

1 **Evidence for the plant recruitment of beneficial microbes to suppress soil-borne**
2 **pathogen**

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18 Summary

- 19 • **Emerging experimental framework suggests that plants under biotic stress may actively**
20 **seek help from soil microbes, but empirical evidence underlying such a ‘cry for help’**
21 **strategy is limited.**
- 22 • **We used integrated microbial community profiling, pathogen and plant transcriptive**
23 **gene quantification and culture-based methods to systematically investigate a three-way**
24 **interaction between the wheat plant, wheat-associated microbiomes and *Fusarium***
25 ***pseudograminearum* (*Fp*).**
- 26 • **A clear enrichment of a dominant bacterium, *Stenotrophomonas rhizophila* (SR80), was**
27 **observed in both the rhizosphere and root endosphere of *Fp*-infected wheat. SR80**
28 **reached 3.7×10^7 cells g^{-1} in the rhizosphere and accounted for up to 11.4% of the**
29 **microbes in the root endosphere. Its abundance had a positive linear correlation with**
30 **the pathogen load at base stems and expression of multiple defense genes in top leaves.**
31 **Upon re-introduction in soils, SR80 enhanced plant growth, both the below- and above-**
32 **ground, and induced strong disease resistance by priming plant defense in the**
33 **aboveground plant parts, but only when the pathogen was present**
- 34 • **Together, the bacterium SR80 seems to have acted as an early warning system for plant**
35 **defense. This work provides novel evidence for the potential protection of plants against**
36 **pathogens by an enriched beneficial microbe via modulation of the plant immune**
37 **system.**

38 **Key words:** crown rot; endophytes; *Fusarium pseudograminearum*; plant microbiome;
39 *Stenotrophomonas rhizophila*; wheat.

40

41 Introduction

42 Plants have been subject to selective pressures from pathogens, pests, and undesirable soil
43 (e.g., nutrient deficient) and weather (e.g., drought) conditions since their evolutionary origin
44 (Chakraborty & Newton, 2011; Liu *et al.*, 2019). Assemblages of host-specific microbiomes
45 in the rhizosphere, root endosphere and other niches, such as the phyllosphere (leaf) and

46 anthosphere (flower) have been reported (Edwards *et al.*, 2015; Álvarez-Pérez *et al.*, 2019;
47 Grady *et al.*, 2019). Emerging evidence indicates that these microbial symbionts, rather than
48 merely acting as tenants, intimately interact with plants and influence their immune systems
49 and multiple processes of plant growth and development (Liu *et al.*, 2019). However,
50 detailed mechanistic evidence for these interactions is missing and how these hugely diverse
51 commensal microbes interact with plants is largely unknown. Addressing these knowledge
52 gaps is urged to support future translational research for the application of microbe-based
53 products in sustainable agriculture.

54

55 The rhizosphere is a hotspot for plant-soil-microbe and microbe-microbe interactions. The
56 microbes and their interactions can largely prevent pathogen outgrowth and extend the plant
57 capacity for disease resistance (Durán *et al.*, 2018). Recent studies revealed that some
58 disease-resistant crop varieties (e.g. tomato and common bean) enrich particular sets of
59 bacterial species in the rhizosphere to suppress pathogens (Kwak *et al.*, 2018; Mendes *et al.*,
60 2018). This suggests that specific soil microbes/microbial functions contribute to protection
61 against plant diseases. Furthermore, it has been shown that diseased plants significantly
62 promoted specific beneficial microbes in their associated microbiomes, which could act as
63 an extended layer of plant defense and a legacy to enhance survival rates of their offspring
64 (Berendsen *et al.*, 2018). However, whether the rhizosphere and root endophytic microbiota
65 are coordinated to modulate plant-pathogen interactions and plant performance is unknown.

66

67 Recent theoretical framework of co-evolution also suggests that recruitment of beneficial
68 microbes upon biotic stress is likely a survival strategy conserved across the plant kingdom
69 although empirical evidence is still scarce and mechanistic pathways remain poorly defined
70 (Liu & Brettell, 2019; Liu *et al.*, 2019). Whether such a cry for help strategy applies to crop-
71 pathogen interactions in field conditions is unknown. Moreover, plants differ in physiology
72 and immune response to pathogen invasions, meaning mechanisms are likely to be plant
73 species-dependent. In this study, we investigated interactions between durum wheat

74 (*Triticum turgidum* var. *durum*) and its microbial symbionts in the rhizosphere and root
75 endosphere upon infection with the fungal pathogen *Fusarium pseudograminearum* (*Fp*).
76 Crown rot (CR) disease caused by *Fp* is a devastating soil-borne disease, which can infect
77 winter cereal crops of all stages and lead to large losses in global cereal production
78 (estimated at \$79 million/year in Australia alone) (Akinsanmi *et al.*, 2004). However, so far,
79 there are no CR-resistant crop varieties and effective control through chemical fungicides is
80 currently not available. Using wheat-*Fp* system, we aimed to explore responses of plant
81 microbiomes to pathogen infection and identify mechanisms underpinning plant-microbiome
82 interactions that may mediate disease resistance of the plant.

83

84 In an effort to achieve our aims, durum wheat plants were sampled from a field experiment
85 where *Fp* inoculations have been historically occurring at the Queensland Department of
86 Agriculture and Fisheries (DAF) research farm at Wellcamp (Australia). Soil and tissues
87 from symptomatic plants that were naturally infected with *Fp* were collected, along with
88 those from asymptomatic plants, to identify key microbial taxa in the plant microbiome that
89 are associated with plant defense responses. We profiled microbial communities in the
90 rhizosphere and roots using bacterial and fungal amplicon sequencing. Plant defense status
91 was evaluated by determining the transcript abundances of defense genes involved in the
92 salicylic acid (SA) and jasmonic acid (JA) signaling pathways. Bacteria were then isolated
93 from *Fp*-infected plants and effects of a disease-enriched bacterium on durum wheat defense
94 and growth were investigated in glasshouse experiments.

95

96 **Methods and Materials**

97 *Fp* treatments in field experiments and sampling

98 Field samples were collected from a wheat trial conducted in 2015 at the Queensland
99 Department of Agriculture and Fisheries (DAF) Experimental Farm at Wellcamp
100 (27°33'54.7"S, 151°51'52.0"E), Queensland, Australia. The soil is a black Vertosol with
101 chemical properties listed in Table S1. Farm management histories including past yield trials,

102 CR trials and fallow periods are shown in Table S2. Plus and minus *Fp* (the causal agent for
103 CR disease) inoculated yield plots (6 m x 2 m) were sown in a randomized complete block
104 design in June 2015. Colonized millet grain inoculum (Percy *et al.*, 2012) was applied in the
105 furrow in a band above the seed at planting. Experimental plots were surrounded by a 7-row
106 buffer zone (2 m each) of non-inoculated durum wheat (Jandaroi variety), running 150 m
107 along the length of the experiment. CR was observed in many plants in the non-inoculated
108 Jandaroi buffer during tillering and as jointing progressed. Diseased plants were scattered
109 amongst asymptomatic plants. *Fp*-infected plant residues remaining from a previous CR
110 experiment (2010) may have been retained in the field and continued to provide a source of
111 inoculum to infect subsequent hosts. The research presented in this study compares samples
112 taken from plants that were asymptomatic and symptomatic for CR in Jandaroi buffer rows,
113 as we aimed to investigate plants naturally infected with *Fp*.

114

115 Thirteen weeks after sowing, both healthy and infected plants were carefully uprooted using
116 a shovel and separated into independent individuals from three different locations of the field
117 in September 2015. Forty healthy and 18 infected plants were collected for microbiome
118 analyses. The top ~10 cm of two to three leaves were cut, transferred into a 15 mL Falcon
119 tube and immediately frozen in dry ice. The leaf along with stem and root (soil attached)
120 samples were stored in dry ice and transported to the laboratory on the same day and
121 preserved at -80°C. Approximately 5 grams of fresh roots (rhizosphere soil attached)
122 collected from a *Fp*-infected plant were kept at 4°C until microbial isolation.

123

124 *Processing of the rhizosphere soil and root samples*

125 Stems, roots and soils were separated before further processing. The procedures included
126 three steps. (i) The most basal internode/base stem (~5 cm that covers an area of brown
127 discoloration of each plant) was cut and scored for disease levels following the protocol
128 below. The stem samples were ground into a fine powder in liquid nitrogen and stored at -
129 80°C for DNA extraction. (ii) Bulk soil was removed from roots by shaking them vigorously.

130 (iii) Rhizosphere soil was then separated from roots by shaking root and soil in 25 mL
131 0.1 M sterile phosphate buffer (7.1 g Na₂HPO₄, 4.4 g NaH₂PO₄·H₂O added to 820 mL
132 deionized water, pH 7.0) in a 50 mL plastic conical centrifuge tube at 200 rpm for 5 min.
133 Roots were then transferred to a new tube for subsequent procedures. The soil suspension
134 was centrifuged at 4,000 g for 15 min and the obtained soil pellet was regarded as the
135 rhizosphere soil and stored at -80°C until genomic DNA extraction. Roots were thoroughly
136 washed under distilled water, followed by surface sterilization to remove microbes on the
137 surface by shaking roots in 25 mL of 4% sodium hypochlorite solution at 200 rpm for 5 min.
138 The roots were then washed three times with sterile phosphate buffer. The last wash was
139 inoculated on a nutrient agar plate incubated for 2 days at 37°C and no viable colonies
140 formed, which indicated that the disinfection procedure was efficient. Roots were air-dried in
141 a laminar hood and stored at -80°C until grinding step in liquid nitrogen leading to a fine
142 powder for DNA extraction.

143

144 *Disease scoring*

145 CR was rated based on the percentage of brown discoloration at the stem base using a 0 to 4
146 scale previously reported (Wildermuth & McNamara, 1994). A score of 0 indicates no
147 visible discoloration, and a score of 1, 2, 3, and 4 describe discoloration of 0~25%, 26% to
148 50%, 51%~75% and 76%~100%, respectively.

149

150 *DNA extraction from soil and plant samples*

151 Genomic DNA (gDNA) was extracted from about 0.2 g plant (stem or root) samples using a
152 Maxwell[®] 16 LEV Plant DNA Kit on a Maxwell[®] 16 Instrument (AS2000) according to the
153 manufacturer's instructions. Soil gDNA was extracted from 0.25 g soil per sample using the
154 PowerSoil[™] DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA) as per the
155 manufacturer's recommendations. Plant and soil DNA concentrations were determined using
156 a Qubit[™] fluorometer with Quant-iT dsDNA HS Assay Kit (Invitrogen). The DNA samples
157 were stored at -20°C until further analysis.

