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Supplemental information

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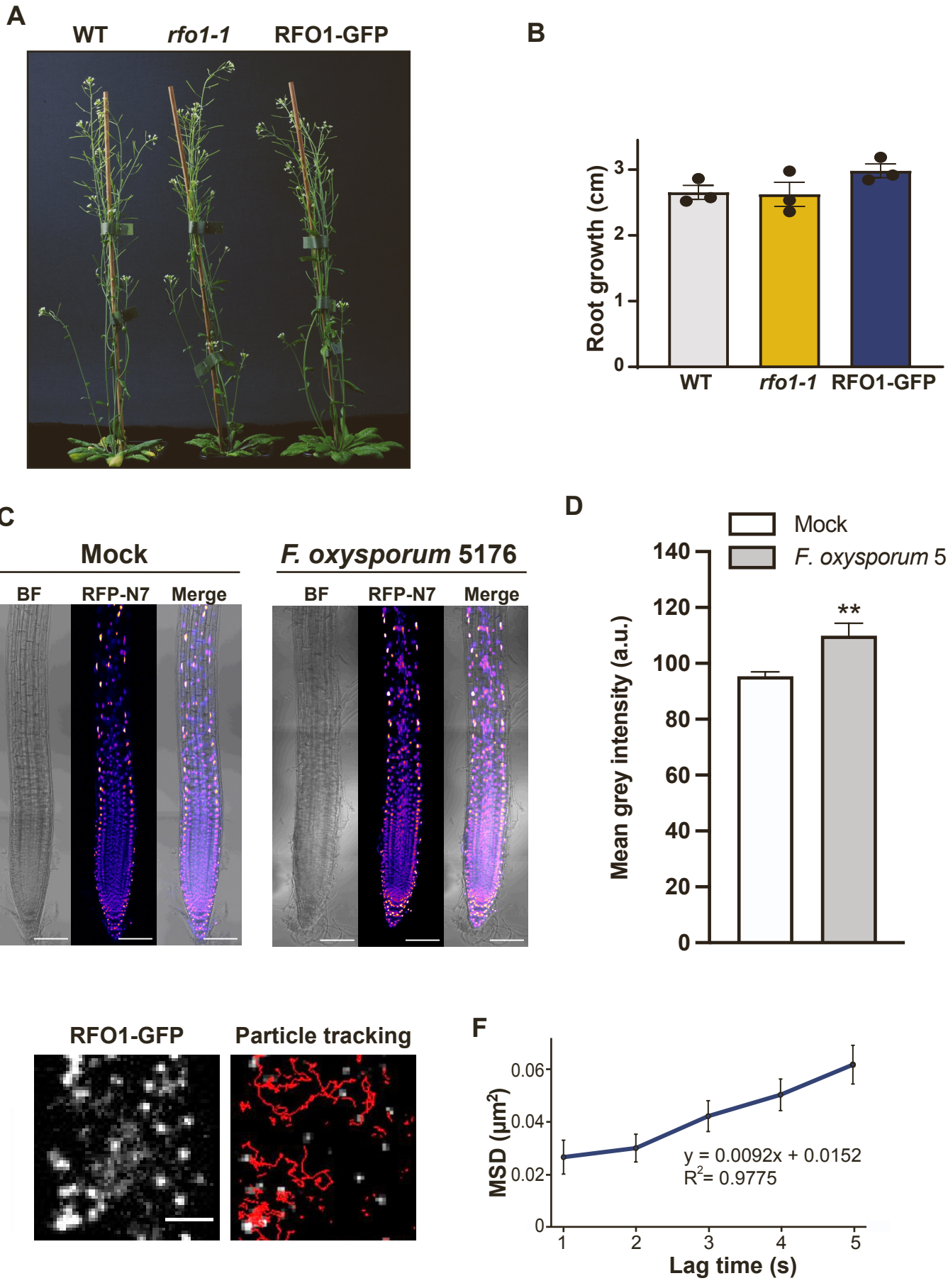

A
WT
rfo1-1
RFO1-GFP
B
Root growth (cm)
0
1
2
3
WT
rfo1-1
RFO1-GFP
C
Mock
F. oxysporum 5176
BF
RFP-N7
Merge
BF
RFP-N7
Merge
D
Mock
F. oxysporum 5176
Mean grey intensity (a.u.)
0
20
40
60
80
100
120
140
**
E
RFO1-GFP
Particle tracking
F
MSD (µm²)
0
0.02
0.04
0.06
y = 0.0092x + 0.0152
R²= 0.9775
1
2
3
4
5
Lag time (s)

Figure S1. Growth phenotypes and *in vivo* RFO1-GFP dynamics at the PM.
(A) Representative image of 8-week old wild type (WT, Col-0), *rfo1-1*, and RFO1-GFP (*rfo1-1* pRFO1::RFO1-GFP) plants. **(B)** Root growth of 8-day old WT, *rfo1-1*, and RFO1-GFP seedlings after 3 dpt to mock plates. Data represent the mean ±SE of >30 seedlings per genotype from 3 independent replicates with a minimum of 10 seedlings per replicate. Two-way ANOVA with Dunnett's multiple comparison test, no significance observed. **(C)** Representative brightfield (BF) and fluorescence images of nuclear RFP from pRFO1::RFP-N7 roots at 2 dpt to mock or *F. oxysporum* 5176 pSIX1::GFP containing plates. Scale bar = 100 µm. **(D)** Quantification of the RFP signal intensity (a.u.) in epidermal cells of the root tip in roots as in (C) . N ≥ 750 cells from 30 roots for mock and 684 cells from 30 roots for *F. oxysporum* 5176 in 3 independent replicates. Unpaired *t*-test, p-value **<0.01. **(E)** Representative spinning disc confocal image of RFO1-GFP particles at the PM of a 5-day old root elongating epidermal cell (left) with particle tracks shown in red after single-particle tracking with Trackmate (right). Scale bar = 5 µm. **(F)** Means squared displacement (MSD) plot of RFO1-GFP particles at the PM as shown in (D) with a linear fit, derived equation, and R^2 value.

