APPENDIX

PHR1 suppresses plant immunity to shape root microbiome through the RALF-FERONIA complex under Pi starvation

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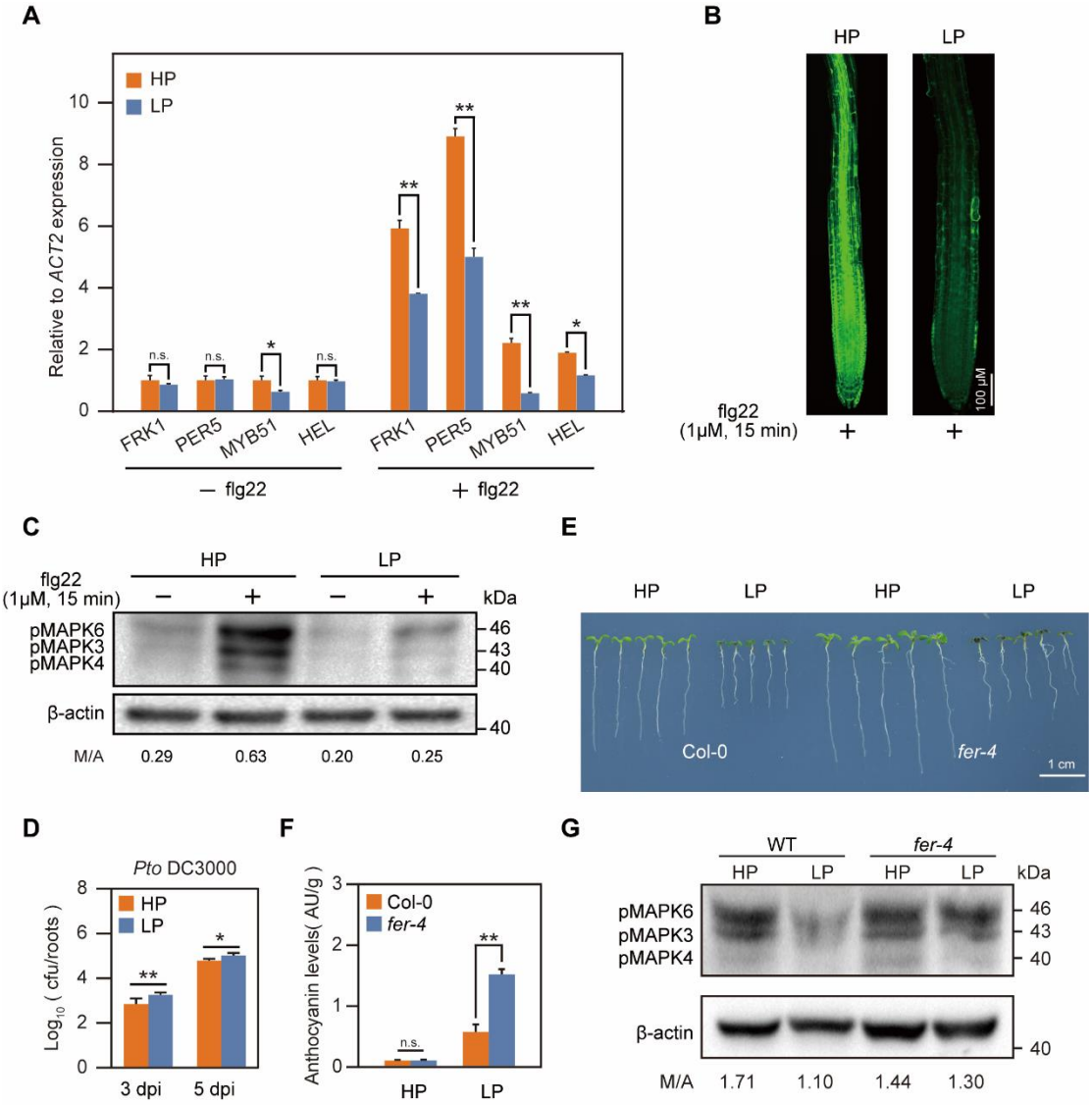
Appendix Table S1



Appendix Figure S1. The PSR negatively regulates the immune response of *Arabidopsis*.

(**A**) The PSR inhibits flg22-induced MAMP-responsive gene expression. WT (Col-0) seedlings grown in either HP or LP media were treated with water or flg22 for 15 mins. Roots were harvested, and the relative expression of MAMP-responsive genes was quantified via RT-qPCR. The data shown indicate the means ± SDs (n = 3, n refers to technical replicates); n.s., not significant; *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test).

(**B**) The PSR inhibits flg22-induced ROS production in WT (Col-0) roots. WT seedlings grown in either HP or LP media were treated with flg22 for 15 mins. Roots were harvested and stained with H_2DCFDA.

(**C**) The PSR inhibits flg22-induced MAPK activation. WT (Col-0) seedlings grown in either HP or LP media were treated with water or flg22 for 15 mins. Roots were

harvested, and MAPK levels were determined with anti-pMAPK. β-actin was used as a loading control.

