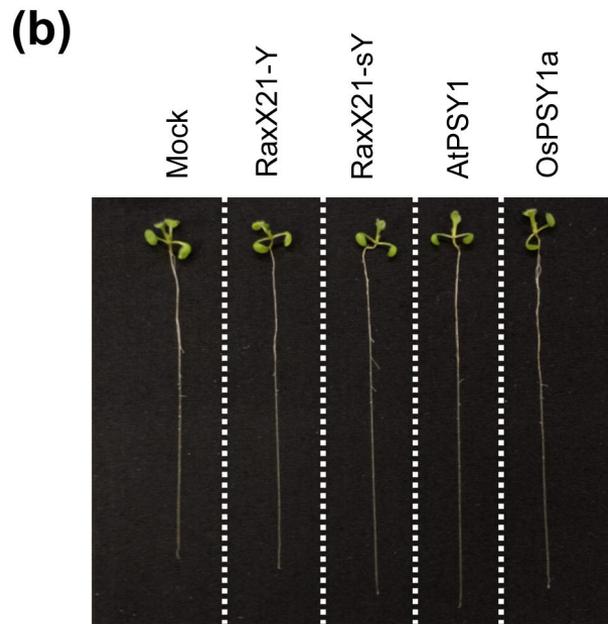
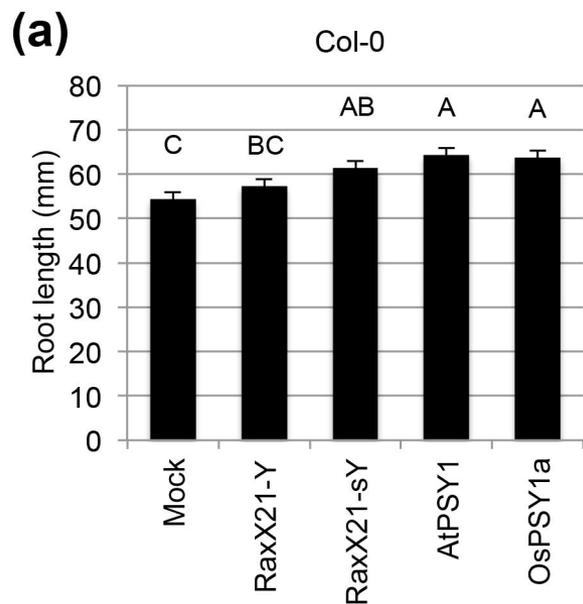
(b)