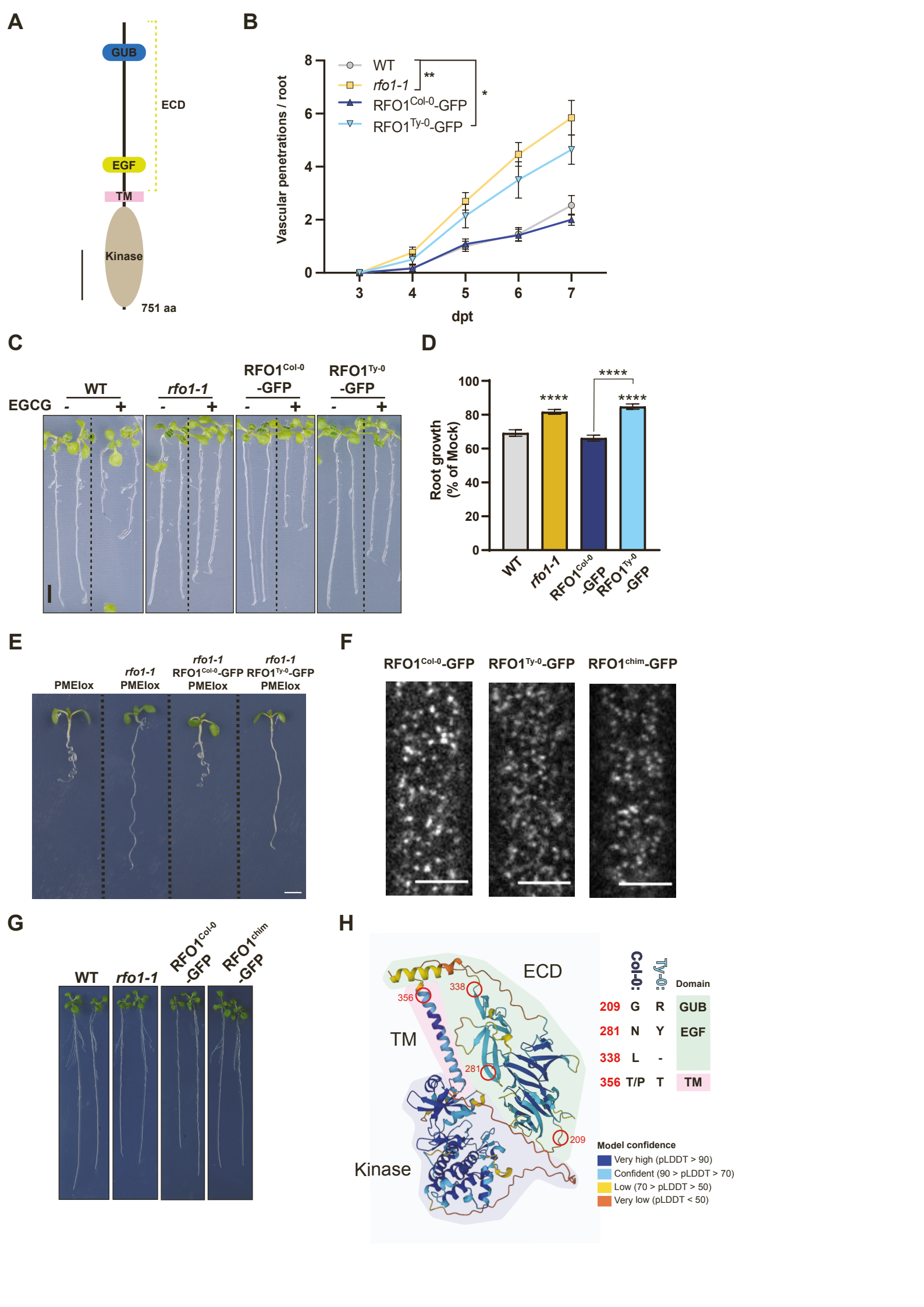

A
GUB
ECD
EGF
TM
Kinase
751 aa
B
WT
rfo1-1
RFO1Col-0-GFP
RFO1Ty-0-GFP
**
*
Vascular penetrations / root
dpt
C
WT
rfo1-1
RFO1Col-0 -GFP
RFO1Ty-0 -GFP
EGCG
D
Root growth (% of Mock)
****
E
PMEIox
rfo1-1 PMEIox
rfo1-1 RFO1Col-0-GFP PMEIox
rfo1-1 RFO1Ty-0-GFP PMEIox
F
RFO1Col-0-GFP
RFO1Ty-0-GFP
RFO1chim-GFP
G
WT
rfo1-1
RFO1Col-0 -GFP
RFO1chim -GFP
H
ECD
TM
Kinase
338
356
281
209
Col-0:
Ty-0:
Domain
209 G R GUB
281 N Y EGF
338 L -
356 T/P T TM
Model confidence
Very high (pLDDT > 90)
Confident (90 > pLDDT > 70)
Low (70 > pLDDT > 50)
Very low (pLDDT < 50)

Figure S2. The plasma membrane localized RFO1^{Ty-0}-GFP does not play a significant role in plant defense against Fo5176 or response to EGCG-induced pectin perturbations.

(A) Scheme of *in silico* predicted protein structure of RFO1 using the Plant Proteome Database (PPDB, http://ppdb.tc.cornell.edu/default.aspx). GUB, galacturonan-binding domain. EGF, calcium binding domain. TM, Transmembrane domain. ECD, Extracellular domain. Scale bar = 100 amino acids (aa). **(B)** Cumulative vascular penetrations observed in wild type (WT; Col-0), *rfo1-1*, RFO1^{Col-0}-GFP (*rfo1-1* pRFO1::RFO1^{Col-0}-GFP), and RFO1^{Ty-0}-GFP (*rfo1-1* pRFO1::RFO1^{Ty-0}-GFP) roots between 3 and 7 dpt to plates containing *F. oxysporum* 5176 pSIX1::GFP microconidia. Data represent the mean ± SE of N ≥ 10 seedlings per genotype from 3 independent replicates. RM ANOVA with Tukey's multi-comparison post-hoc test, p-values *< 0.05, **<0.01. Significance shown compared to WT. **(C)** Representative image of 8-day old WT, *rfo1-1*, RFO1^{Col-0}-GFP, and RFO1^{Ty-0}-GFP seedlings after 48 hours upon mock (-) or 6.25 µM EGCG (+) treatment. Scale bar = 5mm. **(D)** Root growth of EGCG-treated roots relative to mock-treated ones (%), as shown in (B). Bars represent the mean ± SE of >38 seedlings per genotype from 4 independent replicates. One-way ANOVA with Dunnett's multiple-comparison post-hoc test, p-value ****<0.0001. Significance shown compared to WT unless indicated. **(E)** Representative images of 8-day old WT, PMEIox, *rfo1-1*, *rfo1-1* PMEIox, *rfo1-1* RFO1^{Col-0}-GFP PMEIox, and *rfo1-1* RFO1^{Ty-0}-GFP PMEIox seedlings. Scale bar = 5 mm. **(F)** Representative spinning disc confocal image of RFO1^{Col-0}-GFP, RFO1^{Ty-0}-GFP and RFO1^{chim}-GFP particles at the PM of 5 days-old root epidermal cells. Scale bar = 5 µm. **(G)** Representative images of 11-day old WT, *rfo1-1*, RFO1^{Col-0}-GFP, and RFO1^{chim}-GFP seedlings. Scale bar = 10 mm. **(H)** RFO1 protein structure obtained from AlphaFold model (https://alphafold.ebi.ac.uk). Extracellular domain (ECD), transmembrane domain (TM) and Kinase domain (cytosolic) are colored in green, pink and purple, respectively. Per-residue confidence score (pLDDT) of every amino acid is represented by color-code in the legend. In red circles, Col-0 and Ty-0 amino acid variants as reported previously (Diener and Ausubel, 2005) across the RFO1 section used to generate the chimera: ECD (GUB and EGF domains) and TM domains.