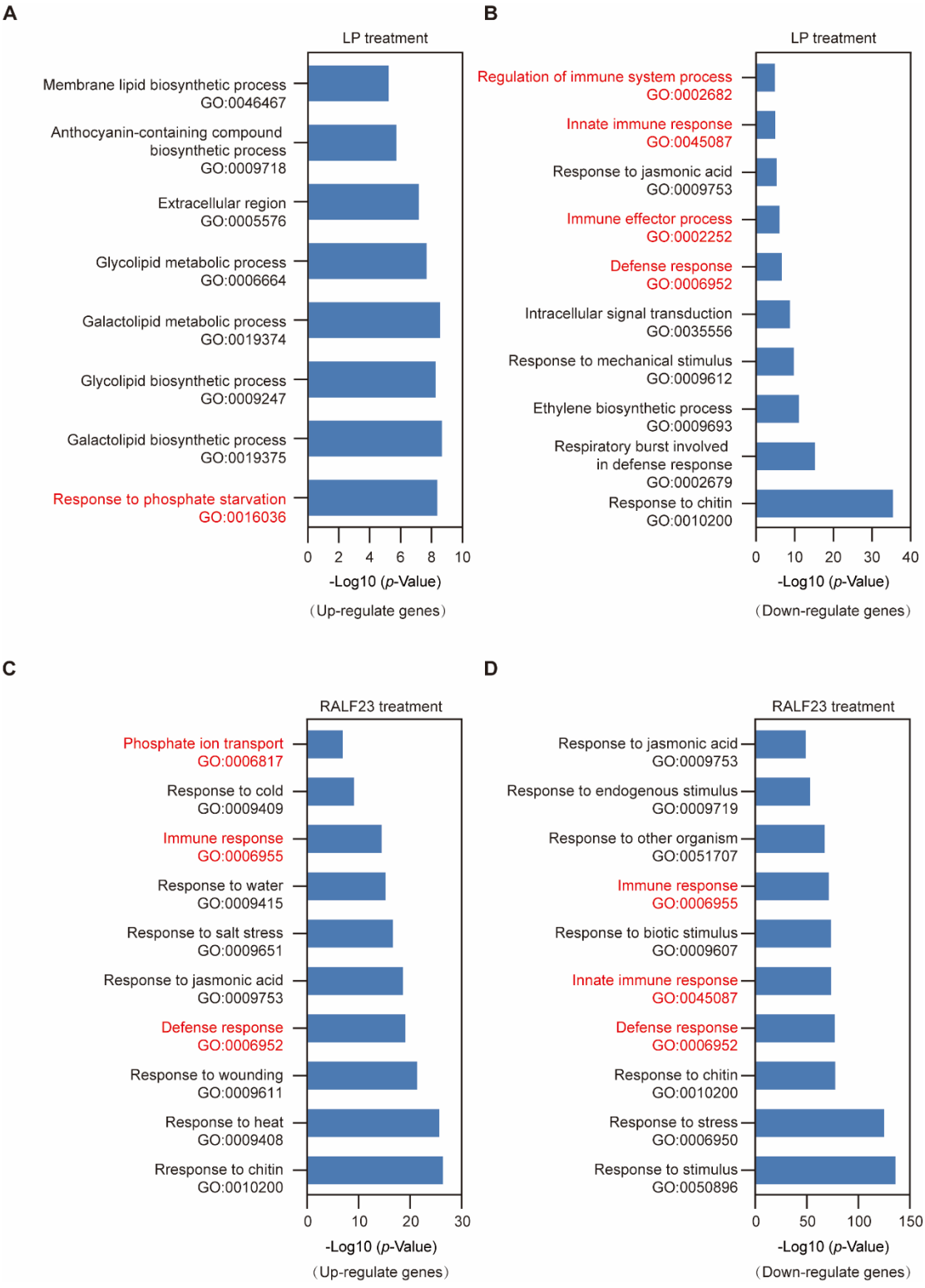
(**D**) Growth of *Pto* DC3000 in WT (Col-0) under Pi-starvation conditions. Seedlings grown in either HP or LP media were inoculated (by soaking) with bacterial suspension (OD_{600} = 0.2) for 2 min, and the number of bacteria from individual root was quantified at 3 days and 5 days after inoculation (dpi), respectively. The data shown indicate the means ± SDs (n=18, n refers to the number of roots per group); The *p* value is for the statistical difference of the log (cfu/root) between HP and LP. *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test).

(**E**) Phenotype of *Arabidopsis* under Pi-starvation conditions. WT (Col-0) and *fer-4* mutants was first grown in 1/2-strength MS media for 3 days and then transplanted to either 1/2-strength MS media consisting of 1.25 mM Pi (HP) or low-Pi media consisting of 10 μM Pi (LP) for another 5 days.

(**F**) Anthocyanin levels were measured spectrophotometrically at 530 and 657 nm. The concentration of anthocyanins was calculated using the formula A530 - 0.25*A657. The data shown indicate the means ± SDs (n = 3, n refers to technical replicates); n.s., not significant; **, $p < 0.01$ (Student's *t* test).

(**G**) MAPK activation in WT (Col-0) and *fer-4* mutant roots. Seedlings grown in either HP or LP media for 5 days and roots were harvested. The MAPK level was determined with anti-pMAPK, and β-actin was used as a loading control.

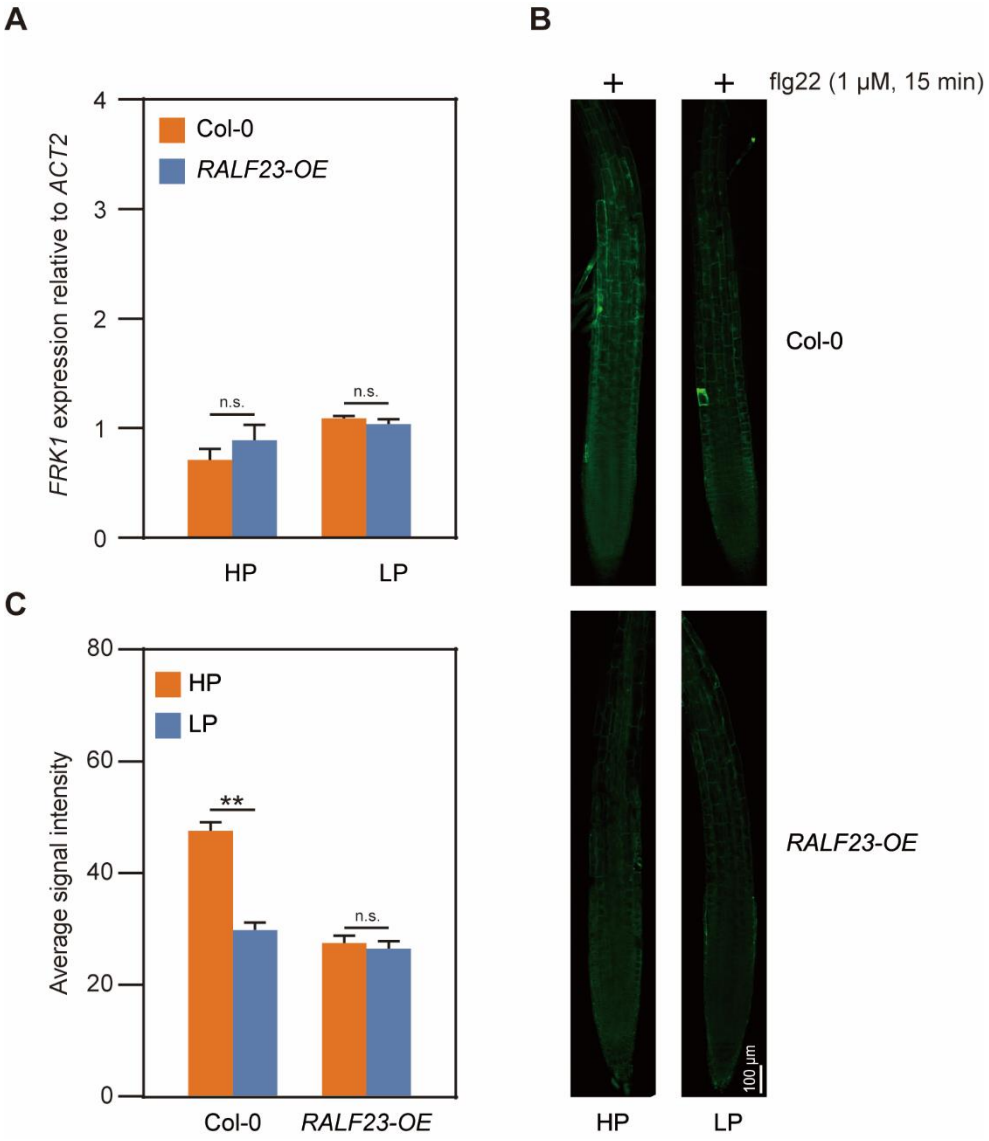
Data information: All experiments were repeated three times with similar results.