PSY

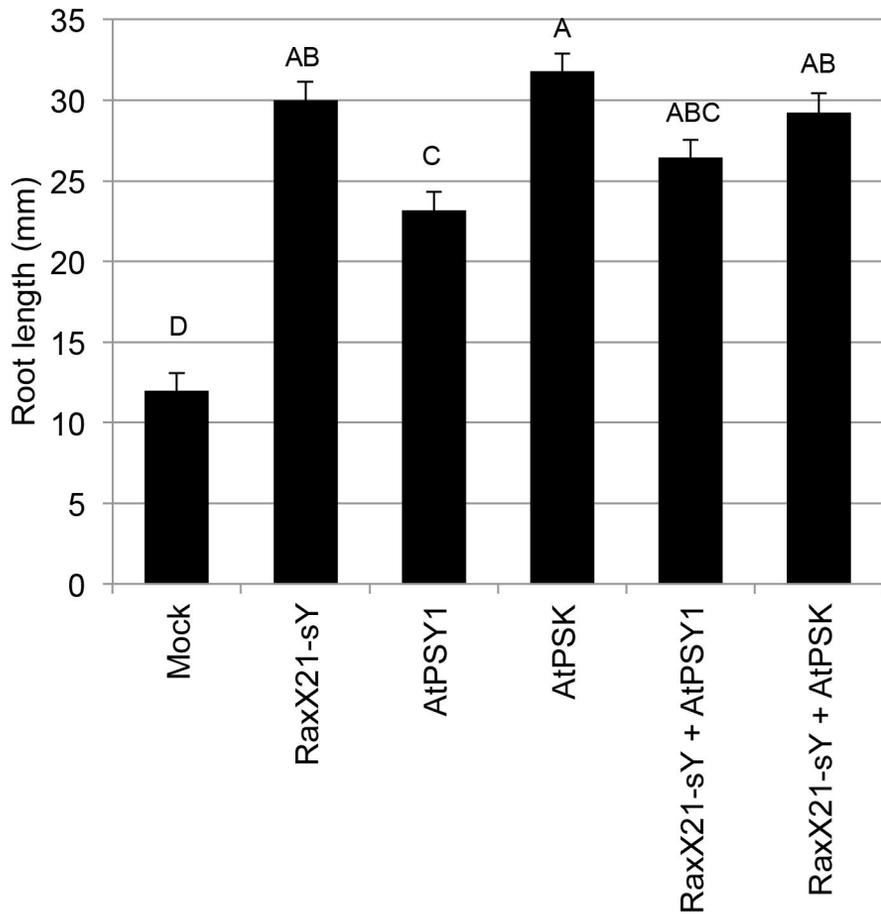


RaxX

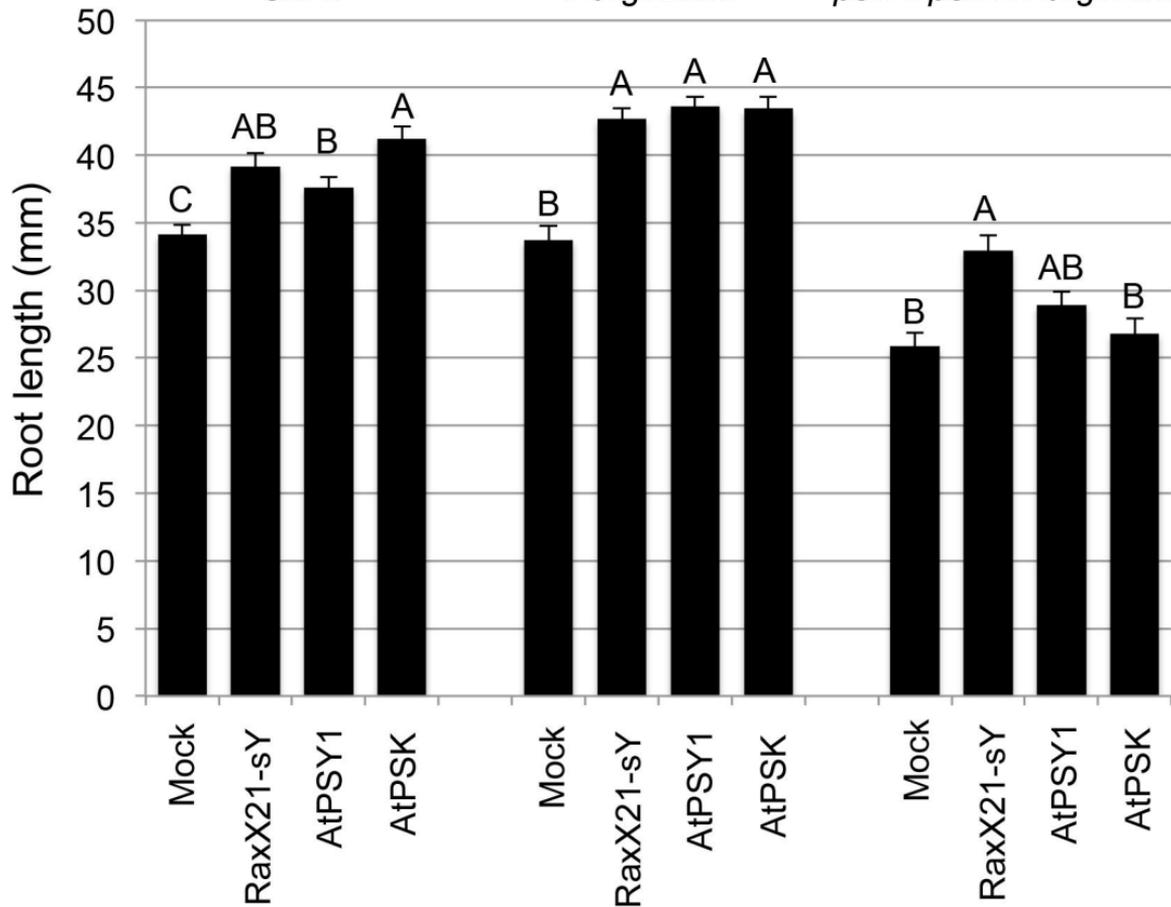