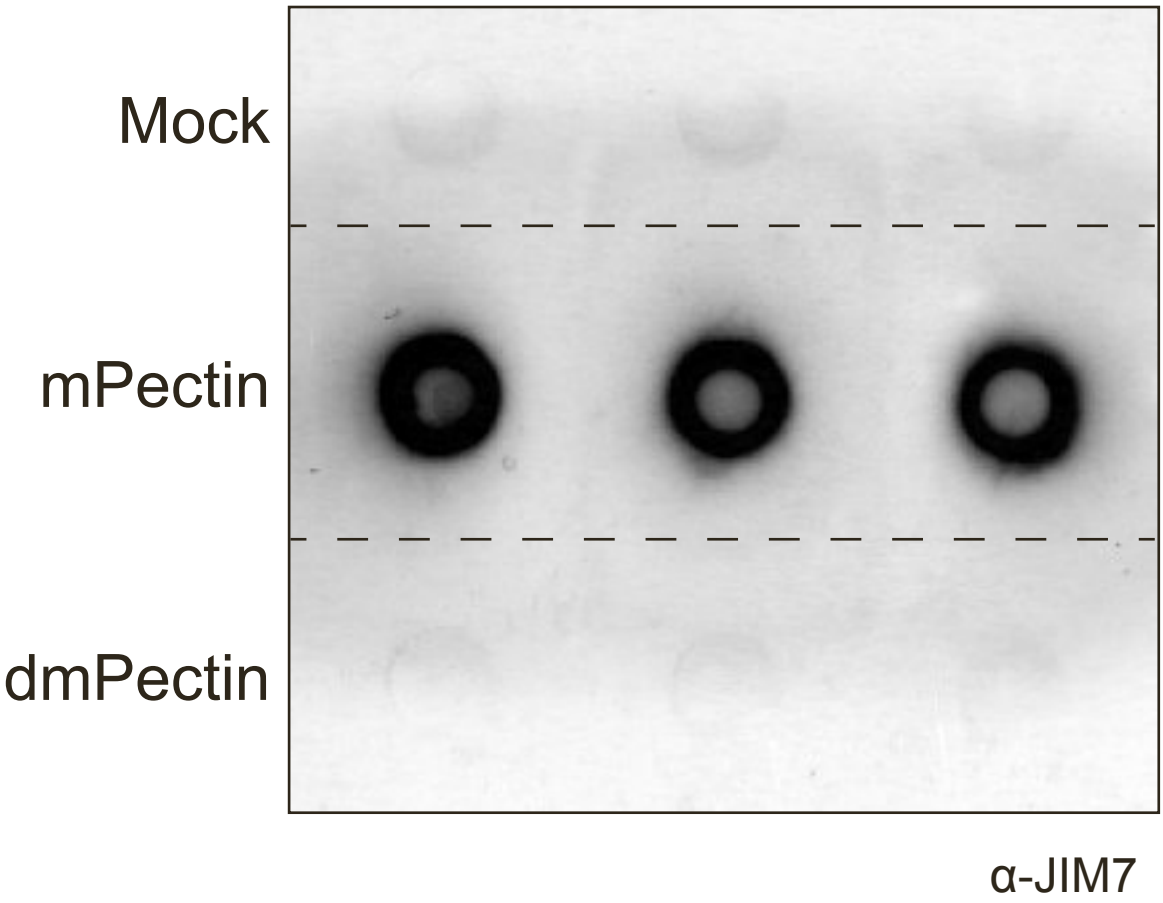

Mock
mPectin
dmPectin
α-JIM7

Figure S3. The dmPectin generated and used in this work is completely demethylated. Dot blot assay of immobilized commercial methylated pectin (mPectin) and demethylesterified pectin (dmPectin) dissolved in 1.5 mM EDTA pH 8 (Mock) against JIM7 (against mPectin) antibody. 3 technical replicates shown.