Appendix Figure S2. The GO enrichment analysis of differentially expressed genes after Pi-stress or RALF23 treatment.

(**A**-**B**) GO enrichment analysis of (**A**) up-regulated genes and (**B**) down-regulated genes after Pi-starvation.

(**C**-**D**) GO enrichment analysis of (**C**) up-regulated genes and (**D**) down-regulated genes after RALF23 treatment.



Appendix Figure S3. RALF23 suppresses flg22-induced immunity.

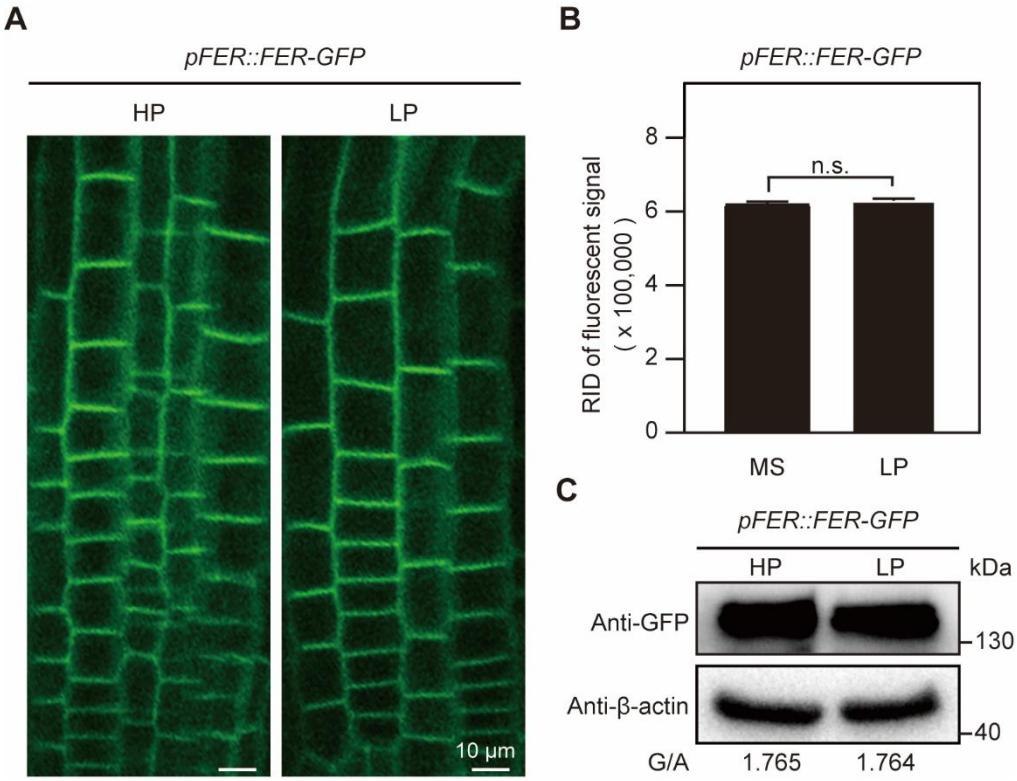
(**A**) *FRK1* expression in Col-0 and *RALF23-OE* roots. Seedlings first grown in 1/2-strength MS media for 3 days and then transplanted to either HP or LP media for 5 days and roots were harvested. The relative expression of *FRK1* was quantified via RT-qPCR. The data shown indicate the means ± SDs (n = 3, n refers to technical replicates); n.s., not significant (Student's *t* test).

(**B**) flg22-induced ROS production in Col-0 and *RALF23-OE* roots. Seedlings grown in either HP or LP media were treated with 1 μM flg22 for 15 mins. Roots were harvested and stained with H_2DCFDA. One representative root was shown.

(**C**) Average H_2DCFDA signal intensity in roots shown in (**B**). The fluorescence intensity was quantified with ImageJ. The data shown indicate the means ± SDs (n =

30, n refers to the number of roots per group); n.s., not significant; **, $p < 0.01$ (Student's *t* test).

Data information: All experiments were repeated three times with similar results.

