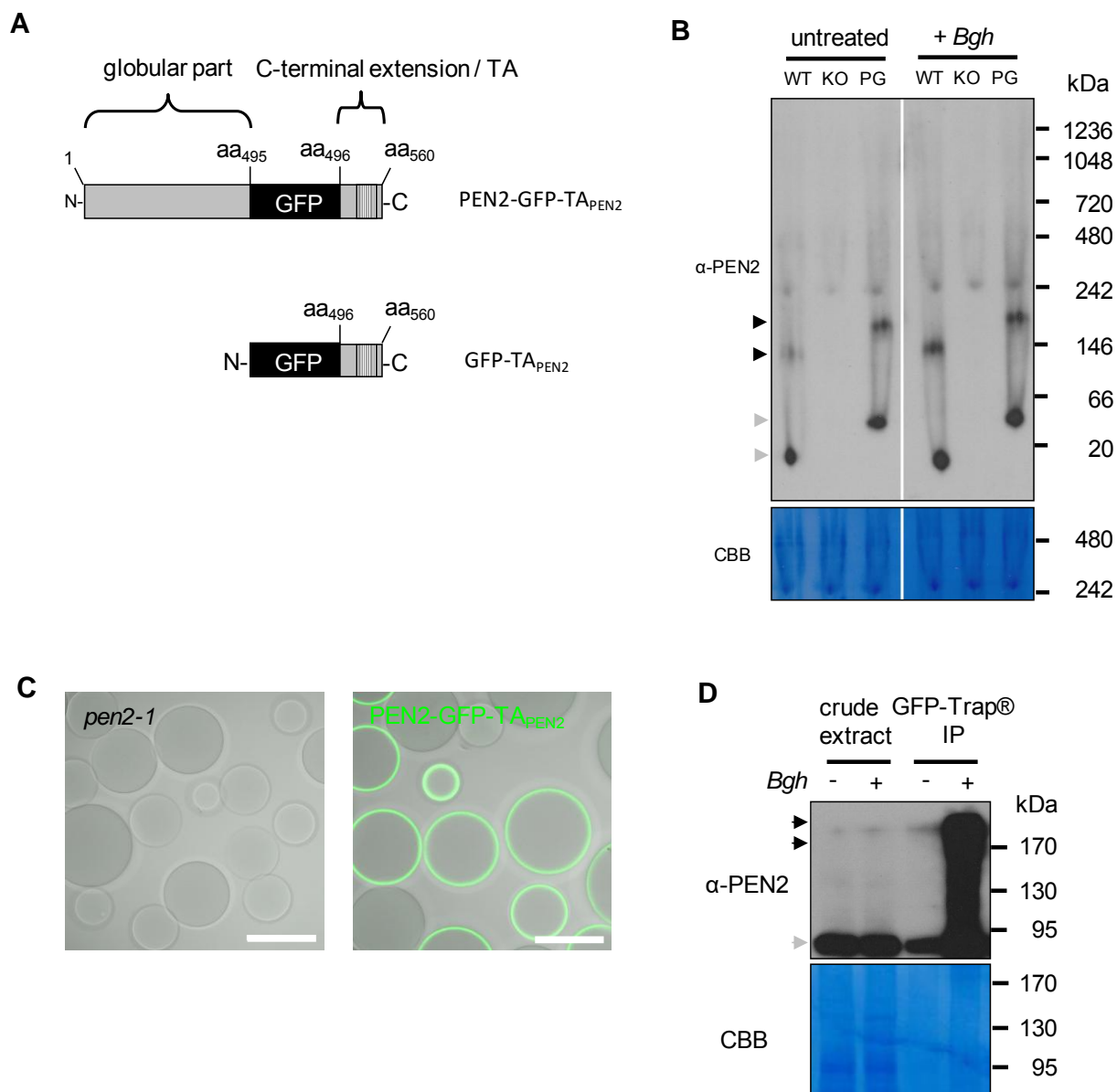


SUPPLEMENTAL MATERIAL

**Supplemental Figure 1. PEN2 shows homomerization capacity.**

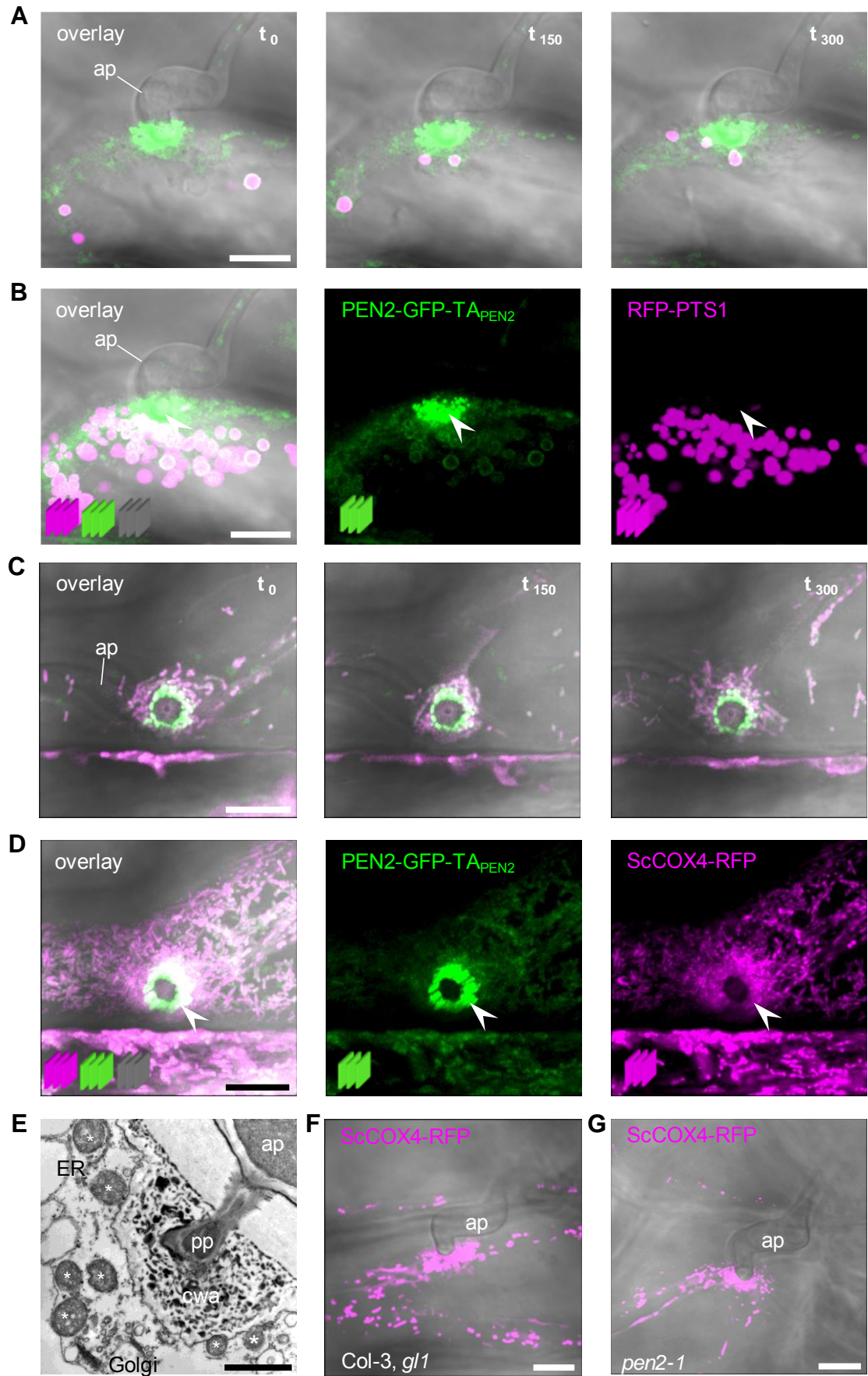
(A) Structural model of PEN2-GFP-TA_{PEN2} and truncated GFP-TA_{PEN2} protein, missing the N-terminal globular part. Dashed part indicates predicted transmembrane domain in the C-terminal extension. Numbers depict amino acid (aa) position in the native PEN2 protein.