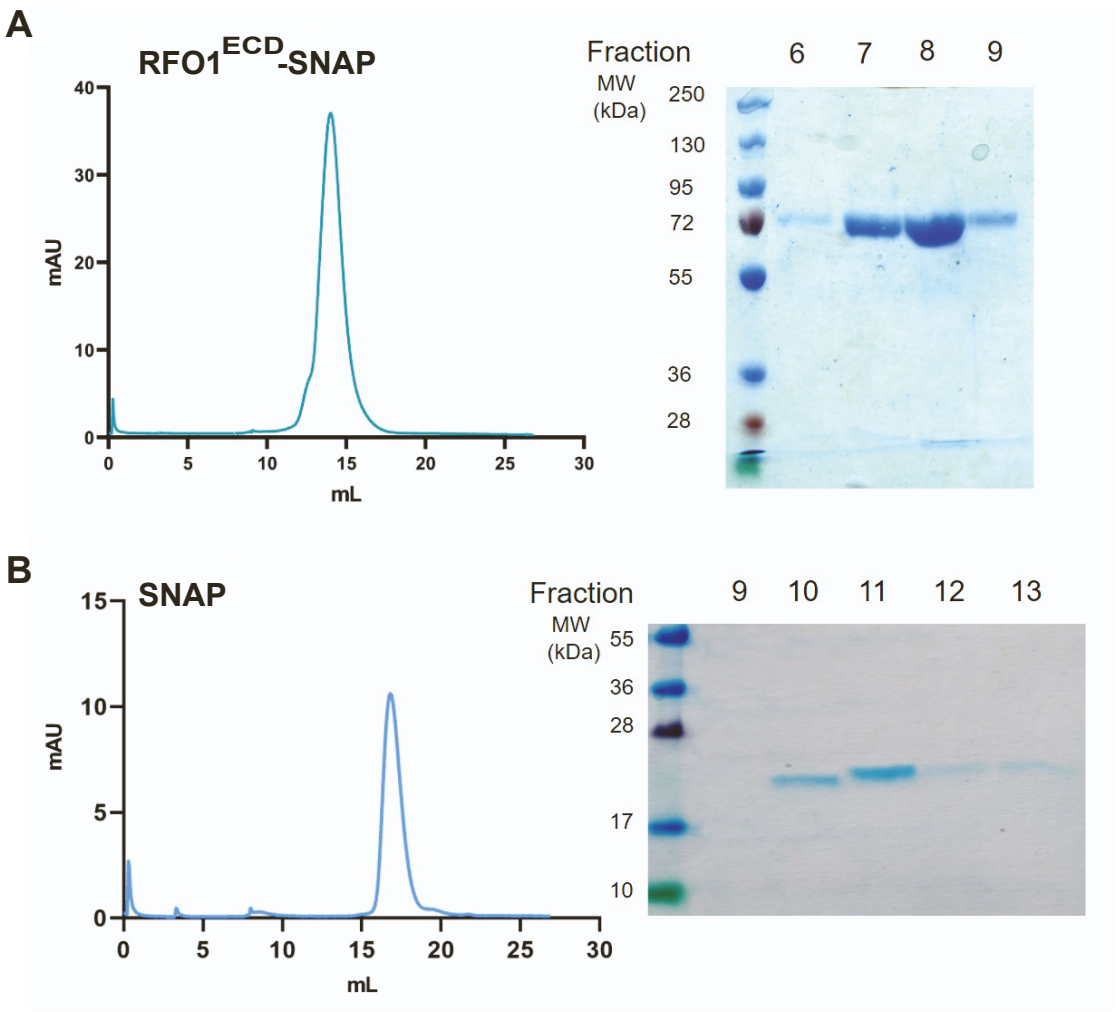
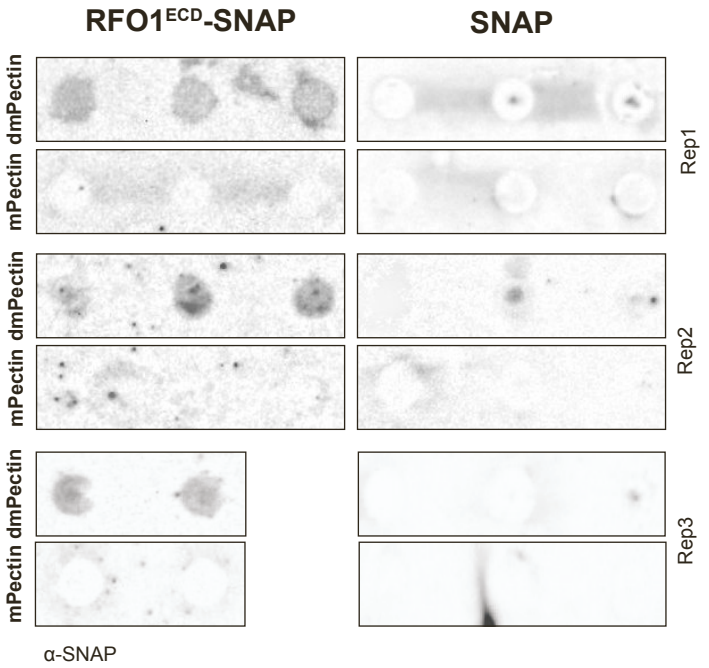

A
RFO1ECD-SNAP
mAU
mL
Fraction
6
7
8
9
MW
(kDa)
250
130
95
72
55
36
28
B
SNAP
mAU
mL
Fraction
9
10
11
12
13
MW
(kDa)
55
36
28
17
10

Figure S4. Size exclusion and SDS-PAGE analysis of RFO1^{ECD}-SNAP and SNAP recombinant proteins from insect cells.
(A) Size exclusion chromatography (SEC, left) of RFO1^{ECD}-SNAP protein and SDS-PAGE (right) of the different fractions corresponding to the SEC elution peak. **(B)** Size exclusion chromatography (SEC, left) of SNAP and SDS-PAGE (right) of the different fractions of the SEC experiment.

A


RFO1ECD-SNAP
SNAP
dmPectin
mPectin
Rep1
Rep2
Rep3
α-SNAP

B


RFO1ECD-SNAP
SNAP
GP

Figure S5. Replicates of dot immunobinding assays of RFO1^{ECD}-SNAP or SNAP recombinant proteins on plant cell fractions and glycoprotein extracts.
(A) Biological replicates (Rep) of dot immunobinding assay of immobilized commercial methylesterified pectin (mPectin) and in-lab de-methylated pectin (dmPectin) probed with RFO1^{ECD}-SNAP or SNAP recombinant proteins. At least two technical replicates were included on each biological one. Protein binding was detected using a αSNAP antibody. The corresponding graph of their quantification is shown in Fig. 3B. **(B)** Representative dot immunobinding assays using plant CW glycoprotein (GP) extracts probed with RFO1^{ECD}-SNAP or SNAP proteins. The experiment was repeated three times with similar results.