158

159 *Fp and total fungi quantification*

160 *Fp* and total fungi colonizing the plant and soil samples were quantified using qPCR. The
161 details of the qPCR system and thermal conditions are described in the Supplementary
162 Materials of this study. The proportion of *Fp*/total fungi DNA to wheat DNA was counted as
163 the relative abundance of *Fp*/fungi in wheat plants (Melloy *et al.*, 2010). *Fp* and fungi were
164 estimated using (i) the *Tri5* gene from the trichothecene cluster responsible for trichothecene
165 production of *Fusarium* species, and (ii) the fungal ribosomal 18S rRNA gene, respectively
166 (Melloy *et al.*, 2010). Wheat actin-binding protein coding sequence was used as the
167 reference gene (Table S3). Quantification of these genes was performed using SYBR Green
168 on a ViiA™ 7 sequence detection system (Applied Biosystems, USA) with two replications
169 for each sample. Amplification specificity for each gene was examined by running melt
170 curve analysis, and the *Fp* amounts were then estimated according to

171
172 Relative Biomass =
$$\frac{E_{Fungal}^{f-Ct}}{E_{Plant}^{f-Ct}}$$

173 where *E_f* is PCR amplification efficiency, which was calculated by LinRegPCR 7.5 based on
174 raw PCR amplification data (Ramakers *et al.*, 2003). The relative abundance of *Fp* in soil
175 was also calculated, by comparing Ct values of each sample against a standard curve that
176 was generated by qPCRs using serially diluted *Tri5* amplicons.

177

178 *Quantification of defense-related genes in the leaf*

179 RNA was isolated from ground leaf samples using Maxwell® 16 Total RNA Purification
180 (Promega) kit on an automated Maxwell® 16 Instrument as per the manufacturer's
181 recommendations. RNA concentrations were quantified using a Qubit™ fluorometer
182 (Invitrogen). cDNA was then synthesized from 2.5 µg RNA in a 20 µL reaction using both
183 random hexamers and oligo(dT) primers provided with the Tetro cDNA Synthesis kit
184 (Bioline™). Ten defense genes were quantified using the SYBR Green qRT-PCR kit on a
185 ViiA™ 7 sequence detection system. These genes are marker genes for JA and SA signaling

186 pathways, characterized in our previous study (Table S3) (Liu *et al.*, 2016). The 18S rRNA
187 gene was used as the housekeeping gene for normalization, and the cDNA was diluted to 1:
188 500 before the amplification of this gene. The reverse transcriptase qPCR (qRT-PCR)
189 system and thermal conditions are described in the Supplementary Materials.

190

191 *Plant and soil microbial community profiling using amplicon sequencing*

192 The universal primers 926F (Engelbrektson *et al.*, 2010) and 1392R (Peiffer *et al.*, 2013)
193 were used for the amplification of bacterial and archaeal 16S rRNA genes in soil samples
194 (Table S3). The primers 799F and 1193R that preferentially amplify archaeal and bacterial
195 DNA in plant materials were used to amplify root endophytic microbes (Table S3). B
196 adaptor was linked to a key (TCAG) and connected to the above template-specific forward
197 primers. A adaptor was linked to a key sequence (TCAG) and a sample-specific molecular
198 identifier, and was then connected to a template-specific reverse primer (Table S3).
199 Amplifications were performed in duplicates for each sample and combined, using the PCR
200 system and conditions described in the Supplementary Materials. PCR products were
201 examined using agarose gel electrophoresis, and microbial amplification products for root
202 DNA samples were recovered from gels to remove the plant mitochondrial DNA-derived
203 amplicons which co-amplify using the 799F and 1193R primers but are of a different length.
204 The obtained 16S rRNA amplicons (~400 bp) were purified using Agencourt AMPure XP
205 beads (Beckman Coulter, Inc.) and quantified with a PicoGreen dsDNA Quantification Kit
206 (Invitrogen). Amplicons were then dual indexed using the Nextera XT Index Kit (Illumina)
207 according to the manufacturer's instructions. Equal concentrations of each indexed sample
208 were pooled and sequenced on an Illumina MiSeq as per the manufacturer's instructions at
209 the University of Queensland's Institute for Molecular Biosciences (UQ, IMB). Additionally,
210 the fungal amplicon sequencing for soil samples was performed at the Western Sydney
211 University using the primer sets FITS7 (Ihrmark *et al.*, 2012) and ITS4 (Glass & Donaldson,
212 1995) as per the standard MiSeq sequencing procedures. Bioinformatic analyses are
213 described in the Supplementary Materials of this study.

214

215 *Bacterial isolation*

216 One gram of roots combined with rhizosphere soil from *Fp*-infected wheat were cut into
217 small pieces and homogenized with autoclaved glass beads (710-1180 μm , Sigma) in a 2 mL
218 centrifuge tube for 3 min using a TissueLyzer (Qiagen). The obtained mixture was serially
219 diluted in 5.0 mL sterile phosphate buffer and 150 μL aliquots of 10^{-4} to 10^{-7} dilutions were
220 spread onto plates containing a range of media: nutrient agar (NA), Pikovskaya's agar,
221 tryptophan soy agar, King's B agar and *Stenotrophomonas* spp. selective medium (32 mg L⁻¹
222 Imipenem added, Adooq Biosciences) (Bollet *et al.*, 1995). Plates were incubated at 30°C in
223 an incubator (Thermoline Scientific) for 2-5 days. Bacterial colonies were then picked based
224 on morphology, color and margin, and further purified by streaking on new NA plates.
225 Purified strains were stored in 25% glycerol at -80°C and sub-cultured on NA plates for the
226 required analyses.

227

228 *Antifungal assays*

229 *Fp* strains CS3427, CS3321, CS3096 (Chakraborty *et al.*, 2010) and A11 (strain in the field
230 trial) were used for this assay. They were cultured on potato dextrose agar (PDA) to a whole
231 plate size, and a mycelial plug of each fungus was placed in the center of a dual media plate
232 ($\frac{1}{2}$ PDA and $\frac{1}{2}$ NA media). Tested bacteria were streaked at four ends of the plate and
233 cultured at 28°C for 7 days to examine antagonistic effects on these *Fp* strains. Two
234 *Burkholderia* bacterial strains were used as positive controls, and three replicates were
235 performed for the assay. Plates that inoculated with *Fp* only served as negative controls.

236

237 *Bacterial identification and whole genome sequencing of SR80*

238 Bacterial strains were identified based on their 16S rRNA gene sequence. They were
239 cultured in nutrient broth for 24 h, pelleted by centrifugation, washed and re-pelleted in PBS
240 buffer twice. Bacteria were then subjected to gDNA extraction using the DNeasy PowerSoil
241 Pro kit (Qiagen). A region of the 16S rRNA gene was amplified using 27F and 1492R

242 primers (Table S3) and sequenced on a capillary sequencer at the Hawkesbury Institute for
243 the Environment (HIE). Sequences were quality-checked, trimmed and BLAST-searched
244 against the nucleotide database of NCBI using Geneious 10.1.3. Sequences of
245 *Stenotrophomonas* isolates were deposited in the NCBI database under GenBank accession
246 numbers MT151295 to MT151301. The identification of different *Stenotrophomonas* spp.
247 was further examined by BOX-PCR (for conditions see Supplementary Materials).
248 Sequences of other bacterial isolates are available under MT158490-MTI158578. Whole
249 genome sequencing of SR80 was conducted using the Illumina MiSeq platform by
250 Guangzhou Magigene Technology Co., Ltd. Sequencing success and quality were checked
251 with FastQC (0.11.8). A total of 11,331,006 high quality reads (Q>35) were obtained. De
252 novo assembling for high quality scaffolds was performed using SPAdes v3.9.0 (Nurk *et al.*,
253 2017). The assembled sequences were deposited in the NCBI database under GenBank
254 accession number SAMN14299480. Hereafter, the components of coding genes, non-coding
255 RNA (ncRNA), and functional annotation using a range of databases including NR,
256 Swissprot, COG, KEGG, GO were performed. A circular map of the genome was obtained
257 using Circos version 0.69 (Krzywinski *et al.*, 2009). The prediction of non-encoding 23S
258 rRNA and 16S rRNA were performed using rRNAmmer 1.2 (Lagesen *et al.*, 2007).

259

260 *Glasshouse experiments to assess effects of SR80 on wheat growth and defense*

261 The biological relevance of SR80 on wheat was investigated in glasshouse experiments. The
262 soil used for wheat cultivation was collected from the Wellcamp site in August 2019 and
263 autoclaved twice to ensure *Fp* elimination. Three treatments including *Fp* only, SR80 only,
264 *Fp* & SR80, and control were applied in pot experiments at sowing time (8×12 cm pots,
265 approx. 200 g soil each). The *Fp* inoculum grown on whole wheat grain was developed
266 using the strain *Fp* A11#04, which was ground, sieved (2 mm) and stored in low humidity at
267 4°C until use. *Fp* was applied using an inoculation method modified from Wildermuth and
268 McNamara, 1994 (Fig.S1). Briefly, six Jandaroi seeds were sown in soil and covered with a
269 thin layer of soil, and topped by a layer of the *Fp* inoculant (1.0 g) in the form of fine powder.

270 For treatments that excluded *Fp*, the autoclaved *Fp* inoculant was applied to reproduce the
271 chemical environment but the pathogen was not present in a living form. For bacterial
272 treatments, 40 mL of the SR80 suspension (in 50 μ M PBS buffer, pH 7.0, OD₆₀₀=1.0) was
273 inoculated to each pot, which provided $\sim 2.4 \times 10^8$ SR80 cells g⁻¹ bulk soil. As control, the
274 same amount of PBS buffer was added to treatment groups that excluded bacterial
275 inoculations. Soils in pots were then watered to field capacity to allow seeds to germinate.
276 Thereafter, soil was watered daily. The height of seedlings that emerged 3 days after sowing
277 was recorded twice daily (10 am and 4 pm) for 15 days. Disease and plant survival rates
278 were recorded once per day. The diameter of the stem base was recorded on the 7th and 14th
279 day after seedling emergence. Approx. 1.5 g of bulk soil was collected from the soil surface
280 from each pot at the 5th, 10th and 15th day for determining the abundances of SR80 and *Fp*.
281 When harvested, wheat shoots were cut, weighed, wrapped in aluminum foil and stored at -
282 80°C for total RNA extraction. Roots were carefully separated from soils, thoroughly rinsed
283 with tap water, air-dried on paper tissues and weighed. Due to total lethality of plants in the
284 *Fp*-treated control group, an additional experiment was conducted, which included the *Fp*
285 and *Fp*+SR80 treatments using the same method as above but with a lower inoculant load
286 per pot (0.6 g). This provided sufficient plant material for RNA extractions and qRT-PCR
287 analyses.