(B) Immunoblot analyses of protein extract from wild-type, mutant and PEN2-GFP-TA_{PEN2} expressing plants confirm formation of homodimers of the native and fusion protein independently of *Bgh* treatment (24 h post inoculation [hpi]). 10 µg protein from each sample was subjected to BN-PAGE and immunoblot analysis using a specific

PEN2 antibody. Black arrowheads indicate protein homodimers, whereas grey arrowheads mark monomers. CBB, Coomassie Brilliant Blue staining; KO, knock-out (*pen2-1*); PG, PEN2-GFP-TA_{PEN2}; WT, wild-type (Col-3, *gl1*; the glabrous mutation is a naturally occurring polymorphism).

(C) Confocal laser scanning microscopy (CLSM) image of immuno-precipitated PEN2-GFP-TA_{PEN2} protein using GFP-Trap® coupled to agarose beads demonstrates GFP-binding activity. Incubation of GFP-Trap® with extract from *pen2-1* mutant plants shows no fluorescence. Bars = 100 μm.

(D) Western blot analyses of immuno-precipitated (IP) PEN2-GFP-TA_{PEN2} from pathogen challenged and untreated plants using GFP-Trap® coupled to agarose beads reveal pathogen-induced formation of PEN2 dimers and oligomers of higher order 18 hpi with *Bgh*. Thirty micrograms of each protein extract and 10 μl of each IP eluate were subjected to SDS-PAGE and immunoblot analysis using PEN2 specific antibody. Black arrowheads indicate protein dimers/ oligomers, whereas grey arrowheads marks monomers. CBB, Coomassie Brilliant Blue staining. Experiments were repeated three times with similar results.




Supplemental Figure 2. PEN2-GFP-TA_{PEN2} aggregate formation is restricted to an immobilized subpopulation of mitochondria.

(A) CLSM time-lapse (5 min, 31 frames) analysis of transgenic leaf epidermal cells indicates no association between PEN2-GFP-TA_{PEN2} aggregates underneath fungal appressorium and mobile RFP-labelled peroxisomes at different time points.

(B) Superimposed time-lapse images confirm different subcellular localization of PEN2-GFP-TA_{PEN2} aggregates and RFP-tagged peroxisomes in the cell 18 hpi with *Bgh*.