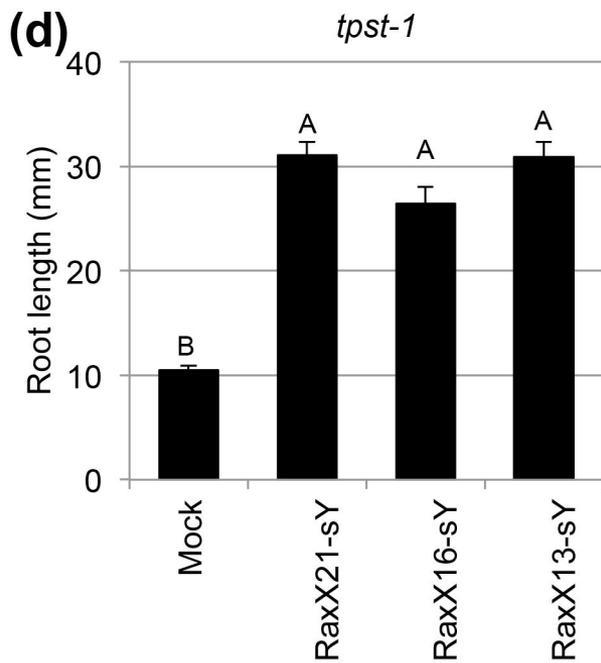
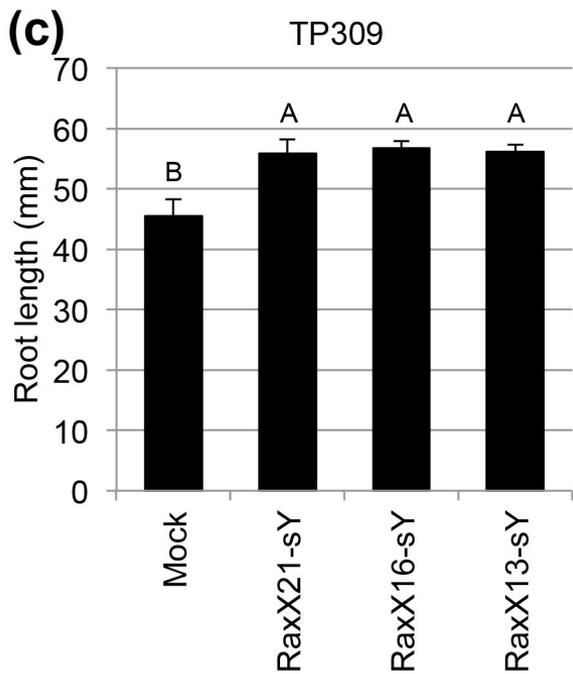