288

289 *Statistical analyses*

290 Statistical analyses were implemented in R3.6.1. Linear model (Pearson correlation) was
291 performed using the package ggpubr (0.1.6) to determine (i) correlations of defense gene
292 abundance with disease severity, and (ii) correlations of SR80 abundance in soil and roots
293 with *Fp* amounts in plants. Data transformation was performed where needed to ensure data
294 normalization before conducting statistical analyses. The effects of *Fp* and SR80 on
295 responses of soil and plant microbial communities were investigated by permutational
296 multivariate analysis of variance (PERMANOVA) using Hellinger transformed OTU
297 abundances. This analysis was performed using the package vegan 2.5-6 (Dixon, 2003).

298 Treatment effects on wheat growth, defense gene expression, alpha diversity of microbial
299 communities, and the abundance of SR80 and *Fp* were analyzed using one-way ANOVA
300 and Tukey post hoc tests.

301

302 **Results**

303 *Plants were naturally infected by Fp and defense signaling pathways were activated in the*
304 *field study*

305 Fifty-eight plants were collected from a field trial at the Wellcamp site. Symptomatology
306 inspections revealed that 40 of these plants were asymptomatic for CR and 18 plants
307 exhibited CR symptoms at different severity levels (Fig.1a). Disease severity of all plants
308 was visually rated based on discoloration of the stem and the level of *Fp* infection was
309 quantified by real-time quantitative polymerase chain reaction (qPCR) targeting the *Tri5*
310 gene (Fig.1b). The two methods provided consistent results (Fig.1b), as plants with visible
311 CR symptoms also had higher pathogen load quantified by qPCR (relative abundance ≥ 1)
312 than the asymptomatic plants (Fig.1a). Moreover, *Fp* load had a linear correlation with the
313 total fungi in base stem tissues (Fig.S2), indicating that *Fp* was the main component driving
314 the increases of fungal abundance in CR-infected plants in the field. Genes involved in plant
315 defense signaling pathways including *PR2* (a beta-1,3-endoglucanase), *PR3* (a chitinase),
316 *PR4a* (wheatwin), *PR5* (a thaumatin-like protein), *PR10* (a wheat peroxidase), *TaPAL*
317 (phenylalanine ammonia lyase) and *Lipase* were highly expressed in top leaves of *Fp*-
318 infected plants (Fig.1c-j). These genes are known to be markers for the activation of JA
319 and/or SA signaling pathways in wheat (Liu *et al.*, 2016). A positive linear correlation was
320 observed between transcript abundances of these defense genes and *Fp* load in the stem
321 tissues (Fig.1c-j, m&n). In contrast, the expression of two genes, *TaNPR1* (wheat
322 nonexpressor of pathogenesis-related genes 1) and *WCI3* (a sulfur-rich/thionin-like protein),
323 were not influenced by *Fp* (Fig.1k&l). Collectively, JA and SA defense signaling pathways
324 were activated in wheat by *Fp* (Fig.1m&n), which indicates successful progression of CR
325 and activation of plant defenses in the field.

326

327 *Microbiome structure differed between healthy and diseased wheat*

328 Wheat roots seem to have acted as a barrier to select soil microbes, resulting in phylogenetic
329 conservation in the rhizosphere and root endosphere (Fig.S3a). These root-associated
330 compartments selected microbial phylotypes within the Proteobacteria, Actinobacteria and to
331 a lesser extent Bacteroidetes and Firmicutes while Acidobacteria, Gemmatimonadetes and
332 Archaea were almost depleted from the root endosphere (Fig.S3a). Meanwhile, a gradient
333 decrease of alpha diversity (e.g. observed species) from bulk soil to the root was observed
334 (Fig.S3b). However, no differences were seen in the alpha diversity for the rhizosphere and
335 root endosphere microbial communities between the healthy and diseased plants (Table S4).

336

337 The microbial composition in the rhizosphere of *Fp*-infected plants significantly differed
338 from that of healthy plants (PERMANOVA, $P=0.0002$) and were marginally different in the
339 root endosphere ($P=0.073$) (Fig.2a&b). Furthermore, the rhizosphere soil fungal community
340 composition significantly differed between healthy and diseased plants ($P=0.001$) (Fig.S4).

341 In the redundancy analyses for the rhizosphere communities (RDA, Fig.2a), bacterial OTU
342 89589 is strongly correlated with the *Fp*-infected plants and is one of the major dominant
343 OTUs in the separation of the diseased plants from healthy plants in the primary axis of the
344 RDA (RDA1), well separated from rest of the OTUs. A consistent pattern was also found in
345 the root endosphere, where OTU 41442 was among the most discriminating OTUs for
346 infected plants (Fig.2b). Further analyses revealed that these two OTUs had 100% nucleotide
347 similarity within the 16S rRNA region amplified, indicating that they were the same
348 bacterial strain (Fig.S5a, b), and belonged to *Stenotrophomonas* spp.

349

350 Further analyses revealed that the abundance of OTU 89589 (in the rhizosphere) positively
351 correlated to the *Fp* load in the stem tissues of infected plants (relative abundance ≥ 1)
352 (Fig.2c). This was less noticeable in asymptomatic plants (relative abundance <1) (Fig.2c).
353 *Fp* infection had contributed to the enrichment of this bacterium to as much as 3.7×10^7 cells

354 per gram of the rhizosphere soil (Fig.2c). Similarly, the abundance of this bacterium in the
355 root endosphere also positively correlated with *Fp* abundance, accounting for up to 11.4% of
356 the bacterial community in roots (Fig.2d). These results suggest that the wheat-associated
357 microbiome had significantly changed in composition in response to *Fp*-infection, and
358 specifically enriched for a *Stenotrophomonas* spp. in the process. We also observed that
359 selection for the bacterium seems to have occurred even when wheat plants were healthy
360 (Fig.2e). Interestingly, the abundance of this bacterium also positively correlated with
361 transcript abundances of all PR defense genes tested (Fig.2f), suggesting that the bacterium
362 is likely to have contributed to the activation of plant defenses in the field.

363

364 *Bacterial isolation, identification and whole genome sequencing*

365 We attempted to isolate *Stenotrophomonas* spp., to investigate its biological implications on
366 wheat performance via controlled *in vivo* and *in vitro* tests. A total of 179 bacterial isolates
367 were recovered from the *Fp*-infected plant rhizosphere and roots using different media,
368 including a selective medium for *Stenotrophomonas* spp. Among these, seven isolates were
369 identified as *Stenotrophomonas* spp. by 16S rRNA Sanger sequencing, and these belonged to
370 four different strains (identified using BOX PCRs, Fig.S5a,b,c). Amplicon sequencing data
371 also revealed that different *Stenotrophomonas* species/strains (OTUs) colonized the
372 rhizosphere and roots, which is in line with the multiple strains obtained by the culture-
373 dependent method. Among the four strains, only the 16S rRNA of *Stenotrophomonas* spp.
374 R80 (hereafter referred as SR80) had 100% nucleotide similarity match with the sequences of
375 OTU 89589 and OTU 41442 (Fig.S5a,b,c). The whole genome of SR80 was then sequenced
376 and analyzed (Fig.S6&7), through which the 16S rRNA region was obtained, which was
377 1,532 bp in length and had 100% sequence similarity to OTU 89589 and OTU 41442
378 (Fig.S6a,b). This suggests that SR80 was the bacterium enriched in the wheat microbiome by
379 *Fp*-infection (Fig.3a,b,c,d, Fig.S6a,b). The SR80 genome had the highest percent of Average
380 Nucleotide Identity (ANI) (97.71%) to a *Stenotrophomonas rhizophila* strain QL-P4 that was

381 isolated from a pine tree leaf, suggesting it is a member of *S.rhizophila* species but possibly a
382 different strain.

383

384 The predicted genome size of SR80 is 4,234,260 bp containing 3,720 detected genes
385 (Fig.S7). Class of genes (COG) annotation was performed on the whole genome and 3,052
386 protein sequences were successfully annotated. Known virulence factors and plant cell wall
387 degrading enzymes were absent, but genes encoding proteins involved in symbiosis, host-
388 cell interaction and activation of plant defense signaling pathways were detected. Genes
389 coding for bacterial flagellin (*flg22*), chitin (*AvrXa21*), and Type I~ Type IV secretion
390 systems were detected (Fig.S8), as were genes *fliR* and *flhB* which code for effector proteins
391 that interact with the plant immune system and induce the plant's primary responses. Genes
392 encoding proteins involved in biofilm formation, chemotaxis, lipopolysaccharide (LPS)
393 biosynthesis, the quorum sensing system and reactive oxygen species (ROS) scavenging
394 (e.g., superoxide dismutase) are also present in multiple copies. These indicate that (i) SR80
395 can cope with plant immune responses that may prevent bacterial colonization, and (ii) this
396 bacterium could be well adapted to the root environment. SR80 was further studied in
397 antifungal tests against four virulent *Fp* strains (including the *Fp* used in the field experiment)
398 on dual media, but no noticeable inhibition was observed (Fig.3c).