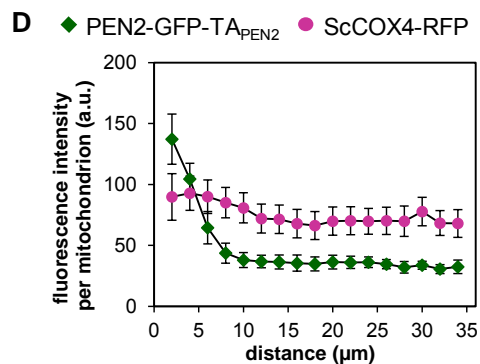
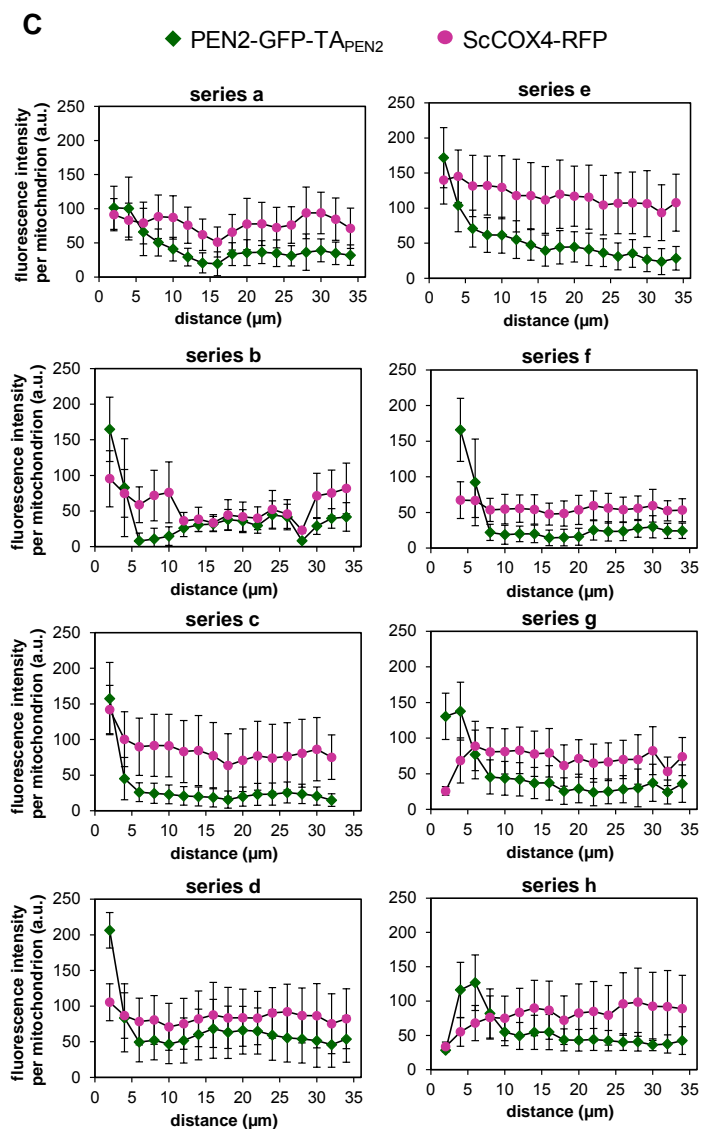
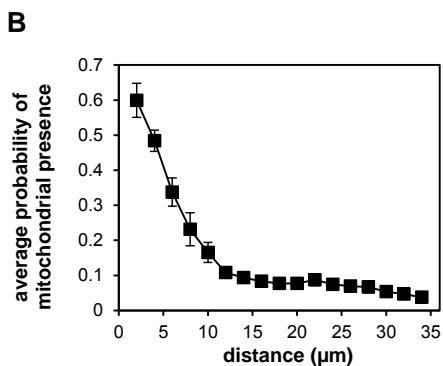
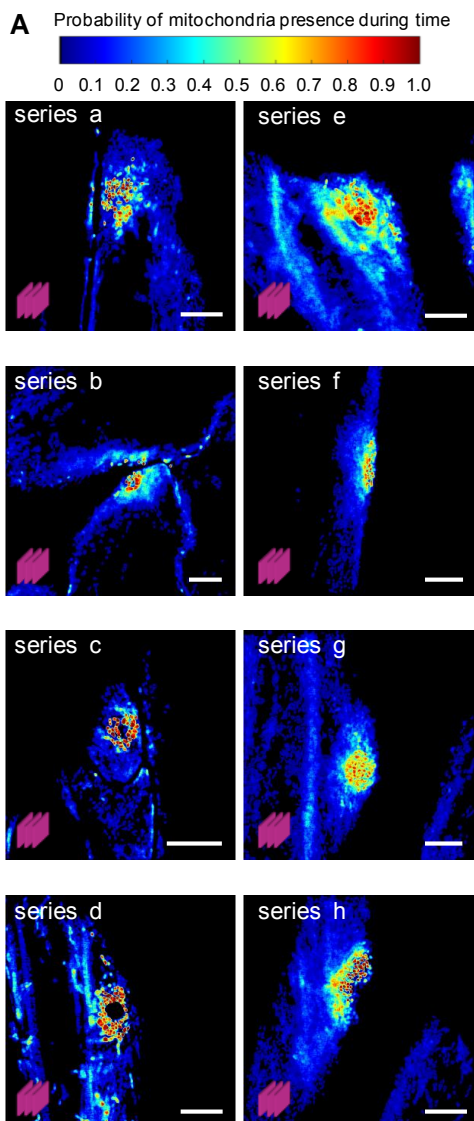
(C) CLSM time-lapse (5 min, 31 frames) experiment demonstrates co-localization of PEN2-GFP-TA_{PEN2} aggregates and a subpopulation of RFP-tagged mitochondria at different time points.

(D) Superimposed time-lapse images confirm co-localization of PEN2 aggregates with immobile mitochondria at pathogen entry site 19 hpi with *Bgh*.

Arrowheads point to PEN2 aggregate formation areas at fungal interaction sites. ap, appressorial germ tube. t, time (s). Maximum projection . Bars = 10 µm.


(E) TEM confirms accumulation of mitochondria at attempted fungal invasion sites in wild-type (Col-3, *gl1*) epidermal cells 19 hpi with *Bgh*. Asterisks mark mitochondria. ap, appressorium; cwa, cell wall apposition; ER, endoplasmic reticulum; pp, penetration peg. Bar = 1 µm.