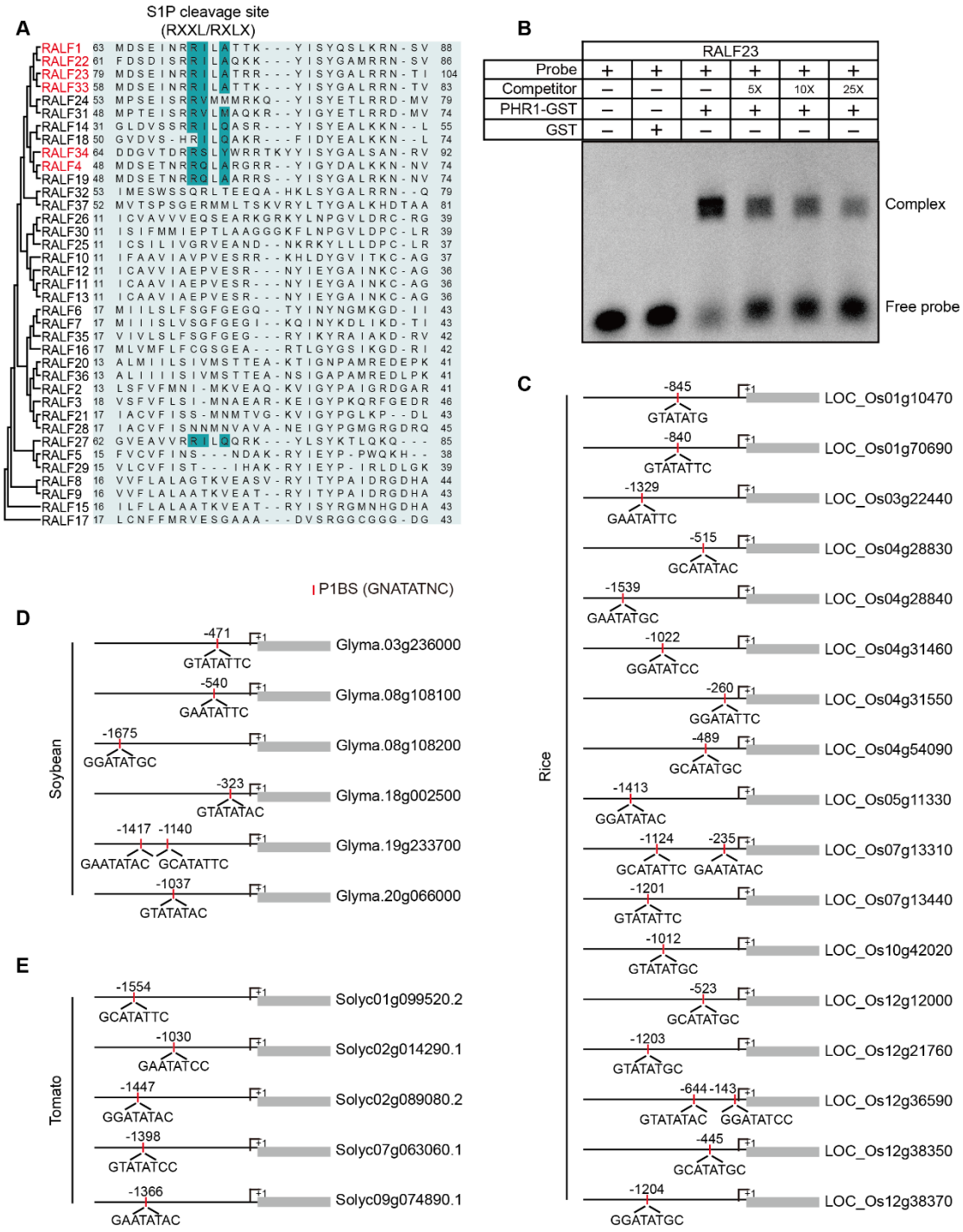


Appendix Figure S4. FER expression in response to Pi starvation.

(**A**-**B**) Fluorescence intensity of FER during the PSR. Native promoter-driven and GFP-labelled FER transgenic *Arabidopsis* lines (*pFER::FER-GFP*) grown in either HP or LP media for 5 days. (**A**), The fluorescence was checked in the root tip. (**B**), The fluorescence intensity was quantified with ImageJ. The data shown indicate the means ± SDs (n = 30, n refers to the number of roots per group); n.s., not significant (Student's *t* test).

(**C**) FER protein accumulation under LP conditions. *pFER::FER-GFP* transgenic seedlings were grown in either HP or LP media for 5 days. FER protein levels were determined with anti-GFP, and β-actin was used as a loading control.

Data information: All experiments were repeated three times with similar results.



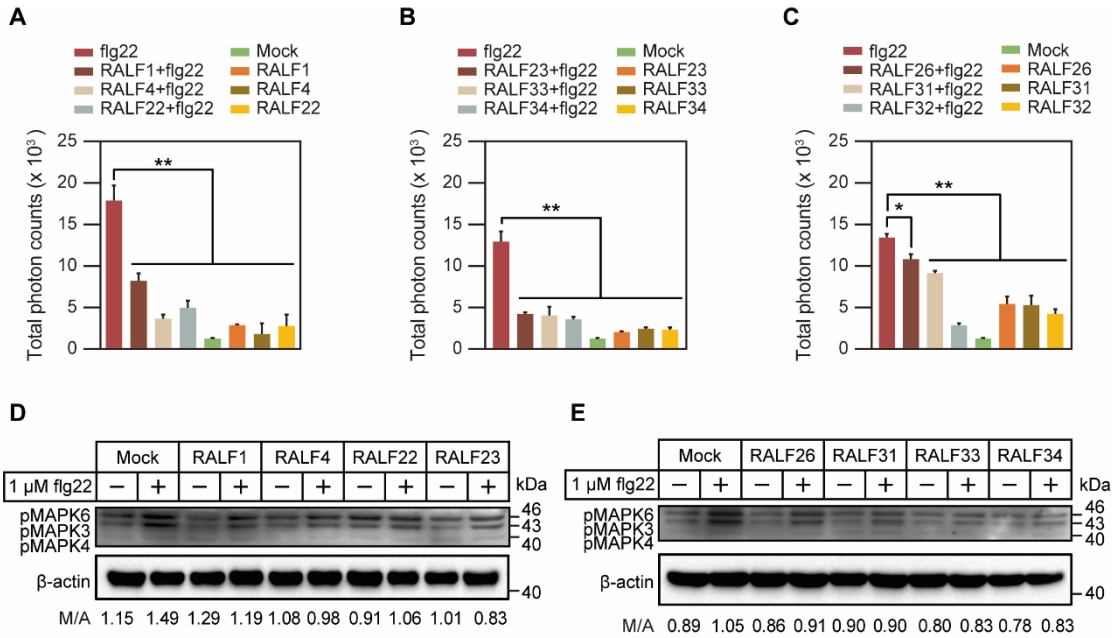
Appendix Figure S5. PHR1 directly binds to *RALF23 in vitro*.

(**A**) Alignment of 37 *Arabidopsis* RALF peptide sequences surrounding the propeptide-peptide transition site. The key residues arginine (R) and leucine (L) needed for S1P recognition are highlighted in green (conserved motif: RXLX/RXXL). RALF peptides that have the S1P recognition motif are highlighted in red, and those RALFs are likely to be cleaved by S1P and bound by PHR1. A phylogenetic analysis of all RALF

members is shown on the left.

(**B**) PHR1 binds to *RALF23 in vitro*. Competitive EMSA showing that the interaction between PHR1 and *RALF23* was probed with fluorescent isothiocyanate (FITC)-labelled DNA directly. Unlabeled probe was used as s competitor. Experiments were repeated three times with similar results.