399 *SR80 promoted plant growth and defense*

400 To evaluate whether SR80 affects wheat growth and defense, we treated the *Fp*-infected
401 variety of durum wheat (Jandaroi) with SR80 in glasshouse experiments (Fig.4). Overall,
402 inoculation with SR80 at the time of sowing significantly promoted plant growth and
403 conferred protection against *Fp* compared with the non-inoculated control (Fig.4a,b,c,d).
404 Within 15 days, plant biomass in both, below-ground (roots, +156%) and above-ground
405 (shoots, +124%), compartments substantially increased by SR80 inoculation (Fig.4b). This is
406 consistent with the observation that the diameter of the stem base was significantly larger at
407 the two time points monitored, although the height of the SR80-inoculated plants was not

408 larger than the control within the timeframe tested (Fig.4c). Moreover, plant survival rates
409 were significantly higher in the SR80-inoculated group than the untreated group when
410 subjected to *Fp* infections. The plants in the untreated control all died from *Fp* infection
411 within 15 days (Fig.4d). qPCR quantification did not reveal a decrease in *Fp* load after the
412 bacterial inoculation (Fig.4f), which is consistent with our plate assay results. The bacterial
413 communities in soils were profiled at three time points (5, 10 and 15 days). We found that
414 the abundance of the added SR80 in soils gradually decreased to a stable level 10 days after
415 seed germination ($\sim 2.4 \times 10^7$ cells g^{-1} soil) (Fig.4e), in agreement with the relative abundance
416 of SR80 in the soil microbiome continuously declining within the timeframe, from 70.0% to
417 40.74% (Fig.S9). The alpha diversity of soil microbial communities gradually increased in
418 all groups within 15 days, with the highest diversity observed in the uninoculated control
419 (Fig.S10).

420

421 *SR80 manipulated plant defense signaling pathways*

422 SR80 did not significantly inhibit *Fp* in soil or on agar plates, indicating that a direct
423 inhibition of the pathogen is not the mechanism for this bacterium to benefit wheat survival
424 and growth. We hypothesized that SR80 modulates expression of genes involved in plant
425 defense. An additional glasshouse experiment was conducted to test this hypothesis, in
426 which plants were inoculated with both the pathogen *Fp* and the beneficial bacterium SR80
427 and evaluated for expression of marker genes associated with plant defense signaling
428 pathways. We found that when SR80 is present in soils, JA and SA signaling pathways were
429 significantly activated in wheat but only in the presence of *Fp*. Expression of genes,
430 including *TaAOS* (+3.1 fold) (Fig.5a), *TaNPR1* (+2.2 fold) (Fig.5b), *Lipase* (+6.0 fold)
431 (Fig.5c), *WRKY78* (+3.5 fold) (Fig.5d), *PR2* (+3.0 fold) (Fig.5f), and *PR3* (+2.3 fold)
432 (Fig.5g), was enhanced by SR80 treatments (Fig.5g). In contrast, expression of *PR4*
433 marginally decreased by the bacterial treatment without *Fp* infection. Other PR genes also
434 showed a decrease although were not statistically significant (Fig.5h).

435

436 **Discussion**

437 Our study demonstrates that CR disease in wheat plants leads to an enrichment of the
438 beneficial bacterium SR80 in the rhizosphere soil and root endosphere. The assembled SR80
439 was able to significantly enhance plant growth at both the above- and below-ground and
440 induce resistance against the CR disease in glasshouse experiments. Despite strains of
441 *S.rhizophila* having been shown to suppress mycelial growth in various fungi at times
442 through the production of volatiles (Kai *et al.*, 2007), SR80 did not directly inhibit *Fp*
443 growth on plates or in soil. However, SR80 can upregulate plant defense signalings (e.g. JA
444 and SA) in shoots with the presence of the pathogen. These findings provide a novel
445 mechanism of tripartite interactions between the devastating fungal pathogen, the plant host
446 and plant-associated microbiota, and revealed a bacterium that may have acted as an early
447 warning system for the onset of plant defense.

448

449 *SR80 intimately interacts with plant immune systems and mediates a disease resistance*

450 SR80 inoculation tends to suppress the expression of PR-related defense genes in wheat
451 shoots without *Fp* presence in soils. Consistent with this finding, suppression of the plant
452 root immunity by non-pathogenic *Pseudomonas* species has recently been observed in
453 *Arabidopsis thaliana*, which may have facilitated the bacterial colonization on roots (Yu *et*
454 *al.*, 2019). These suggest that quenching plant immune responses is an emerging strategy for
455 plant-associated bacteria to prevent constitutive activation of plant immune responses.
456 Rhizosphere and endosphere microbiota are rich in microbe-associated molecular patterns
457 (MAMPs), which can trigger the first layer of plant immune defense that restricts pathogen
458 reproduction, MAMP-triggered immunity. SR80 undoubtedly possesses a range of
459 components acting as MAMPs (e.g. flagellin), but how the bacterium avoids eliciting strong
460 immune responses in plants is unclear. Recent conceptual and experimental framework
461 indicates that plant-associated commensal microbes can alleviate/avoid plant immune
462 responses by (i) producing enzymes to scavenge reactive oxygen species (ROS) production
463 of the plant (Sessitsch *et al.*, 2012), (ii) producing organic acids to quench local plant

464 immune responses (Yu *et al.*, 2019), and (iii) modifying, degrading or changing the structure
465 of MAMPs (Liu *et al.*, 2020). Our whole genome annotation data suggest that SR80 contains
466 multiple genes encoding key components that are functioning by these mechanisms.

467

468 Interestingly, upon challenge with *Fp*, pre-colonization by SR80 on wheat seedlings induced
469 a much stronger plant defense that increased plant survival rates relative to untreated plants.

470 This suggests that SR80 possesses elicitors that triggered Induced Systemic Resistance (ISR)
471 and ‘primed’ the plant for faster and more pronounced responses to pathogen attack. Both
472 the SA and JA signaling pathways of the plant were activated, which is quite unusual
473 because SA and JA signaling pathways are mostly antagonistic, although non-canonical
474 mechanisms of synergism have been reported (Thaler *et al.*, 2012). In monocots, the
475 relationships between the disease signaling pathways and the nutrient acquisition behavior of
476 the pathogen is less understood.

477

478 *Fp* behaves as a hemi-biotroph as there is an extended initial phase that plants show no
479 symptoms, after which necrotic lesions develop. Two oxalotrophic strains of the same genus
480 as SR80, *Stenotrophomonas*, were isolated from the tomato rhizosphere and have been
481 shown to upregulate *PR-I*, a marker gene for the SA pathway (Marina *et al.*, 2019).
482 However, these strains did not influence the expression of *PDF1.2*, a marker gene for
483 JA/ethylene (ET) signaling pathway (Marina *et al.*, 2019). In line with our findings, a strain
484 of plant growth promoting *Stenotrophomonas maltophilia*, isolated from the rhizosphere of
485 sorghum, increased synthesis of a range of enzymes associated with defense against fungal
486 pathogens when inoculated on wheat, including peroxidase, β -1,3 glucanase, polyphenol
487 oxidase and phenylalanine ammonia lyase (Singh & Jha, 2017). Overall, our findings
488 suggest that SR80 can play a key role in the three-way interactions with the host plant
489 immunity and the fungal pathogen via a mechanism that enhances the plant defence and
490 growth.

491

492 *SR80 was enriched in root environments upon Fp infection*

493 In this study, we used amplicon sequencing and qPCR for the analysis of plant microbiomes,
494 which can detect differences in microbial community structure and estimate the abundance
495 of microbial taxa in soils. Our results revealed that SR80 was thriving at high abundances
496 around roots of the *Fp*-infected plants (up to 3.7×10^7 cells g^{-1} rhizosphere soils). *S.*
497 *maltophilia* was also present at high titers in root samples of oilseed rape, about 1.1×10^7
498 colony forming units (cfu) g^{-1} wet weight root (Berg *et al.*, 1996). A minimum of 10^5 cfu g^{-1}
499 root has been reported to be required for the onset of ISR by certain beneficial microbes
500 (Pieterse *et al.*, 2014). Accordingly, the quantity of SR80 accumulated in the rhizosphere and
501 roots of the infected wheat is likely have reached sufficient numbers to induce plant defense
502 responses and confer a protective barrier to restrict *Fp*. This is consistent with upregulated
503 defenses in wheat plants under both the glasshouse and field conditions by the presence of
504 SR80. However, we noticed that the developed CR symptoms persisted throughout the life
505 cycle of the plant in the field and glasshouse experiments, which suggests that the
506 recruitment of SR80 unlikely cures an existing infection, but may instead increase the plant
507 tolerance to the disease.

508

509 Recent studies in dicot plants including *A.thaliana* and pepper (*Capsicum annum* L. cv.
510 Bukwang) are in line with our findings as attack of foliar tissues by pathogens or insect
511 herbivores resulted in plant-mediated changes in the rhizosphere microbial communities
512 (Kong *et al.*, 2016; Berendsen *et al.*, 2018). Particular microbial taxa affiliated to
513 *Stenotrophomonas* spp. were similarly enriched in the diseased plants, which suggests that
514 this genus is commonly influenced by different plant biotic stresses. *Stenotrophomonas* spp.
515 including *S. rhizophila* possess a strong capacity to colonize root tissues (Hayward *et al.*,
516 2010), which allows the bacterium to intimately interact with the host plant. Under the
517 conditions tested in our study, wheat preferentially selected certain microbial phylotypes (e.g.
518 Gammaproteobacteria) including SR80, resulting in a continuous increase of SR80
519 abundance in the wheat microbiome as habitats shifted from bulk soil to the root endosphere.

520 In fact, there is evidence to suggest that healthy plants assemble different microbial
521 populations to assist their growth and development (Yuan *et al.*, 2015; Qiao *et al.*, 2017;
522 Imchen *et al.*, 2019). CR disease also modulated the wheat microbiome towards
523 accumulation of more SR80 in the rhizospheric and endophytic microbial communities,
524 suggesting that disease infection can be a critical driver for the plant microbiome assembly,
525 and a cry for help strategy can be triggered to allow plants to actively seek help from soils to
526 cope with biotic stresses.

527

528 It is worth pointing out that not all plant diseases induce microbial changes in the plant
529 microbiome. For example, infection by the necrotrophic pathogen *Botrytis cinerea* in
530 *A.thaliana* did not clearly promote microbes in the rhizosphere although it did activate the
531 plant JA-dependent defense pathway (Berendsen *et al.*, 2018). This indicates the pattern of
532 disease-induced changes in the plant microbiome is defined by the particular interactions
533 occurring between a specific plant species and a pathogen. Furthermore, enrichment of
534 particular microbes in plant microbiomes can at times promote the fungal spore germination
535 and virulence (Partida-Martinez & Hertweck, 2005; Seneviratne *et al.*, 2007).
536 *Burkholderia* spp. for example, forms a symbiotic relationship with the pathogenic fungus
537 *Rhizopus*, the causative agent of rice seedling blight (Partida-Martinez & Hertweck, 2005).
538 This bacterium produces a toxin that is required for the pathogen to colonize its rice host. In
539 this case, the enrichment of a bacterium helps in the establishment of a fungal disease.