(F) And (G) single CLSM images of transgenic plants expressing RFP-tagged mitochondria either in wild-type Col-3, *gl1* or *pen2-1* mutant background demonstrate mitochondria accumulation underneath the fungal appressorium 20 hpi with *Bgh*. ap, appressorium. Bars = 10 µm.



Supplemental Figure 3. PEN2-GFP-TA_{PEN2} forms mitochondrial aggregates underneath attempted *Bgh* penetration sites that exhibit a high fluorescence intensity and reduced mobility.

For these analyses, eight time-lapse CLSM datasets of Arabidopsis *pen2-1* mutant plants co-expressing PEN2-GFP-TA_{PEN2} and ScCOX4-RFP at 24 hpi with *Bgh* were used.

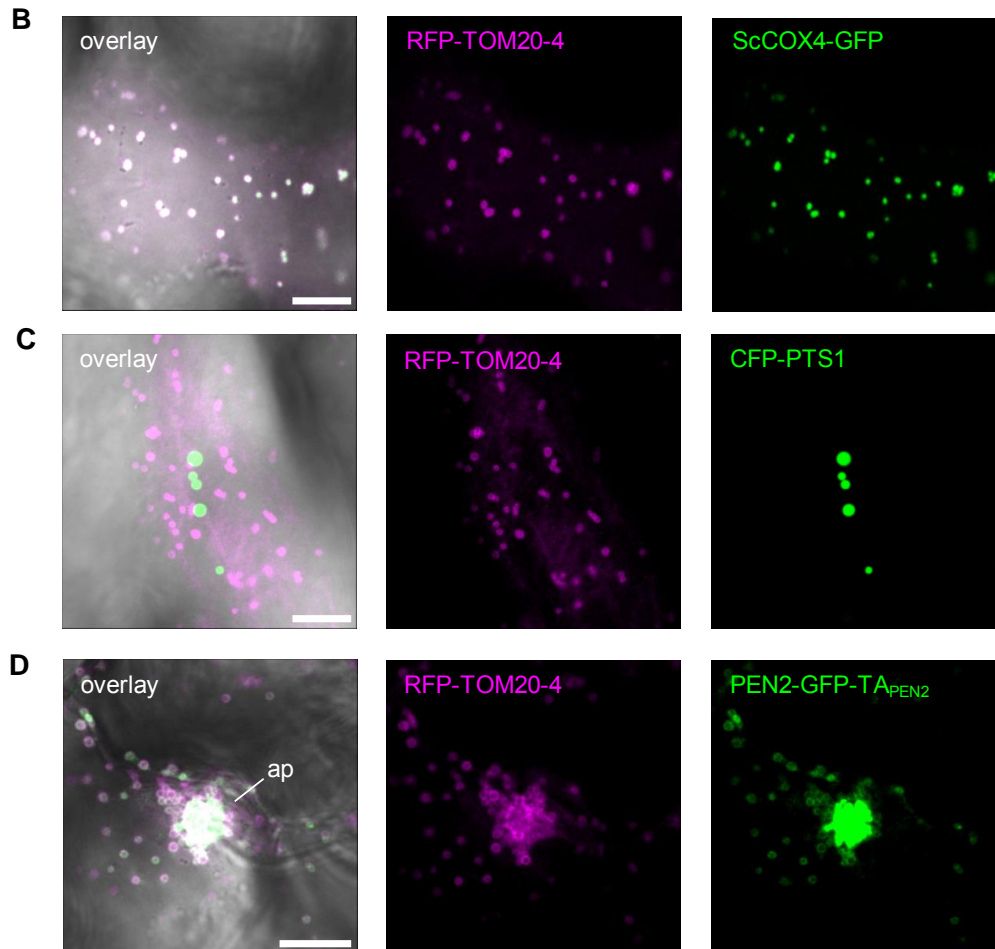
(A) Pseudo-color coded probability maps for the presence of a mitochondrion at each pixel over time. High values around the attempted penetration site correlate with immobilized mitochondria in the video sequences. Maximum projection . Bars = 10 μ m.

(B) Average probability for the presence of a mitochondrion with distance from the attempted penetration site, measured in concentric 2 μ m annuli centered on the attempted penetration site (mean \pm s.e.m, n=8).