(**C**-**E**) PHR1-binding site (P1BS, GNATATNC) prediction within 2000 bp of certain RALF promoters in rice (**C**), soybean (**D**), and tomato (**E**). The positions are relative to the start codon (+1).

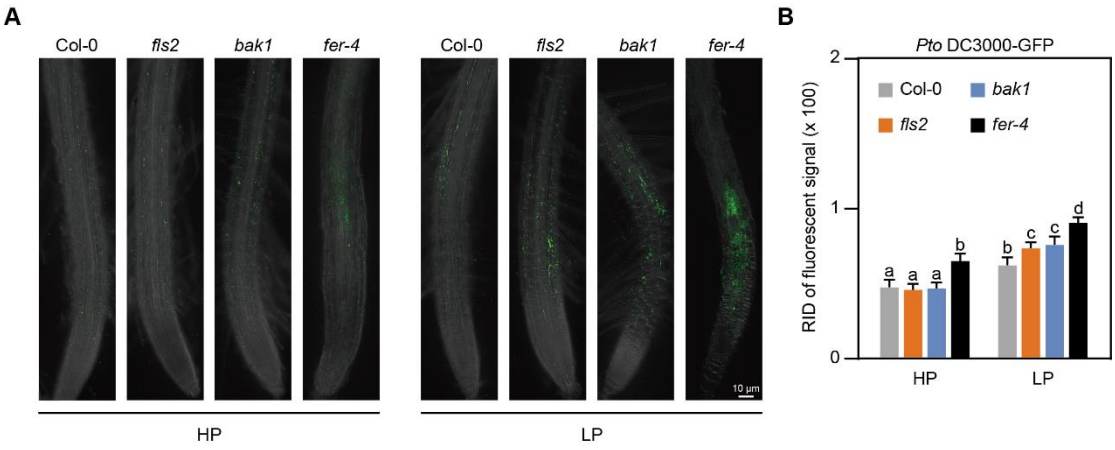


Appendix Figure S6. Potential S1P-cleaved RALF peptides inhibit flg22-induced PTI.

(**A-C**) RALFs inhibited flg22-induced ROS production in Col-0 leaves. Four-week-old leaves of Col-0 were treated with water, 1 μM flg22, 1 μM RALF or both flg22 and RALF. ROS levels were then determined. The data shown indicate total ROS counts during a 30 mins period ± SEs (n = 3, n refers to technical replicates). *, $p < 0.05$; **, $p < 0.01$ (one-way ANOVA).

(**D-E**) RALFs inhibit flg22-induced MAPK activation. Seven-day-old seedlings of Col-0 were treated with water, 1 μM flg22, 1 μM RALF or both flg22 and RALF for 15 mins. The MAPK level was determined with anti-pMAPK, and β-actin was used as a loading control.

Data information: All experiments were repeated three times with similar results.

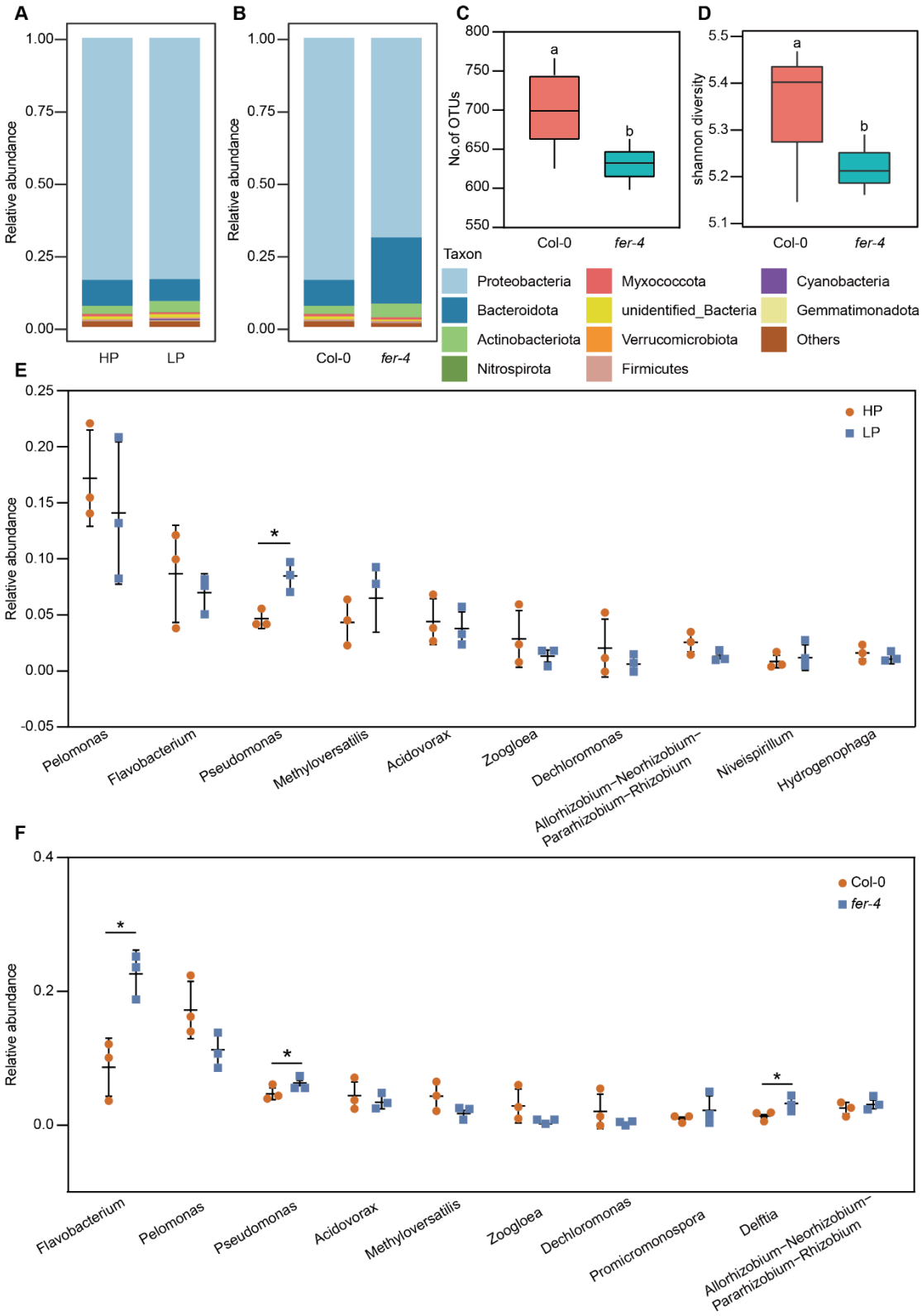


Appendix Figure S7. The FLS2-BAK1 pathway regulates the growth of *Pto* DC3000 under Pi-starvation condition.