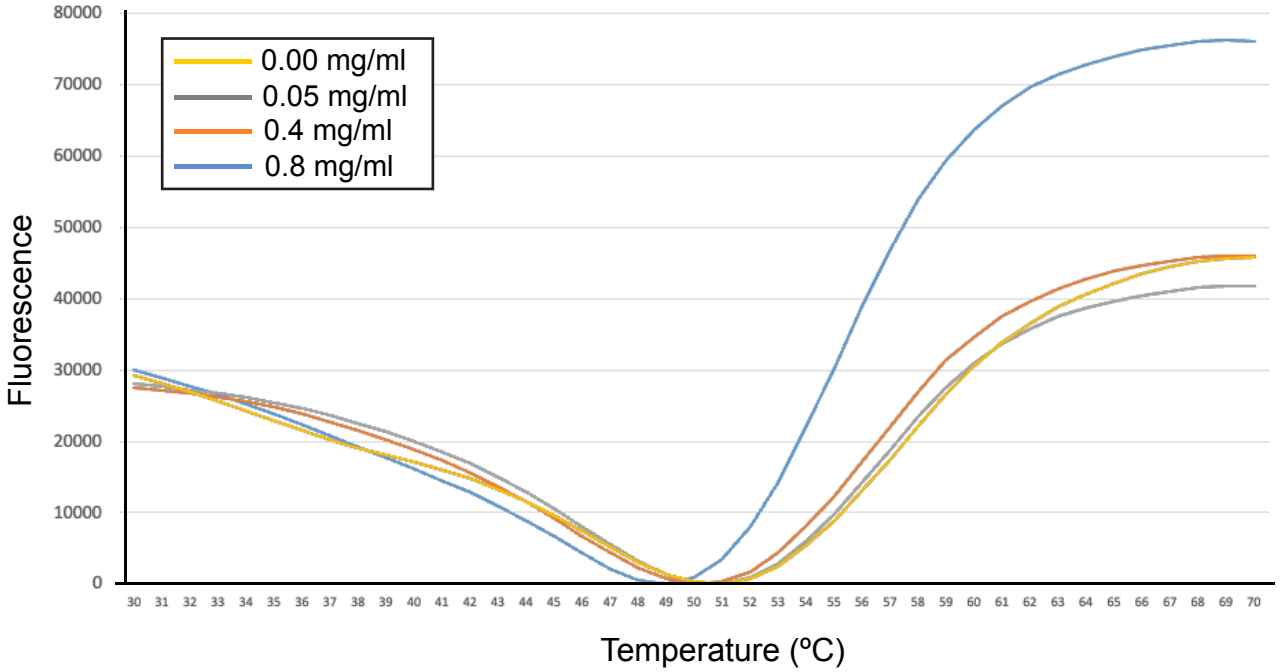

0.00 mg/ml
0.05 mg/ml
0.4 mg/ml
0.8 mg/ml
Fluorescence
80000
70000
60000
50000
40000
30000
20000
10000
0
30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70
Temperature (ºC)

Figure S6. Representative thermal shift profiles of RFO1^{ECD} in the presence of different concentrations of dmPectin. The lack of increase in the melting temperature of RFO1 in the presence of different concentrations of dmPectin suggests that RFO1 does not form high affinity-stable complexes with dmPecin. The experiment was repeated 3 times, with 4 technical replicates each one.

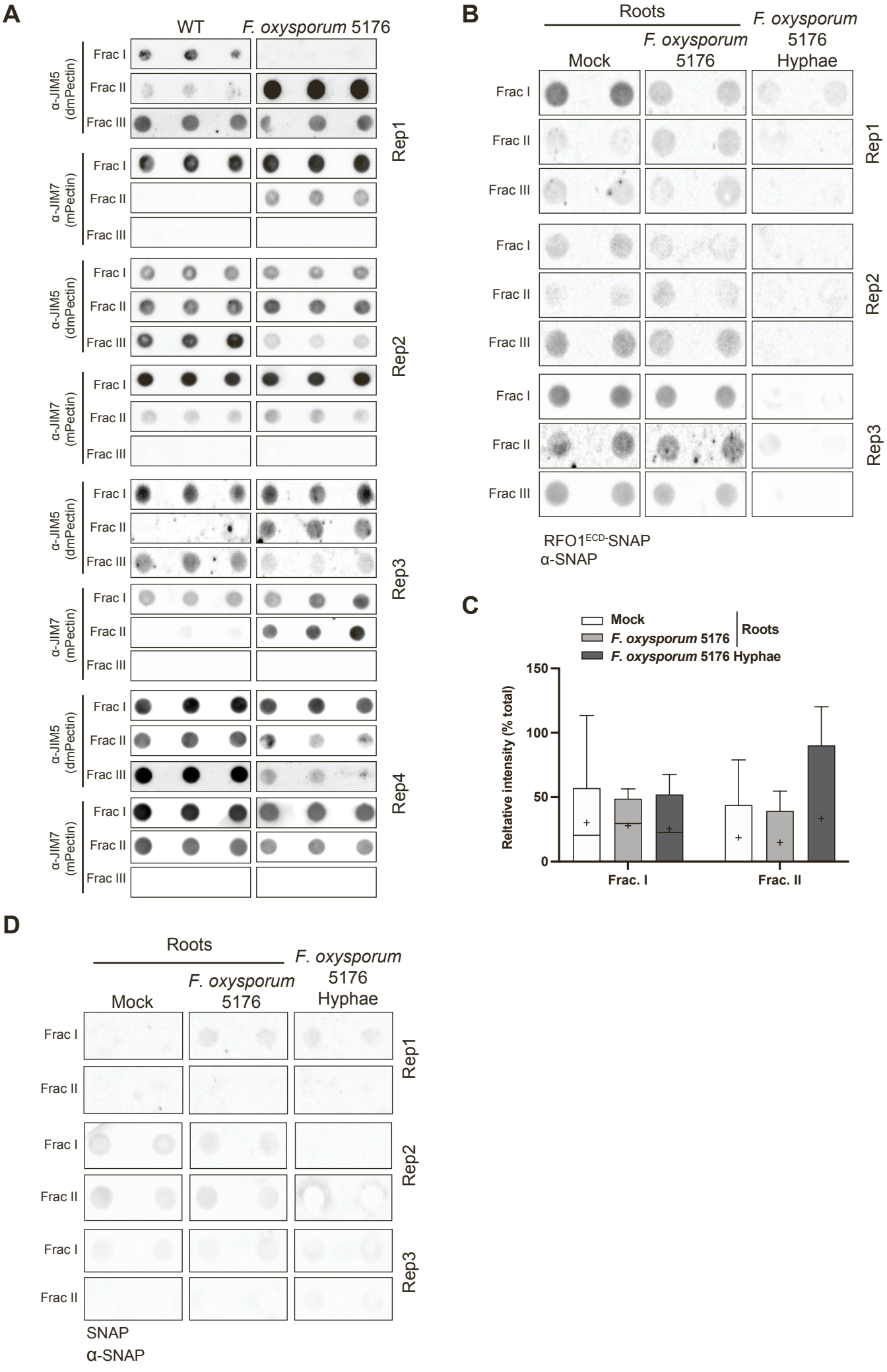
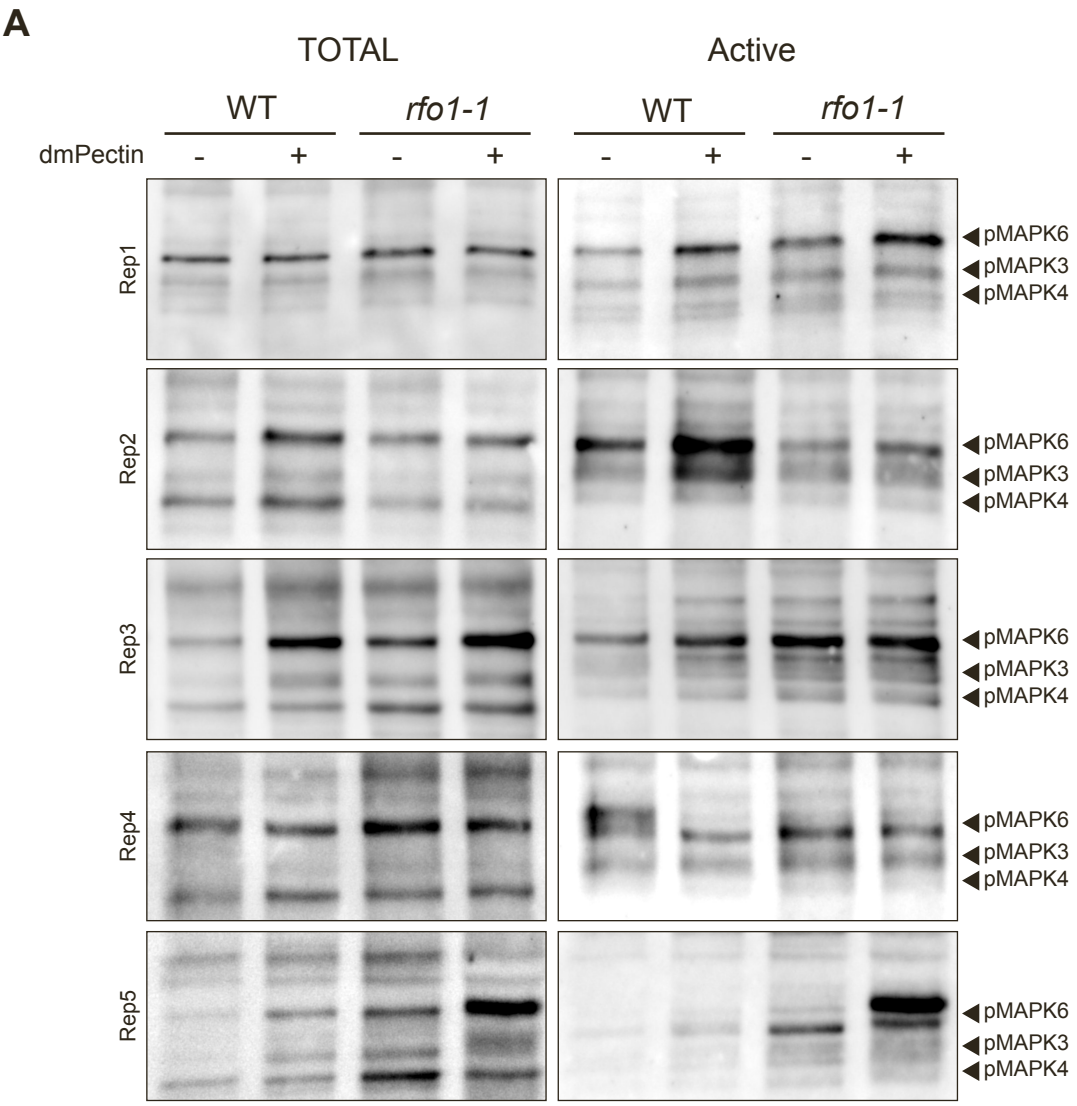