(C) Quantification of fluorescence intensities of mitochondria expressing PEN2-GFP-TA_{PEN2} and the mitochondrial marker ScCOX4-RFP in eight individually captured time series 24 h after *Bgh* inoculation indicates higher GFP fluorescence close to the penetration site, whilst the RFP signal remains relatively constant. Data points represent average (\pm S.D.) fluorescence intensities of each mitochondria present in concentric 2 μ m annuli centered on the attempted penetration site and measured over 60 time frames at 5s intervals.

(D) Average signal from PEN2-GFP-TA_{PEN2} and ScCOX4-RFP with distance from the attempted penetration site (mean \pm s.e.m, n=8).

A [RFP-TOM20-4]...¹³⁰APELHTGGTAGPSSNSAKTMKQKKTSEFKYDVFGWVILASYVVAWISFANSQTPVSRQ RFP-TOM20-4
 [PEN2-GFP]...⁵⁰³SKKEEKESYGKQLLHSVQDSQFVHSIKDSGALPAVLGSLFVVSATVGTSLFFKGANN PEN2-GFP-TA_{PEN2}
 [PEN2-GFP]...⁵⁰³SKKTSEFKYDVFGWVILASYVVAWISFANSQTPVSRQ PEN2-GFP-TA_{TOM20-4}



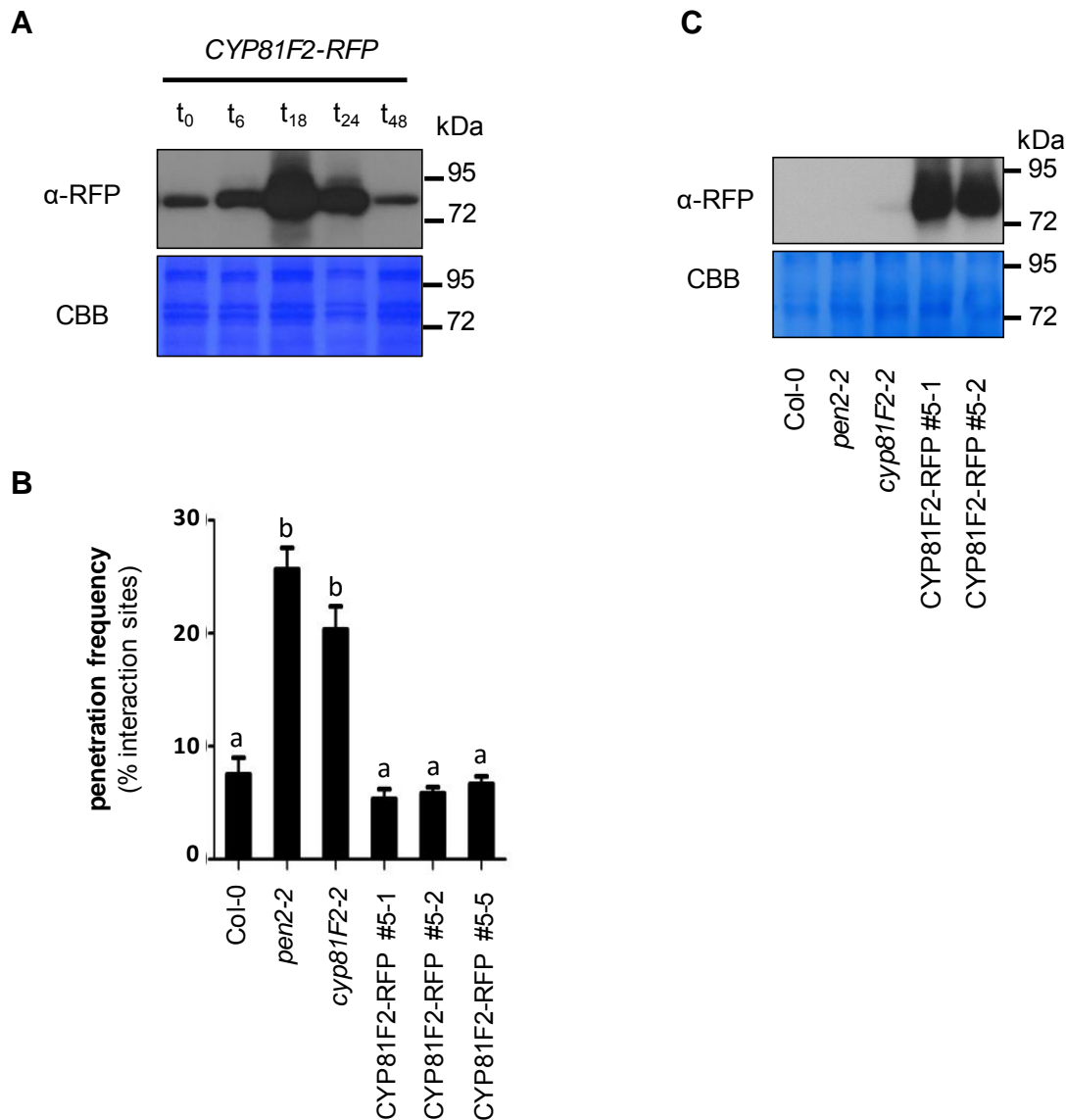
Supplemental Figure 4. The C-terminal-anchored protein TOM20-4 is specifically localized in the outer membrane of mitochondria.