(**A**) Col-0, *fls2*, *bak1* and *fer-4* mutants seedlings grown in either HP or LP media were inoculated (by soaking) with a GFP-labelled *Pto* DC3000 suspension (OD_{600} = 0.2) for 2 min. The number of bacteria from individual root was quantified at 3 dpi and the fluorescence was checked in the root tip. One representative root was shown.

(**B**) The fluorescence intensity was quantified with ImageJ. The data shown indicate the means ± SDs (n = 30, n refers to the number of roots per group). Different lowercase letters indicate statistical significance (one-way ANOVA).

Data information: All experiments were repeated three times with similar results.



Appendix Figure S8. LP treatment recruits specific rhizosphere microbiome.

(**A**) Relative abundance of bacteria phyla in rhizosphere samples under HP or LP conditions (n = 3, n refers to biological replicates).

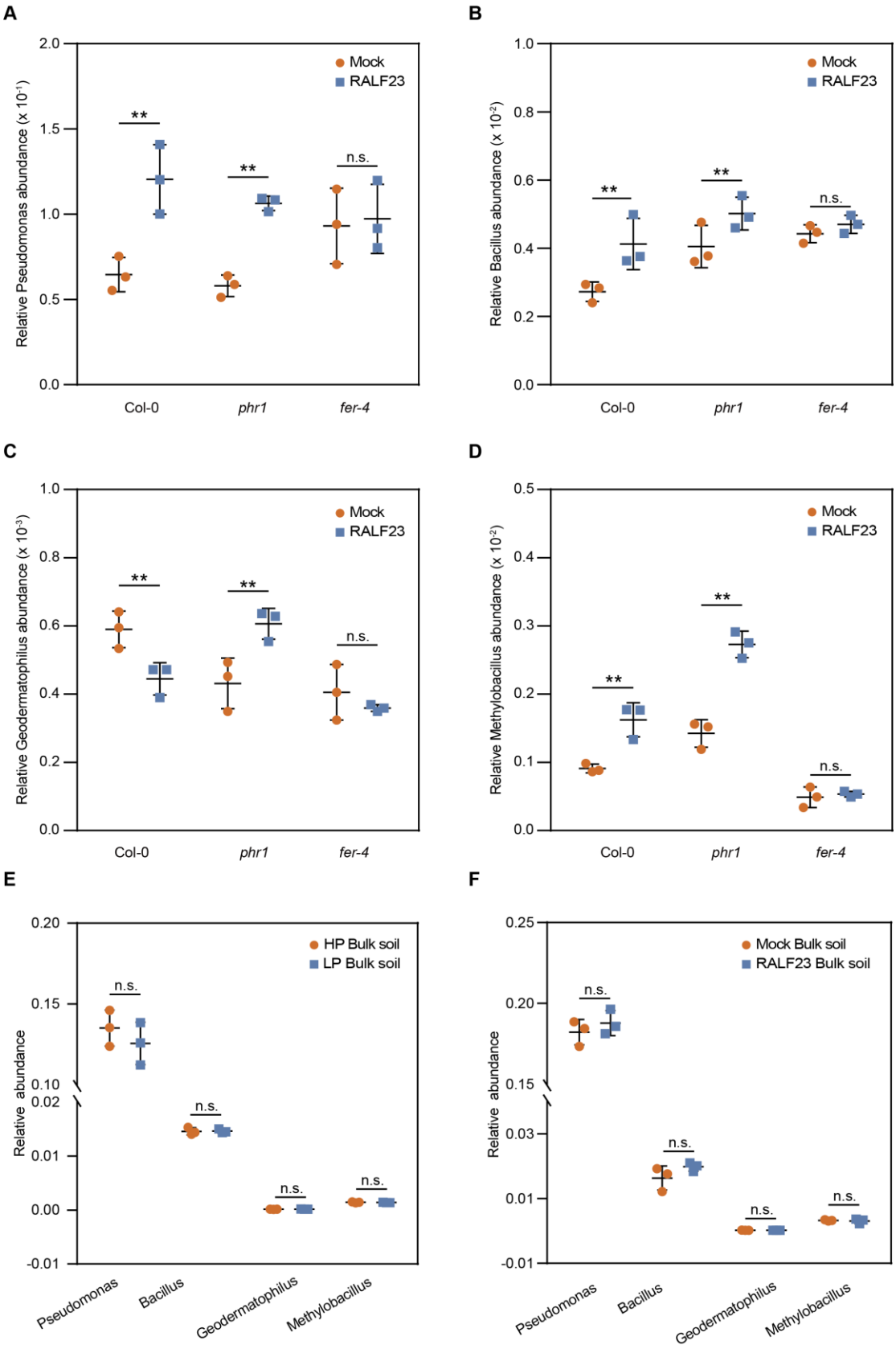
(**B**) Relative abundance of bacteria phyla in Col-0 or *fer-4* rhizosphere samples (n = 3, n refers to biological replicates).

(**C**) Number of OTUs in Col-0 or *fer-4* rhizosphere samples (n = 3, n refers to biological replicates). The different lowercase letters indicate statistical significance (Student's *t* test).

(**D**) Shannon diversity indexes in Col-0 or *fer-4* rhizosphere samples (n = 3, n refers to biological replicates). The different lowercase letters indicate statistical significance (Student's *t* test).

(**E**) Relative abundance of bacteria genera in rhizosphere samples under HP or LP conditions (n = 3, n refers to biological replicates). *, $p < 0.05$ (Student's *t* test).