540

541 Collectively, our findings suggest that infection of wheat plants by CR disease results in the
542 recruitment of a beneficial rhizospheric microbe that has the potential to help plant growth
543 and protect plants via modulation of plant defense capacity. The enriched microbe acts as an
544 early warning agent, rapidly activating the JA and SA signaling pathways upon pathogen
545 invasion in plants. This work advances the current understanding of plant-microbe
546 interaction research and supports the coevolution theory of mutualism between the plant and
547 microbes.

548

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667

668 *Acknowledgements*

669 The authors would like to acknowledge the financial support from the Australian Research
670 Council (DP1094749; DP170104634; DP190103714). We thank Dr Friday Obanor for

671 providing us *Fp* strains and Dr Anna Balzer for helping with plant sampling and soil
672 collection. We also thank Associate Professor Mark Dieters for providing wheat seeds and
673 Dr Laura E. Brettell for proofreading this article.

674

675 *Author contributions*

676 H.L., P.S., L.C.C conceptualized the idea, which was further refined by B.K.S; H.L., L.C.C.,
677 and C.P. did the plant and soil sampling; H.L. and J.L. processed the plant and soil samples
678 and did the measurements for glasshouse treatments; H.L. analyzed the data and wrote the
679 first draft with significant inputs from B.K.S; all authors have critically read and revised the
680 manuscript.

681

682 *Data availability*

683 Data of this study are available at [10.6084/m9.figshare.12464465](https://doi.org/10.6084/m9.figshare.12464465). Other relevant data are
684 available upon request.

685

686 **Figure captions**

687 **Fig.1** The effects of *Fp*-infection on wheat stem and expression of defense genes. An
688 example of healthy and *Fp*-infected wheat stems (internode 1) (a). *Fp*-infected wheat stems
689 had a brown discoloration (relative abundance of $Fp \geq 1$) while the healthy stems were
690 white/light green (Fp abundance < 1). A Pearson correlation of *Fp* abundance with visually
691 rated disease levels at stem base (b). Effects of *Fp* infection on the transcription of ten genes
692 associated with jasmonic acid (JA) and salicylic acid (SA) signaling pathways in wheat (c-1).
693 Linear correlation tests were performed for these defense genes with the *Fp* load present in
694 the stem base. Each grey circle represents an independent measurement ($n = 58$). Red dashes
695 show significant regression and the overlaid shaded area represents 95% confidence intervals.
696 Both the JA and SA signaling pathways were activated by the *Fp* infection (m), and a
697 heatmap summarizing the correlation of the expression of defense genes in leaves with the
698 disease severity in wheat (n).

699

700 **Fig.2** Effects of *Fp* infection on wheat-associated microbial communities. Redundancy
701 analysis summarizing variations in composition of microbial communities in the rhizosphere
702 soil (a) and root endosphere (b) attributed to crown rot. Grey crosses shown in (a) and (b)
703 represent bacterial OTUs detected in the rhizosphere and root endosphere. Pearson
704 correlations of the SR80 abundance in the rhizosphere soil (c) and root endosphere (d) with
705 *Fp* abundance in wheat stems. Gradient increases in relative abundance of SR80 from the
706 bulk soil to the rhizosphere, and to the root endosphere (e). A table summarizing the
707 correlation of SR80 abundances with defence gene expression in leaves (f). Asterisks
708 indicate significant correlations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

709

710 **Fig.3** Morphology of SR80 isolated from wheat rhizosphere soil and root endosphere.
711 Colony morphology of SR80 grown for 24 h on trypticase soy agar (TSA) (a). Bacterial cell
712 morphology under a light microscope (b). Slight inhibition of the bacterium on *Fp* growth on
713 a dual media (1/2 PDA+1/2 TSA) (c). Bacteria used as positive controls at the bottom right
714 were two strains of *Burkholderia* spp.

715

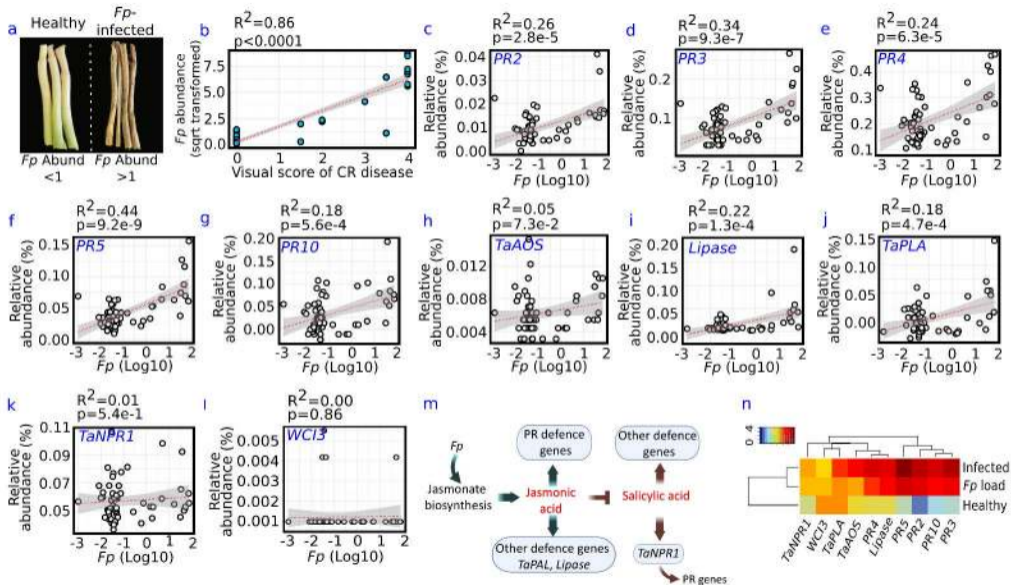
716 **Fig.4** Effects of SR80 inoculation on wheat growth and survival. Wheat phenotypes 14 days
717 post treatment applications (a). Control: no inoculation, SR80: inoculation with SR80 only,
718 *Fp*: inoculation with *Fp* only, SR80+*Fp*: dual inoculation with SR80 and *Fp*. Biomass
719 accumulation of wheat shoots and roots 15 days after the treatment applications (b). Increase
720 in plant height and stem size over 15 days from the application of treatments (c). Wheat
721 survival rates under different treatments (d). Changes in abundance of SR80 (e) and *Fp* (f)
722 abundance in soils. Asterisks indicate significant differences between treatments
723 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Error bars in (b)- (f) represent standard errors
724 of the mean (n=6).

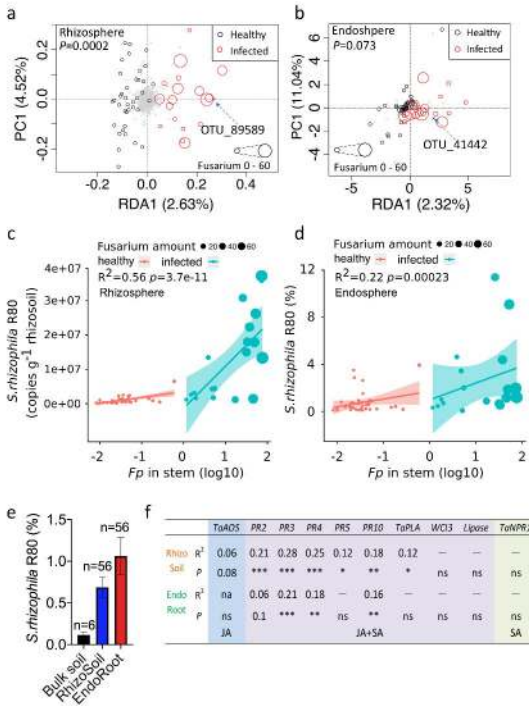
725

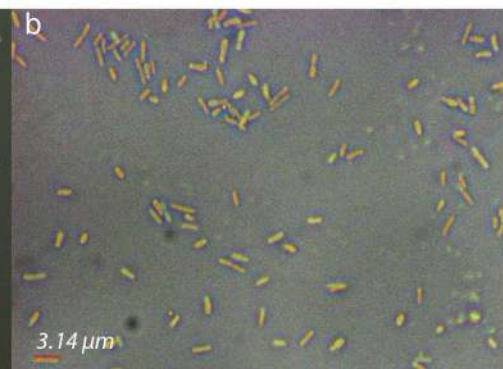
726 **Fig.5** Effects of SR80 and *Fp* inoculations on the transcription of genes associated with JA
727 and SA signaling pathways. Glasshouse experiment 1 (left) and 2 (right) were separated by
728 black dash lines on each graph. Asterisks indicate significant differences between treatments
729 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Error bars represent standard errors of the
730 means (n = 6).