A
WT
F. oxysporum 5176
α-JIM5 (dmPectin)
α-JIM7 (mPectin)
Frac I
Frac II
Frac III
Rep1
Rep2
Rep3
Rep4
B
Roots
F. oxysporum 5176 Hyphae
Mock
F. oxysporum 5176
RFO1ECD-SNAP
α-SNAP
C
Mock
F. oxysporum 5176
F. oxysporum 5176 Hyphae
Roots
Reltative intensity (% total)
150
100
50
0
Frac. I
Frac. II
D
Roots
F. oxysporum 5176 Hyphae
Mock
F. oxysporum 5176
SNAP
α-SNAP

Figure S7. Replicates of dot immunobinding assays of plant and *F. oxysporum* 5176 CW extracts.

(A) Biological replicates (Rep) of dot immunobinding assay of immobilizied plant CW fractions from 4 dpt mock and *F.oxysporum* 5176-infected WT roots probed with JIM5 (anti-dmPectin) or JIM7 (anti-mPectin) antibodies. Three technical replicates were included on each biological one. The corresponding graph of their quantification is shown in Fig. 4B. **(B)** Three replicates (Rep) of dot immunobinding assay using root CW fractions as described in (A) and *F. oxysporum* Hyphae CW fractions probed with $RFO1^{ECD}$-SNAP recombinant protein. Protein binding was detected using a αSNAP antibody. The corresponding graph of their quantification is shown in Fig. 4C. **(C)** Quantification of dot immunobinding assays intensities as a percent of mean gray values per CW fraction probed with SNAP. Box plots: centerlines show the medians; means marked by +; box limits indicate the 25^{th} and 75^{th} percentiles; whiskers extend to the minimum and maximum. N=3 independent replicates. Two-way ANOVA with Tukey's multiple comparison test, no significance observed. **(D)** Dot immunoblotting assay replicates (Rep) used for the quantification depicted in (C).