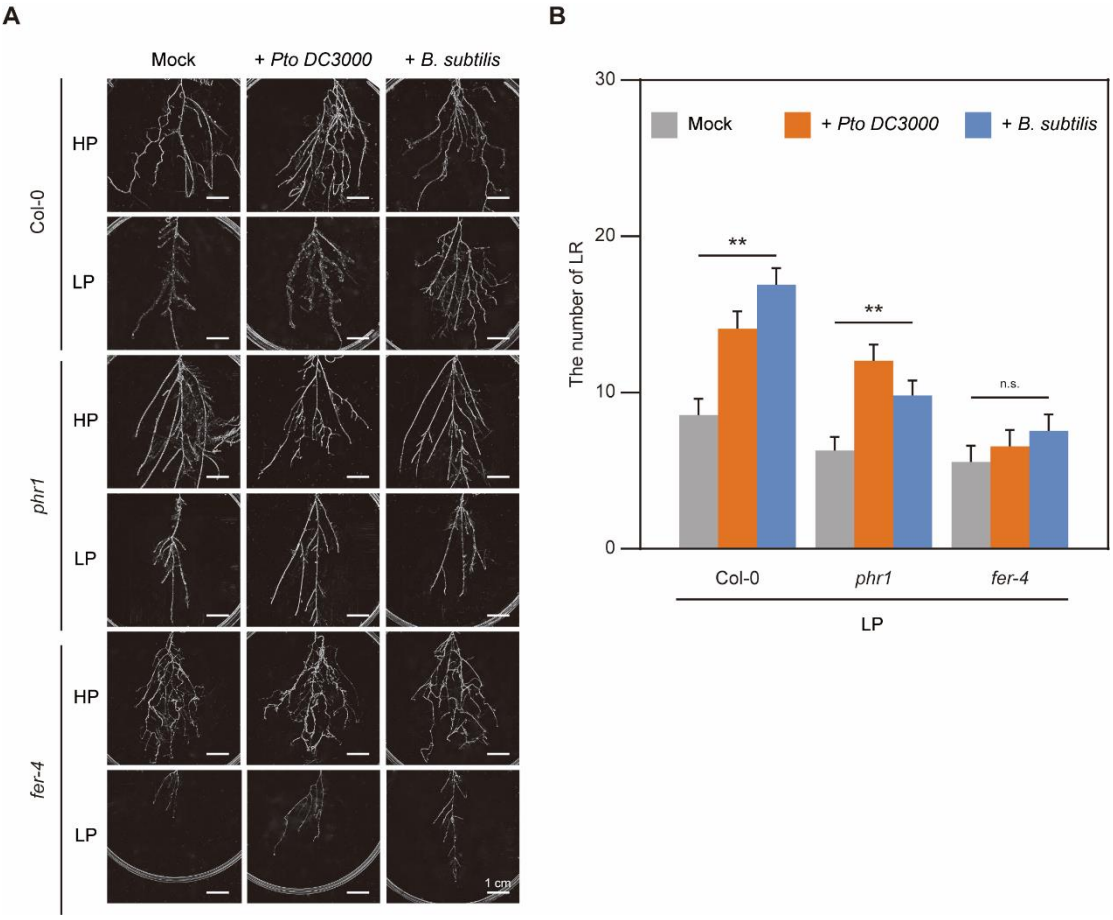
(**F**) Relative abundance of bacteria genera in Col-0 or *fer-4* rhizosphere samples under HP conditions (n = 3, n refers to biological replicates). *, $p < 0.05$ (Student's *t* test).



Appendix Figure S9. RALF23 promotes the bacterial abundance under Pi-sufficient conditions.

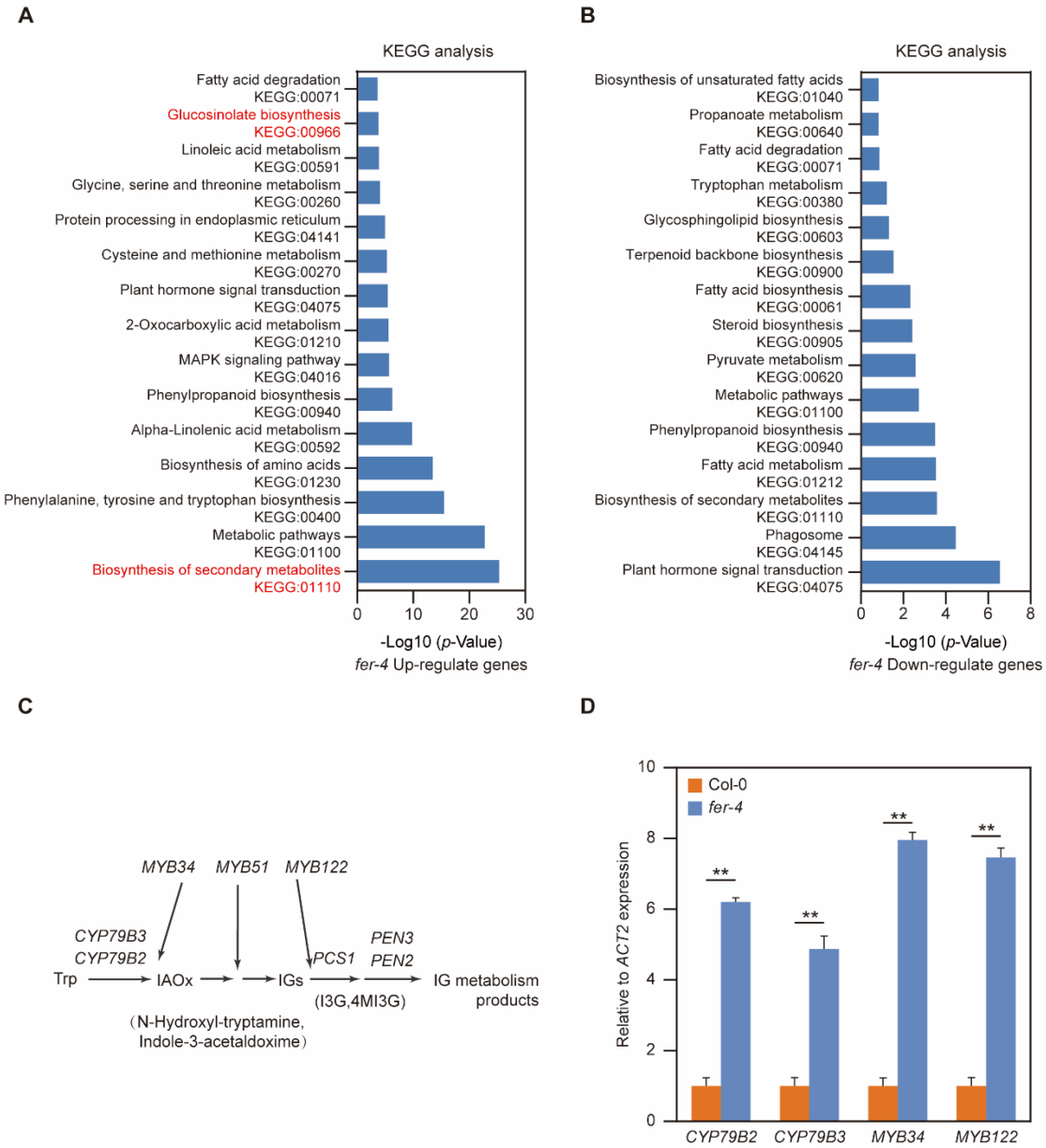
(**A**-**D**) Relative *Pseudomonas* (**A**), *Bacillus* (**B**), *Geodermatophilus* (**C**) and *Methylobacillus* (**D**) abundance in rhizosphere samples after treatment with *B. subtilis* expressing empty vector (Mock) or RALF23 (RALF23) under Pi sufficient conditions (n = 3 biological replicates). n.s., not significant; *, $p < 0.05$, **, $p < 0.01$ (Student's *t* test).