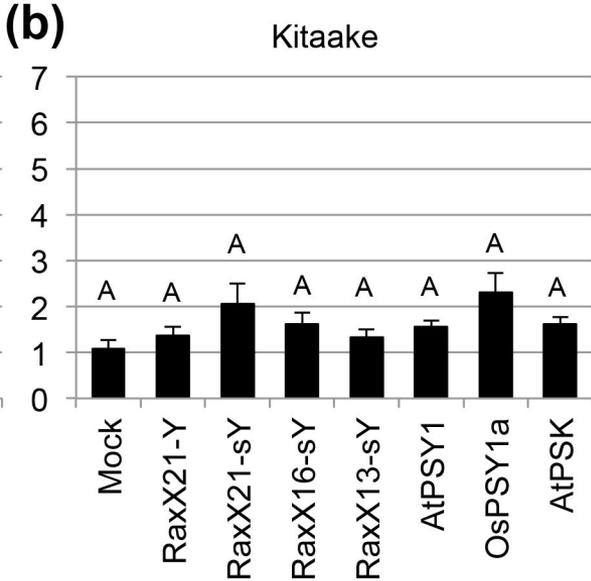
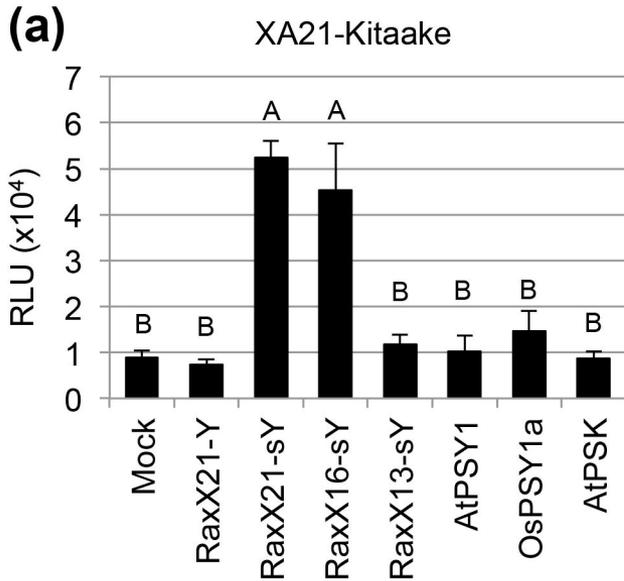