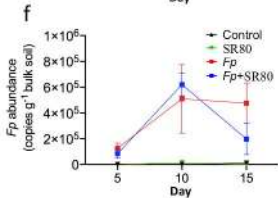
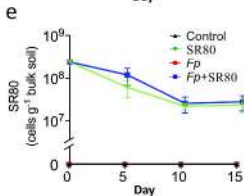
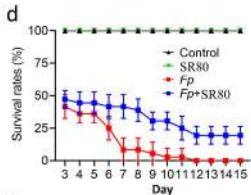
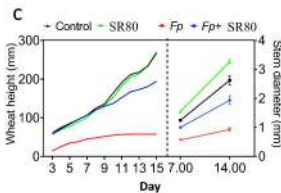
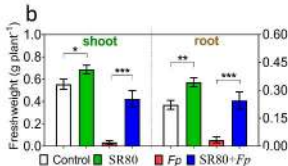
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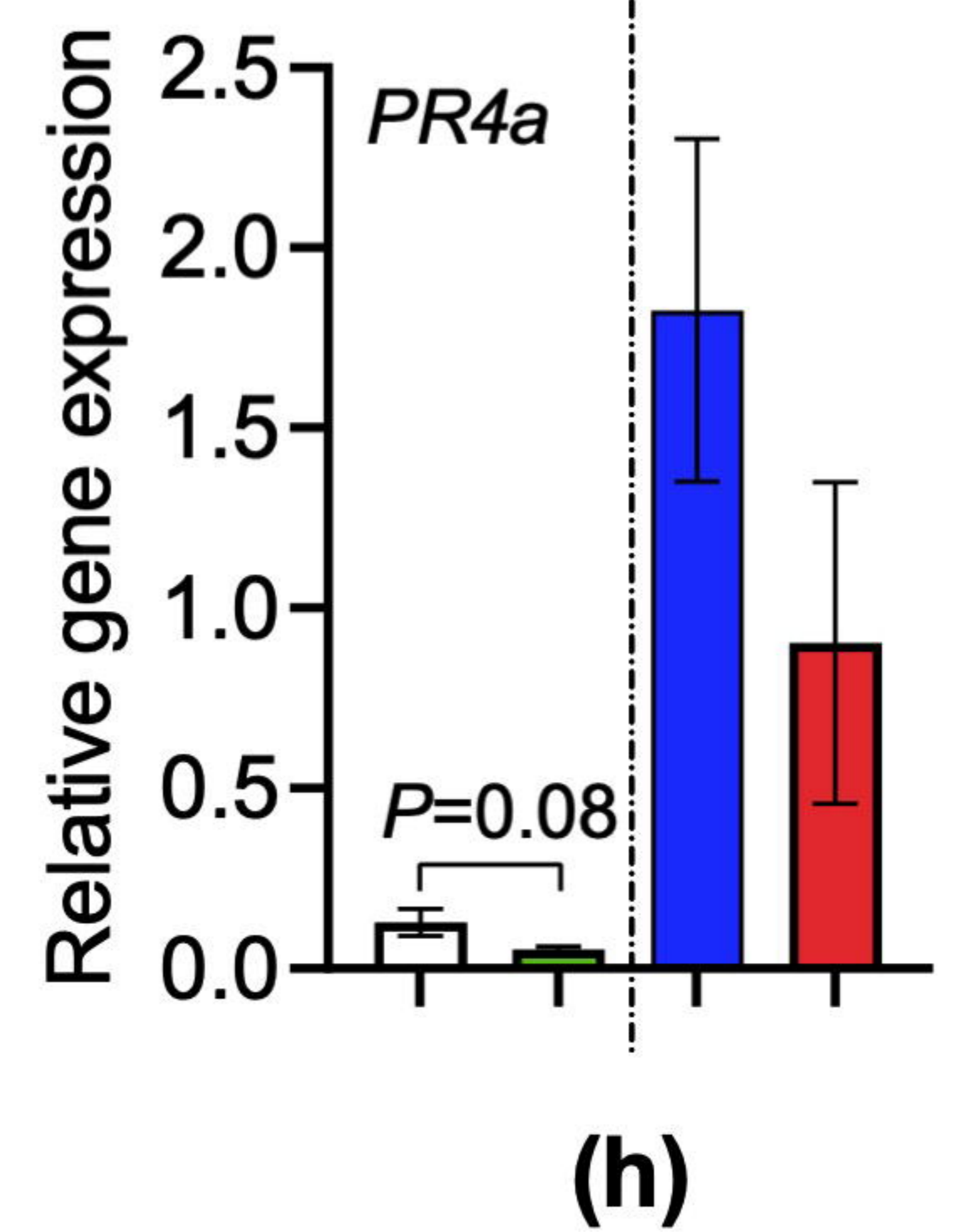
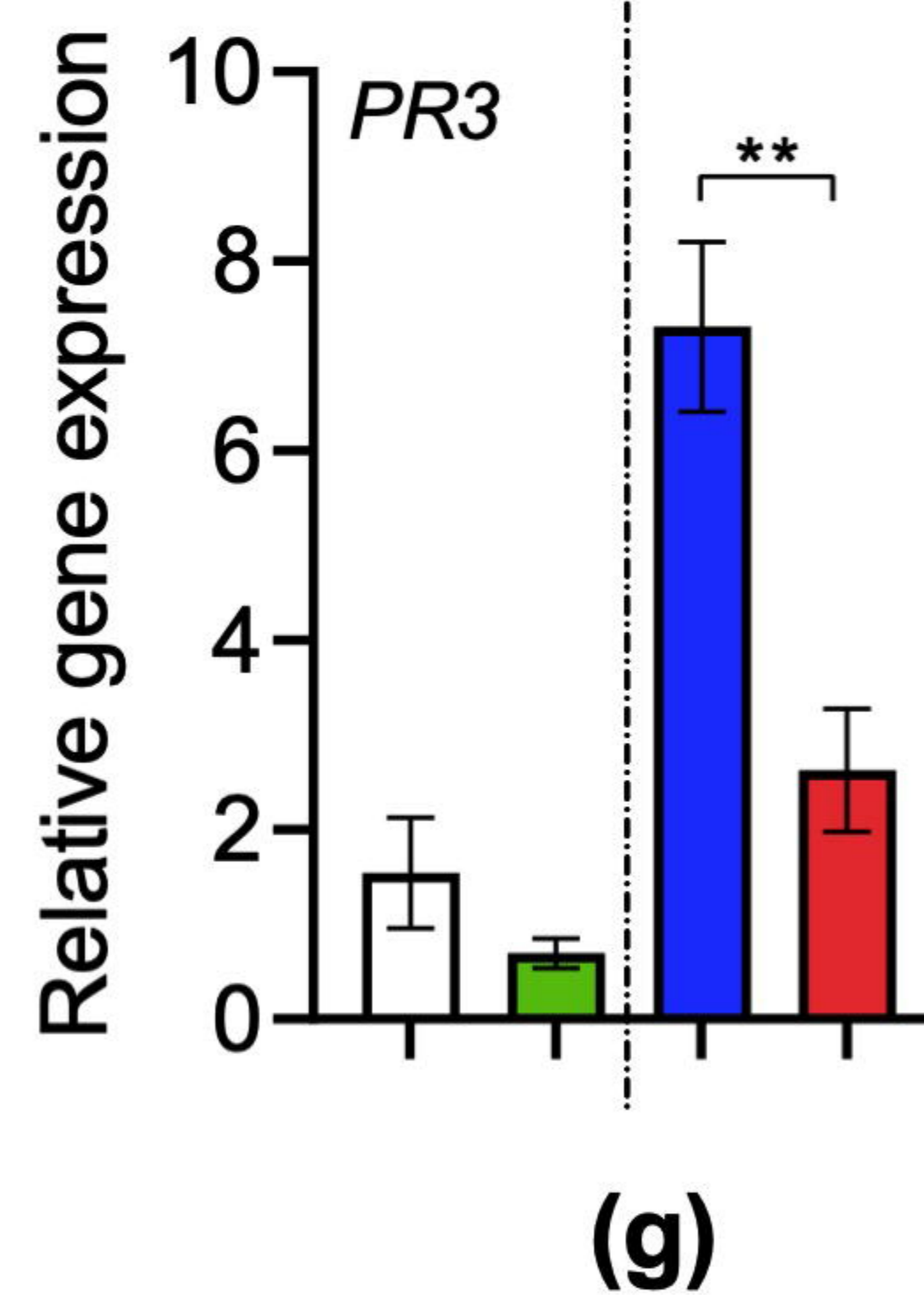
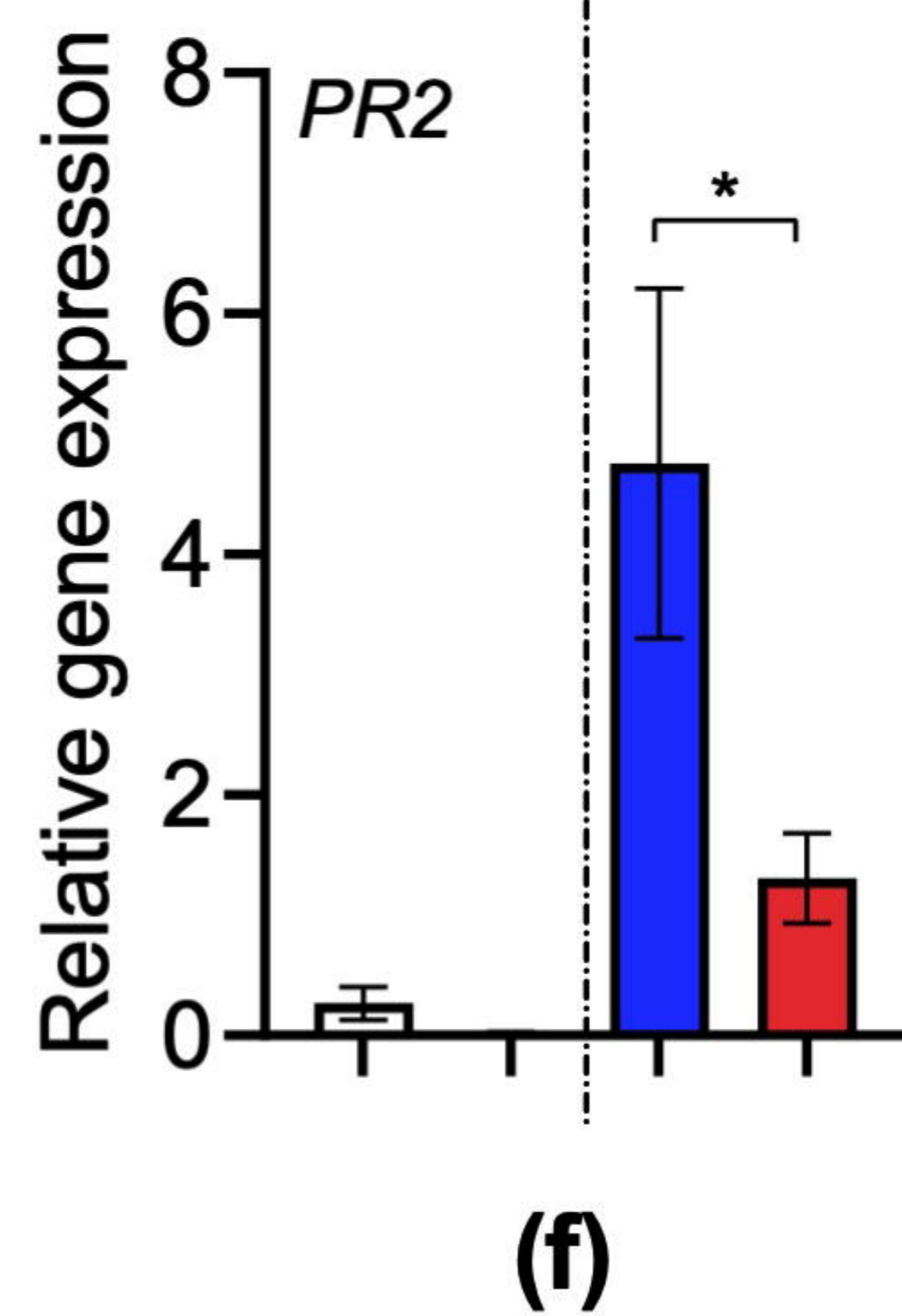
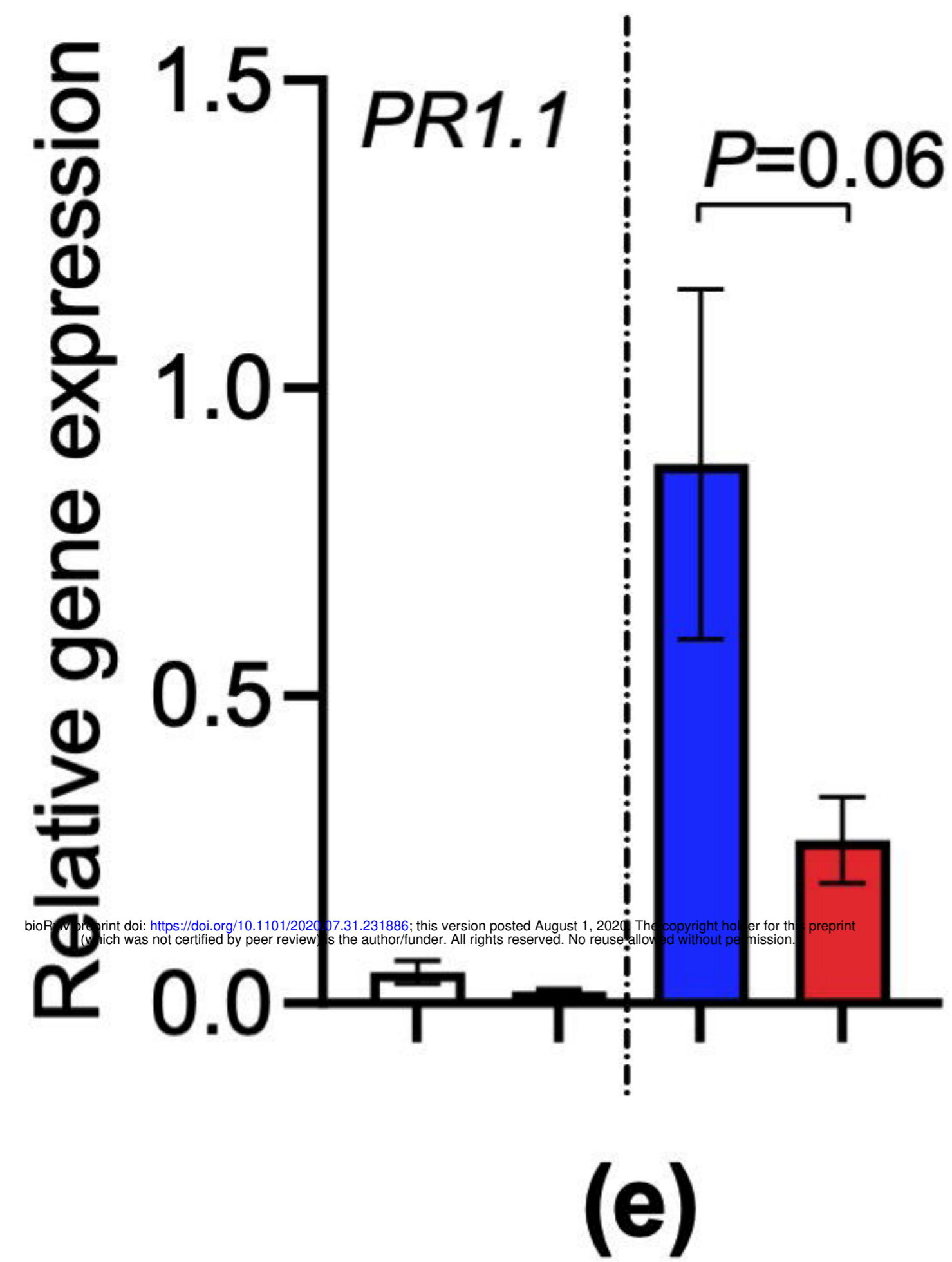
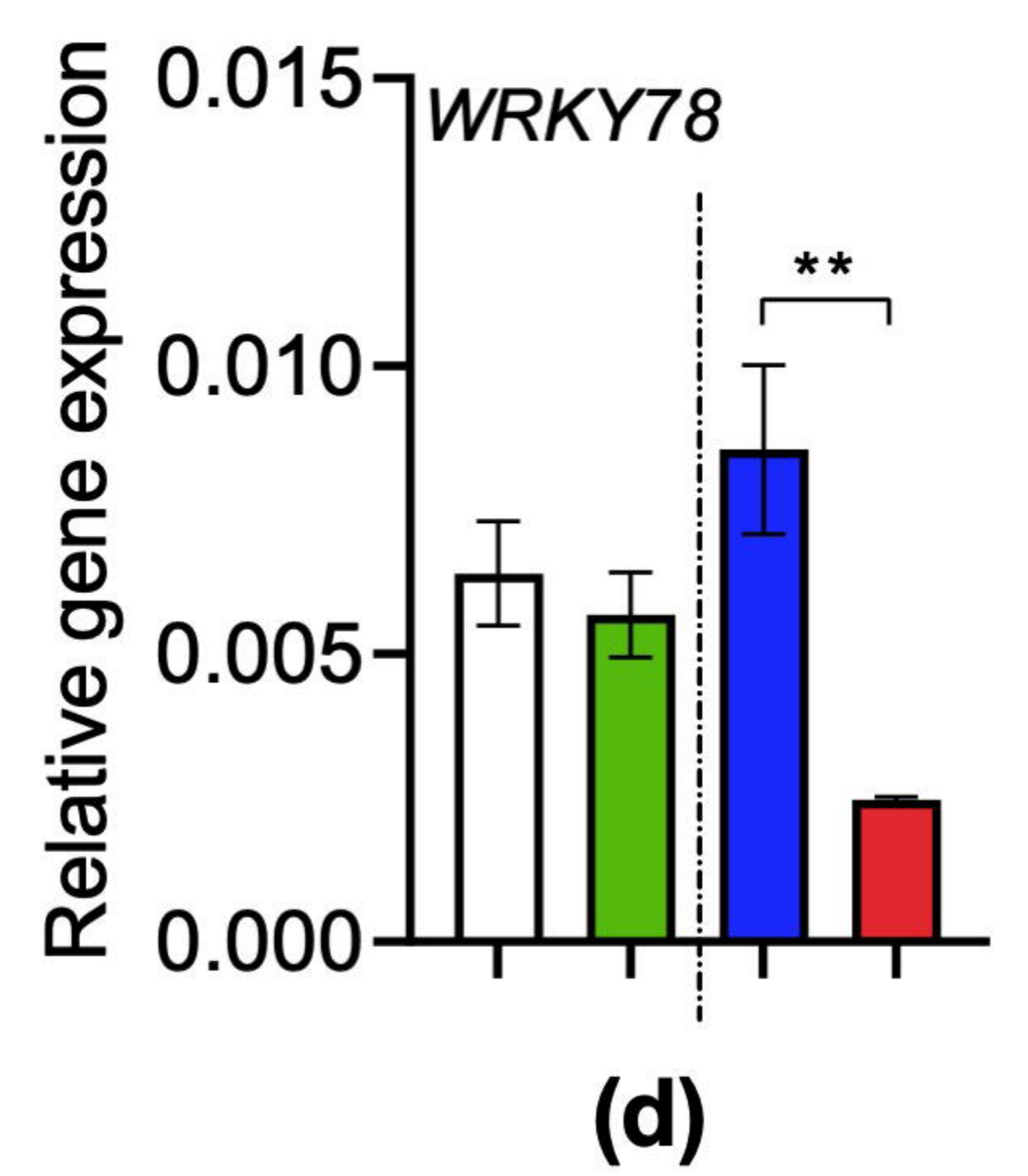
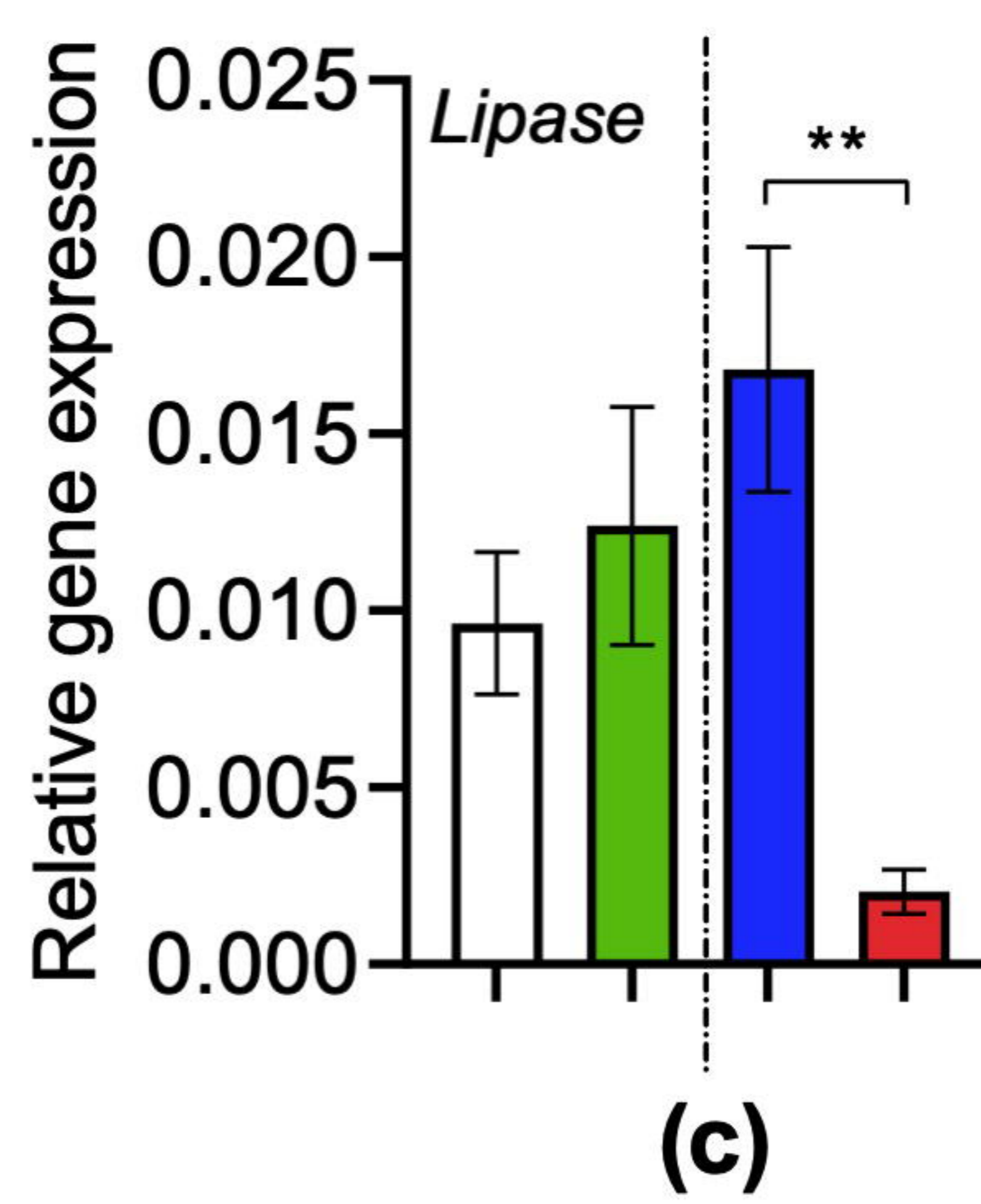