(**E**-**F**) Relative *Pseudomonas*, *Bacillus*, *Geodermatophilus* and *Methylobacillus* abundance in bulk soil sample after LP treatment (**E**) or treatment with *B. subtilis* expressing RALF23 (RALF23) (n = 3, n refers to biological replicates) (**F**). n.s., not significant (Student's *t* test).



Appendix Figure S10. **Bacterial colonization promotes LR growth.**

(**A**-**B**) *Arabidopsis* seeds were germinated on 1/2-strength MS media for 3 days and then transplanted to either HP or LP media for another 5 days. The seedlings were then transferred to vermiculite and inoculated with *Pto* DC3000 or *B. subtilis*. The roots were imaged 4 weeks after inoculation to analyze their morphology (**A**). The number of LR (lateral root) under LP condition was analyzed (**B**). The data shown indicate the means ± SDs (n = 10, n refers to the number of seedlings per group). n.s., not significant; **, $p < 0.01$ (Student's *t* test).



Appendix Figure S11. FER is involved in regulation of Trp-derived glucosinolates metabolites synthesis.

(**A**-**B**) KEGG enrichment analysis of (**A**) up-regulated genes and (**B**) down-regulated genes in *fer-4* mutants compare to Col-0.

(**C**) Trp-derived glucosinolates metabolite pathways in *A. thaliana*.

(**D**) The FER inhibits glucosinolates metabolite genes expression. Col-0 and *fer-4* mutants seedlings were grown in HP media for 5 days. Roots were harvested, and the relative expression of glucosinolates biosynthesis genes was quantified via RT-qPCR. The data shown indicate the means ± SDs (n = 3, n refers to technical replicates); **, $p < 0.01$ (Student's *t* test).

Appendix Table S1 qPCR Primers

Protein	Sequence 5'-3'
PHL1-F	AGCCACCTGTTTCCACCG
PHL1-R	CCAATCTTGCCATTCACTTT
WRKY6-F	CCGCCTCCTAATGGTTCCA
WRKY6-R	TGCATCAGTGAGTTGTGGTTGTT
WRKY45-F	GTTTCATGGGGTCGACAACT
WRKY45-R	CTGCTTTTTGGCCGTACTTC
WRKY75-F	CAAGGAGCCAAGTGGATATTCT
WRKY75-R	CTCCTAGGGAACTTGTTGTTCTT
PHT1;1-F	GGTTCCTATATGCGGCTCAA
PHT1;1-R	GCTAACCTCAGCCTCACCAG
PHT1;3-F	GGTCTACGTGCCATGGAATATC
PHT1;3-R	CTGCGTCTGTCTTGGTCTTATC
PHT1;4-F	TGATAAGCTCGGGAGGAAGA
PHT1;4-R	TGGTTGCGGATAAAGGGTAG
PHT1;5-F	CGCCGATATCCCATGACAAG
PHT1;5-R	GACCTAATGCGACGACGTTTG
PHT1;6-F	ACGTTATACATCATGGCAGGAATCAAT
PHT1;6-R	AAGCTCCTCAAGTGATTTCCCATTAGT
PHT1;7-F	TGGAGGATATCCATGCTCTGTCT
PHT1;7-R	CGCGGCTTCTGGAAAATTAG
PHT1;8-F	TTACCCGAAGTAAACCGTATGAGAA
PHT1;8-R	AATACGTCACCAAGATTCCAGCAA
PHT1;9-F	TGCAAAGAGTCATGTCCGTATC
PHT1;9-R	CGGCGAGAGAAGAGTTTGTATG
FRK1-F	TATATGGACACCGCGTATAGTG
FRK1-R	ATAAAACTTTGCGTTAGGGTCG
PER5-F	GAGACGCTTCTGAATACG
PER5-R	GTCGATGTCGCTTGAGTT
MYB51-F	AATGACAAGGCGAAGAAG
MYB51-R	GAGGTTGGTGCGAAGGAA
HEL-F	GGTGGTCGAACTTGTCCC
HEL-R	ACGGCTTATCAGCATCCC
RALF1-F	CTTACGATTCTCGTCGTCTTCATCATCTC
RALF1-R	CGTGGCAGCCTGAACCATTGTCT
RALF4-F	ACAAACCGTCGTCAACTC
RALF4-R	ATCATTTAGCGAGCGTAC
RALF22-F:	TTCGGAGATTCGCTAGATTTCGTGAG
RALF22-R	TCAACGCCTGCACCTAGTGATGGT
RALF23-F	TCCATCTTTCAGTGGCATTT
RALF23-R	GGCGACGGACCAGTTATG
RALF33-F	TCGCCGCCGTAACCTCCCAATC

RALF33-R	ACGCCTGTTGATCTCAGAGTCCATCTCG
RALF34-F	CTTCTTCGCTCTAGTTTCC
RALF34-R	CGTCGTCTTCCTCCGTAA
CYP79B3-F	CAATCAAGAGGCTTATGTTCGG
CYP79B3-R	TAGATCCAATCCCGTAAGCATC
CYP79B2-F	GAAAGTTGTGATGACGGAACTC
CYP79B2-R	GCATTTCCACAGTAATGCCTAG
MYB34-F	TAGCTTGGCGGGACGAAC
MYB34-R	CCTAGCGGAACCGGATGA
MYB122-F	ATGGTGAAGGCGGTTGGC
MYB122-R	TGAATGGCGTGGAGGTTG