A
TOTAL
Active
WT
rfo1-1
WT
rfo1-1
dmPectin
- + - + - + - +
Rep1
Rep2
Rep3
Rep4
Rep5
pMAPK6
pMAPK3
pMAPK4

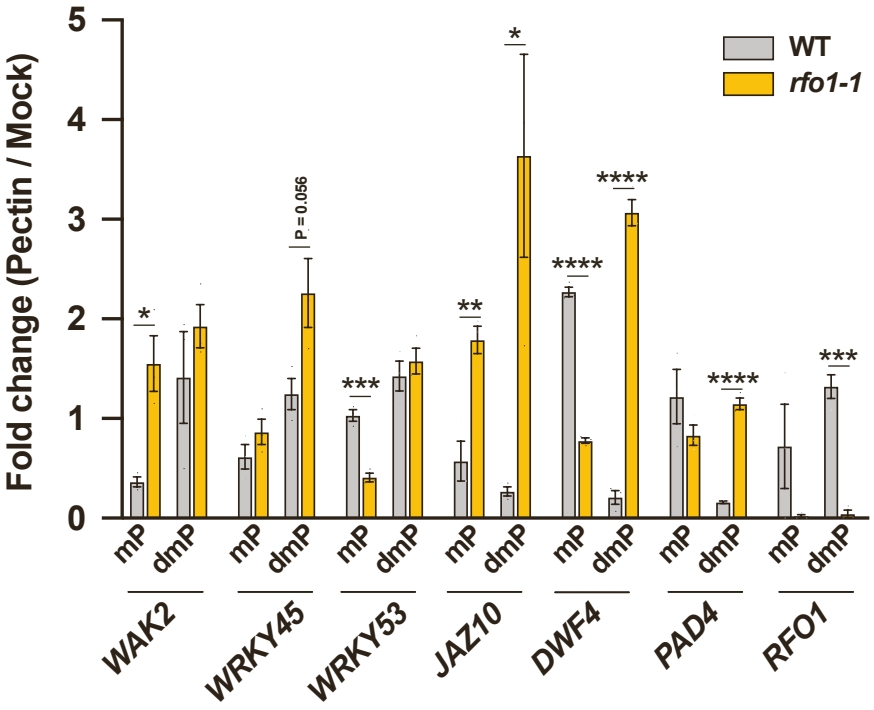

B
Fold change (Pectin / Mock)
0
1
2
3
4
5
WT
rfo1-1
P = 0.056
mP
dmP
WAK2
WRKY45
WRKY53
JAZ10
DWF4
PAD4
RFO1

Figure S8. RFO1 participates in the downstream signaling triggered by different degrees of pectin methylesterification. (A) Biological replicates (Rep) of MAPK activation assays. Each membrane shows total and phospho-activated (Active) MAPK6, MAPK3, and MAPK4 in 8-day old roots of wild type (WT; Col-0) and *rfo1-1* plants treated with Mock (-) or 10 µg/ml of dmPectin (+) for 30 min. The corresponding graph of their quantification is shown in Fig. 5A. **(B)** Fold change variation of *WAK2, WRKY45, WRKY53, JAZ10, DWF4, PAD4* and *RFO1* expression in 8-day old roots of WT and *rfo1-1* plants upon mPectin (mP) or dmPectin (dmP) treatment. Data represent the mean ± SE of N = 3 independent replicates. Data were analyzed by t-test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Asterisks in black indicate the difference with WT.

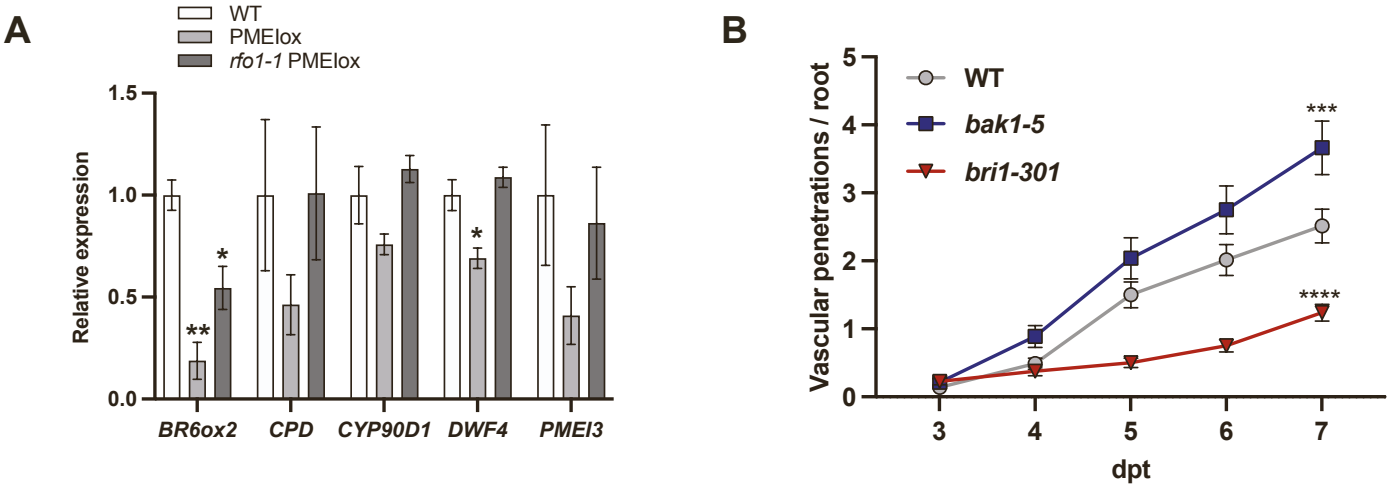

A
WT
PMEIox
rfo1-1 PMEIox
Relative expression
1.5
1.0
0.5
0.0
**
*
*
BR6ox2
CPD
CYP90D1
DWF4
PMEI3
B
Vascular penetrations / root
5
4
3
2
1
0
WT
bak1-5
bri1-301
***
****
3
4
5
6
7
dpt

Figure S9. RFO1 activates BR signaling, which participates in defense against Fo. *bri1-301* is more resistant than wild type to *F. oxysporum*5176 vasculature colonization. (A) *BR6ox2*, *CPD*, *CYP90D1*, *DWF4* and *PME3* gene expression relative to *GAPDH*, in 8 days-old WT, PMEIox and *rfo1-1* PMEIox plants, normalized to mock WT gene expression. Data represent the mean ± SE of N = 3 independent replicates. Unpaired *t*-test (two-tailed) against WT mock per gene. **(B)** Cumulative vascular penetration of *F. oxysporum*5176 per root of wild type (WT; Col-0), *bri1-301* and *bak1-5* at different days post-transfer (dpt) to *F. oxysporum*5176 pSIX1::GFP microconidia-containing plates. Data represent the mean ± SE of N = 80 seedlings per genotype from four independent biological replicates. Two-way ANOVA with Tukey's multiple comparison test, p-values *** < 0.001, **** < 0.0001 at 7 dpt. See Table S1 the complete statistical analysis.

Table S1. Statistical analysis of root vascular penetrations upon *F. oxysporum* 5176 pSIX1::GFP infection (linked to Figure S7B).