(A) Protein sequence of the C-terminal TA of TOM20-4, PEN2-GFP-TA_{PEN2} and the chimeric construct PEN2-GFP-TA_{TOM20-4}. The predicted transmembrane domain is underlined. The replaced TOM20-4 protein sequence is marked in blue in the chimeric construct. Numbers indicate protein position in the native proteins.

(B) and (C) CLSM images of double transgenic leaf epidermal cells expressing RFP-TOM20-4 and either GFP-tagged mitochondria (B) or CFP-labelled peroxisomes (C) reveal association of TOM20-4 only with mitochondria.

(D) CLSM image of PEN2-GFP-TA_{PEN2} and RFP-TOM20-4 double transgenic epidermal cells show co-localization of both proteins in the periphery of mitochondria 24 hpi with *Bgh*.

ap, appressorium. Bars = 10 μ m.



Supplemental Figure 5. The CYP81F2-RFP fusion protein complements the *cyp81F2* penetration phenotype.

(A) Immunoblot analysis using for each sample 30 μ g protein extract and RFP specific antibody indicates pathogen-induced expression of CYP81F2-RFP 18 hpi with *Bgh*. Please note that whole leaf extracts were used and that the basal level of CYP81F2-RFP detectable at t_0 does not allow detection by CLSM in unchallenged plants (Figure 5A). This indicates that basal CYP81F2-RFP levels are beyond the detection level by CLSM. The significant increase of the CYP81F2-RFP immunoblot signal at t_{18} and t_{24} correlates with cell-autonomous ER-associated CYP81F2-RFP fluorescence in individual epidermis cells that are under pathogen attack. This suggests a dramatically high level of triggered CYP81F2-RFP production in attacked cells allowing detection by CLSM (Figure 5B) and in immunoblot analysis with crude leaf extracts, as shown here.

(B) *cyp81F2-2* mutant complementation studies of CYP81F2-RFP show full complementation capacity of the fusion protein scored at 72 hpi with *Bgh*. Different letters indicate significantly different classes (99% confidence intervals) determined by one-way analysis of variance (ANOVA) with Tukey post test. Depicted results represent two biological replicates with 100 interaction sites analysed on three different leaves, each. Error bars indicate standard error of the mean.

(F) Corresponding immunoblot experiments of all tested plant lines using 30 µg protein extract and RFP-specific antibody indicate equal protein expression levels for both lines. Experiments were repeated three times with similar results. CBB, Coomassie Brilliant Blue staining; t, time (h).

Supplemental Table 1. Oligonucleotide primers used in this study

List of all primers and restriction sites used in this study.