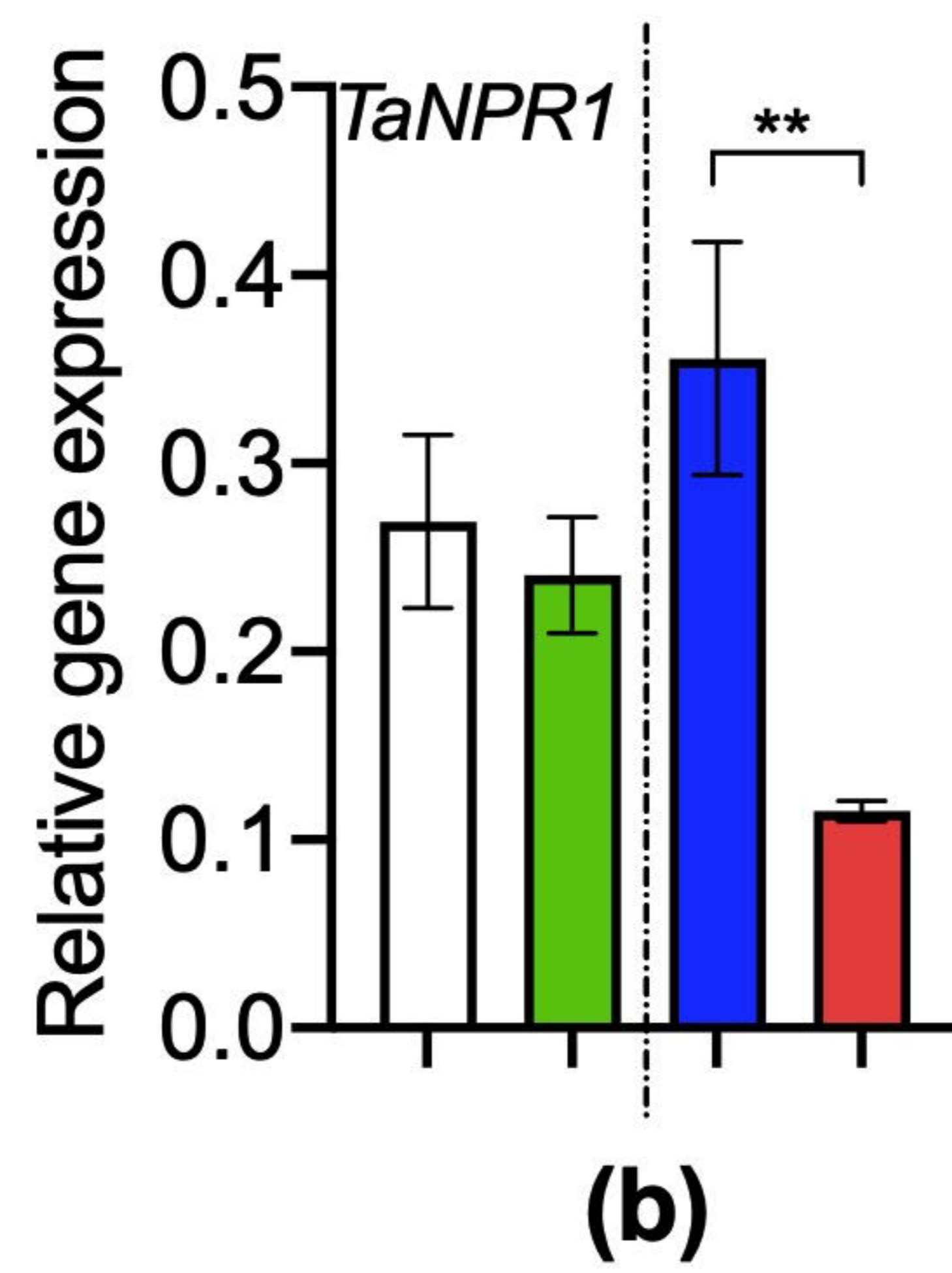
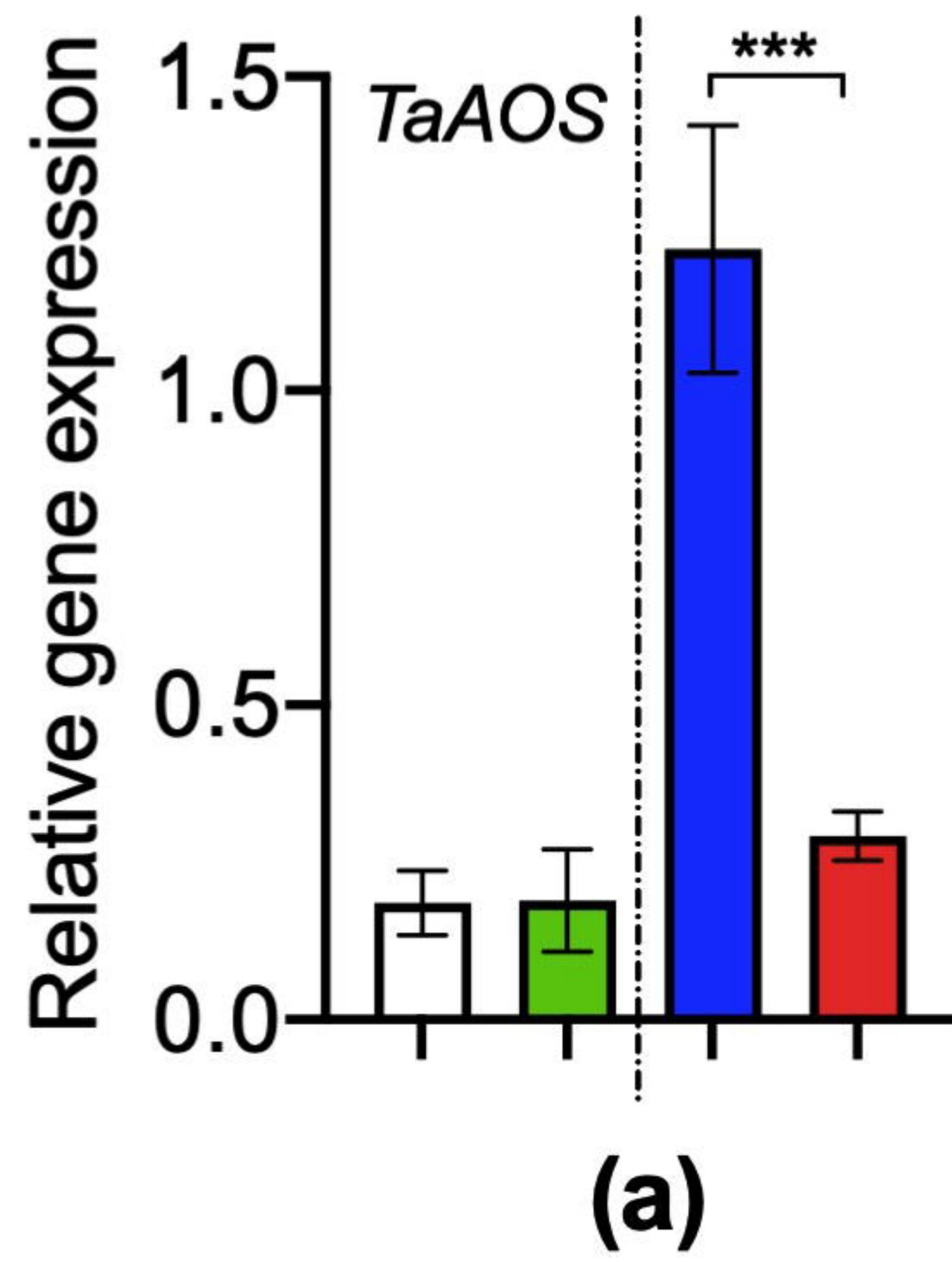
732 **Fig.6** Schematic drawing illustrating biological implications of SR80 on plant growth and
733 defense. The bacterium SR80 can modulate wheat growth and defense, which seems to be
734 mediated by the plant immune system. The plant favors the enrichment of the bacterium
735 from the rhizosphere soils and to the root endosphere. Moreover, when exposed to *Fp*, the
736 wheat plant favors the enrichment with even more SR80 in the rhizosphere. Such an active
737 recruitment process is accompanied by an enhanced defense gene expression in leaves.
738 Future research can investigate (i) whether plant leaves enrich the bacterium (process 1), and
739 (ii) whether allelopathic effects lead to the enrichment of the bacterium in the neighboring
740 threatened plants (process 2&3).











Control
 SR80
 SR80+Fp
 Fp

Stenotrophomonas rhizophila R80 comes to wheat's rescue