Statistical analysis of root vascular penetrations upon *F. oxysporum* 5176 (Fo5176) pSIX1::GFP infection. Two-way ANOVA with post-hoc Tukey test for multiple comparisons corresponding to root vascular penetration events (p-value < 0.05 *, 0.01 **, 0.001 ***, 0.0001 ****). Day 3 post treatment is not included because there were no statistically significant differences (p-value > 0.05).

Two-way ANOVA (Tukey test)	**p-value**
4 dpt	
WT vs. *bak1-5*	**ns**
WT vs. *bri1-301*	**ns**
bak1-5* vs. *bri1-301	**ns**
5 dpt	
WT vs. *bak1-5*	**ns**
WT vs. *bri1-301*	****
bak1-5* vs. *bri1-301	*****
6 dpt	
WT vs. *bak1-5*	*
WT vs. *bri1-301*	*****
bak1-5* vs. *bri1-301	*****
7 dpt	
WT vs. *bak1-5*	****
WT vs. *bri1-301*	*****
bak1-5* vs. *bri1-301	*****

Table S2. Primers used in this work.

		NAME	SEQUENCE	#
CLONING	pRFO1:RFO1-GFP	pRFO1_FW	CATGATTACGAATTCGAGCTCCGTCGCGGTCAGATGTTGAAC	1
		pRFO1_RV	CTTAGAGGATCCCGCACTCGAGGCCGTCTCGTTGGAATTTGGAAG	2
	pRFO1:RFO1^{Ty-0}-GFP	RFO1_FW	CACCATGAAGAGAAGGAGACTTTTTTTCTC	3
		RFO1_RV	CCATGTTCGTTGAGGAACCAGC	4
	pRFO1:RFO1^{eckt}-GFP	RFO1_TY_FW	CACCATGAAGAGAAGGAGACTTTTTTTCTTGT	5
		RFO1_TM_FW	GTTCTAGGTTTTCCACTGTTGTTCTT	6
		RFO1_TM_RV	AAGAACAACAGTGGAAAACCTAGAAC	7
	pRFO1::RFP-N7	pRFO1_N7_GFP_FW	CGAATTGGAGCTGCGGCCGCGAATTCCGTCGCGGTCAGATGTTGAAC	8
		pRFO1_N7_GFP_RV	CCTCGCCCTTGCTCACCATGCCGTCTCGTTGGAATTTGGAAG	9
		RFP_FW	ATGGTGAGCAAGGGCGAGG	10
		RFP_RV	GGCGCCCGCCGCCGCCGCCGCTCCCTTGTACAGCTCGTCCATGC	11
		N7_FW	GGAGCGGCGGCGGCGGCGGGCGCCGAATTCAAGCGTGAAGAGCAAGC	12
		N7_RV	TTTCATCTTCATCTTCATATTCTAGATCACTCTTCTTCTTGATCAGCTTCTGTGTCG	13
GENOTYPING	GFP	GFP_FW	ATCTTCTTCAAGGACGACGG	14
		GFP_RV	CTGTTGTAGTTGTACTCCAGCT	15
	PMEIox	attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT	16
		attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT	17
	RFO1	SALK_077975_LP	TACCAACCAAGCTCAATCACC	18
		SALK_077975_RP	TATGAATGATTTGCGTTGGTG	19
		SALK_LB	CGCTTTCTTCCCTTCCTTTCTC	20
RT-PCR	BR6ox2	BR6ox2_FW	CAATAGTCTCAATGGACGCAGAGT	21
		BR6ox2_RV	AACCGCAGCTATGTTGCATG	22
	CPD	CPD_FW	CCCAAACCACTTCAAAGATGCT	23
		CPD_RV	GGGCCTGTCGTTACCGAGTT	24
	CYP90D1	CYP90D1_FW	CTCATTACCCTTGCCGTCAAA	25
		CYP90D1_RV	CAGCTTCATGTTTTCTTCCGTTAG	26
	DWF4	DWF4_FW	CAACAGCAAAACAACGGAGCG	27
		DWF4_RV	TCTGAACCAGCACATAGCCTTG	28
	GAPDH	GAPDH_FW	AGGTGGAAGAGCTGCTTCCTTC	29
		GAPDH_RV	GCAACACTTTCCCAACAGCCT	30
	JAZ10	JAZ10_FW	CGCCAGGTCTAGTACCGAAC	31
		JAZ10_RV	TGCTGCTTCATTAGCGACCT	32
	PAD4	PAD4_FW	CACCGCACTTTGGCTTCTATC	33
		PAD4_RV	AGTAAGTTCCAAAGGGCCAG	34
	PME3	PME3_FW	ACGGTAGCACCACTTTCCAC	35
		PME3_RV	ATCAGAACCCACACGGAGAG	36
	RFO1	RFO1_FW	ATGAAGAGAAGGAGACTTTTTTTCTCTGTTC	37
		RFO1_RV	ACCTCGTACCAGTCGTTGAG	38
	WAK2	WAK2_FW	AACTGCCCATCTGGTTACCG	39
		WAK2_RV	CTCTGTGTTCTTCCGGTGCT	40
	WRKY45	WRKY45_FW	GGAGGGAAGATGTGCATTTGTG	41
		WRKY45_RV	GAACAATCCATTCCCCAGGAG	42
	WRKY53	WRKY53_FW	GCGACAAGACACCAGAGTCA	43
		WRKY53_RV	ACCGTTGGATTGAACCAGTC	44

Movie S1: Dynamic Localization of RFO1-GFP at the plasma membrane. Related to Figure 1F-G

The movie depicts the dynamic behavior of RFO1-GFP expressed in *rfo1-1* in *Arabidopsis* root epidermal cells acquired using Spinning disc confocal microscopy. Foci identified by Trackmate using the LAP tracker to be analyzed are indicated in the right panel. This movie corresponds to Figure 1F and G.

Movie S2: Dynamic Localization of RFO1-GFP at the plasma membrane when dmPectin levels at the cell wall are altered. Related with Figure 6.

The movie depicts the dynamic behavior of RFO1-GFP expressed in *rfo1-1* in *Arabidopsis* root epidermal cells acquired using Spinning disc confocal microscopy. The pectin methylation level at the cell wall was modulated by introducing the line iPMEIox which was activated using estradiol (Mock; middle panel) to reduce the dmPectin levels. This effect was compensated by adding dmPectin (right panel). The control line is shown in the left panel. Scale bar = 5 µm. This movie corresponds to Figure 6.