Construct	Primer name	Restriction site	Sequence
PEN2-RFP-TA _{PEN2}	PEN2-RFP-F	AccIII	5'-CAAGCCTCCGGAATGGCCTCCTCCGAGGACG-3'
	PEN2-RFP-R	KpnI	5'-CTTGGGCCTCGGTACCGGCGCCGGTGGAGTGGCGGCCCTC-3'
GFP-TA _{PEN2}	GFP-PEN2-Cterm-F		5'-AACCAAAGATGAGTAAAGGAGAAGAAC-3'
	GFP-PEN2-Cterm-F2	XhoI	5'-TCTCGAGGCAACCAAAGATGAGTAAAGG-3'
	35S-Term-R	(EcoRI)	5'-CTATAAGAACCCTAATTCCTTATCTG-3'
PEN2-GFP-ΔC3	F411-30-F2	(SnaBI)	5'-TGATCCGAGTGATCCAGATGATGTC-3'
	PEN2-GFP-ΔC3-R	EcoRI	5'-GGAATTCTCATCCTTTGAAGAACAGAGAAGTACC-3'
PEN2-GFP-ΔC4	F411-30-F2	(SnaBI)	5'-TGATCCGAGTGATCCAGATGATGTC-3'
	PEN2-GFP-ΔC4-R	EcoRI	5'-GGAATTCTCATTTGAAGAACAGAGAAGTACC-3'
PEN2-GFP-ΔC5	F411-30-F2	(SnaBI)	5'-TGATCCGAGTGATCCAGATGATGTC-3'
	PEN2-GFP-ΔC5-R	EcoRI	5'-GGAATTCTCAGAAGAACAGAGAAGTACCAAC-3'
PEN2-GFP-K556G	F411-30-F2	(SnaBI)	5'-TGATCCGAGTGATCCAGATGATGTC-3'
	PEN2-GFP-K556G-R	EcoRI	5'-GGAATTCTCAATTATTAGCTCCTCCGAAGAACAGAG-3'
PEN2-GFP-ΔTM	F411-30-F2	(SnaBI)	5'-TGATCCGAGTGATCCAGATGATGTC-3'
	PEN2-GFP-ΔTM-R1		5'-GAAGAACAGAGAAGTGTCTTTAATCGAATGAAC-3'
	PEN2-GFP-ΔTM-R2		5'-ATTAGCTCCTTTGAAGAACAGAGAAGTGTCTTTAATCG-3'
	PEN2-GFP-ΔTM-R3	EcoRI	5'-CTGCAGGAATTCATATTATTAGCTCCTTTGAAGAACAG-3'
PEN2-GFP-TA _{TOM20-4}	PEN2-GFP-TOM20Cterm-F	AatII	5'-TTCGACGTCTAAAAAGACCAGTGAGTTCAAG-3'
	PEN2-GFP-TOM20Cterm-R	EcoRI	5'-CGAATTCTTACTGCCTTGACACCGGCG-3'
RFP-TOM20-4	TOM20-4-F	NotI	5'-AGCGGCCGCAATGGATATGCAGAATGAAAACG-3'
	TOM20-4-R	EcoRI	5'-CGAATTCTTACTGCCTTGACACCGGCG-3'
	RFP-F	HindIII	5'-TCAGAAGCTTATCAACAATGGCCTCCTCCGAGGA-3'
	RFP-R	EcoRI	5'-TCAGAATTCTTAGGCGCCGGTGA-3'
Np-CYP81F2-RFP	CYP81F2-F	XhoI	5'-CCTCGAGATGGATTACGTTTTGATTGTTTTGC-3'
	CYP81F2-R	NotI	5'-CAGCGGCCGAGCCAAGAGATTAGTCATAATGGGA-3'
	RFP-F	NotI	5'-GCTGGAGCGGCCGCTGGCGCAGGGGCAATGGCCTCCTCCGAGGACGTC-3'
	RFP-R	EcoRI	5'-TCAGAATTCTTAGGCGCCGGTGA-3'
	Np-CYP81F2-F	AscI	5'-AAGGCGCGCCTCATATTTACCTATTTTGGTTTTGG-3'
	Np-CYP81F2-R	SnaBI	5'-CCGACGAATCTCGTCTTTACGTACGGAGAGGAATC-3'
Y2H PEN2 full-length	PEN2-TOPO-F		5'-CACCATGGCACATCTTCAAAGAACATTTTC-3'
	PEN2-TOPO-R		5'-ATTATTAGCTCCTTTGAAGAACAGAGA-3'
Y2H PEN2-N-term	PEN2-TOPO-F		5'-CACCATGGCACATCTTCAAAGAACATTTTC-3'
	PEN2-N-term-R		5'-ATCAAACCTCAAGAAGTCTTTCAACCATAACG-3'
Y2H PEN2-C-term	PEN2-C-term-F		5'-CACCATGCAAGAAGACGATTCTTCG-3'
	PEN2-TOPO-R		5'-ATTATTAGCTCCTTTGAAGAACAGAGA-3'