tpst-1

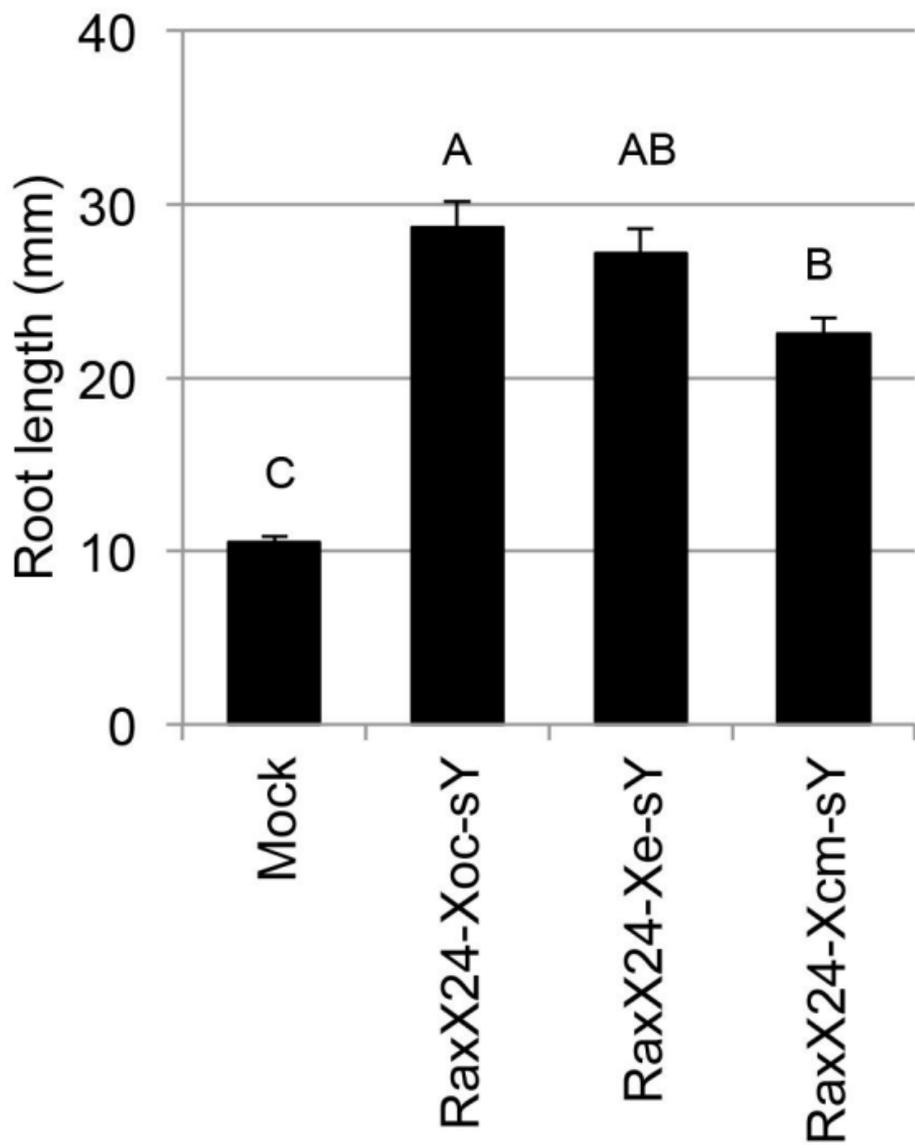


Col-0

*At1g72300**pskr1/pskr2/At1g72300*

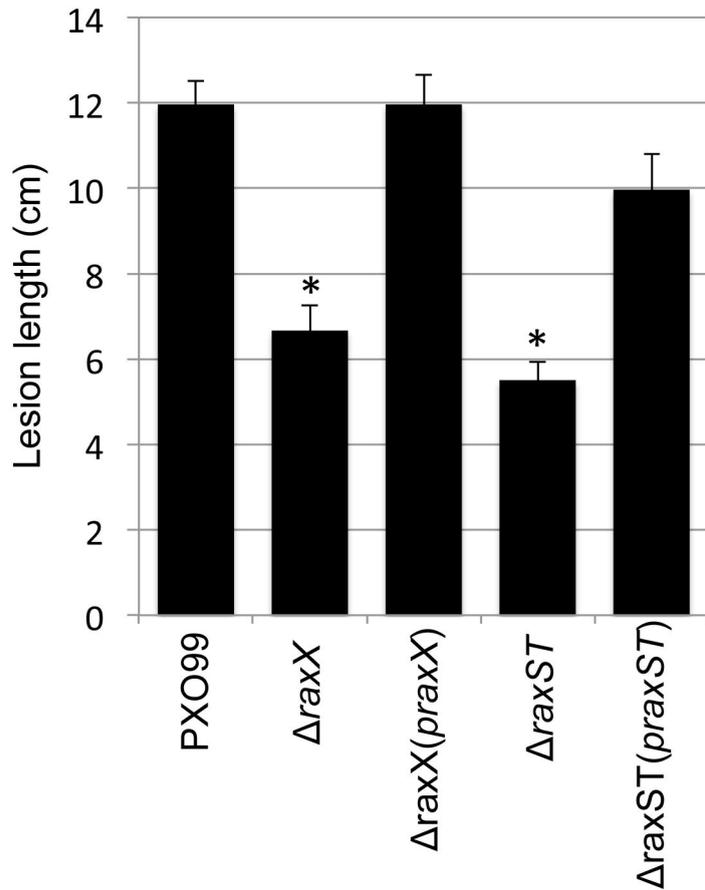


tpst-1



(a)

TP309

**(b)**

XA21-TP309

