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Supplemental Information

Transcription activator-like effector protects bacterial endosymbionts from entrapment within fungal hyphae

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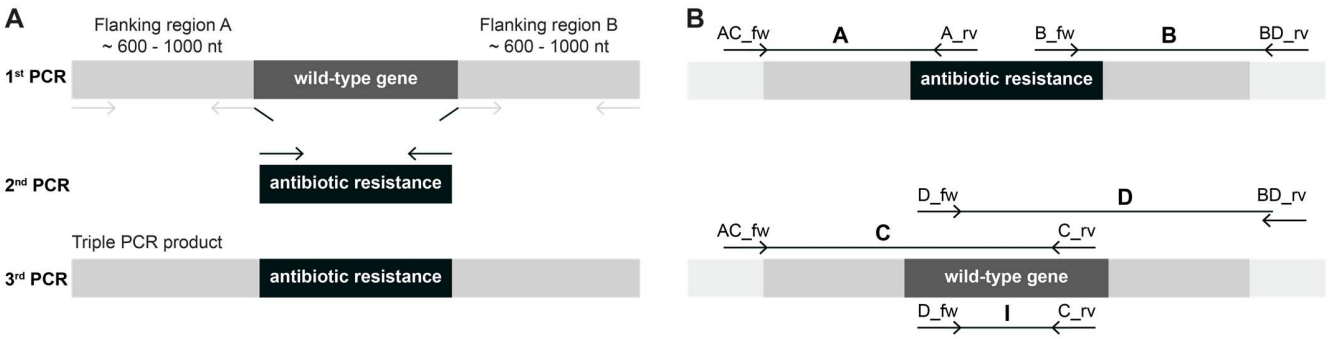
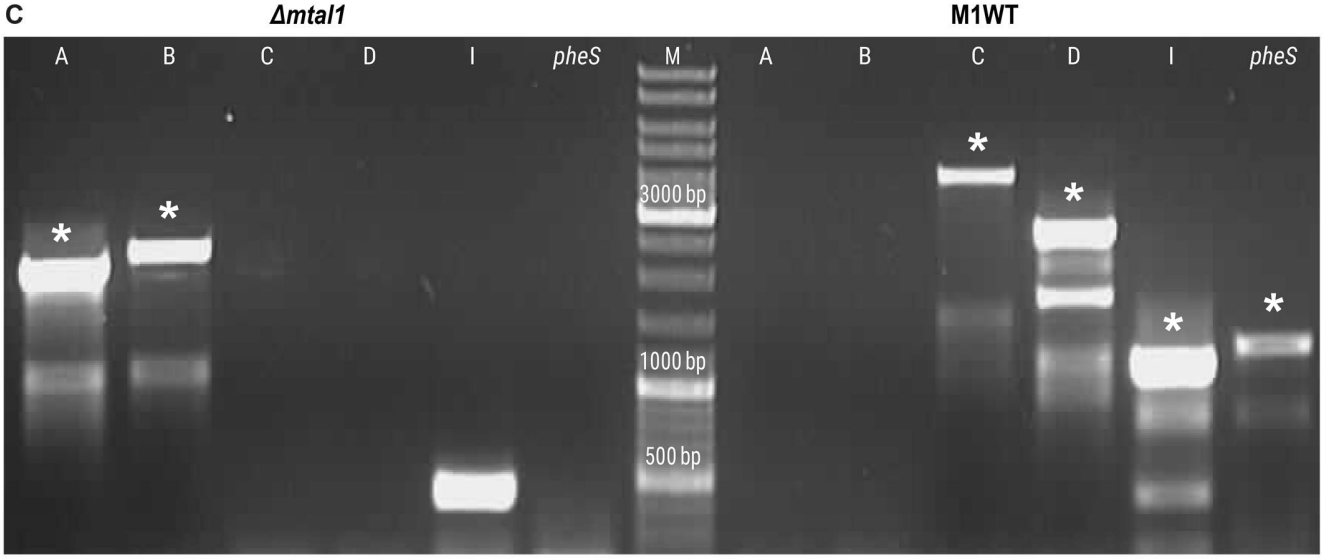
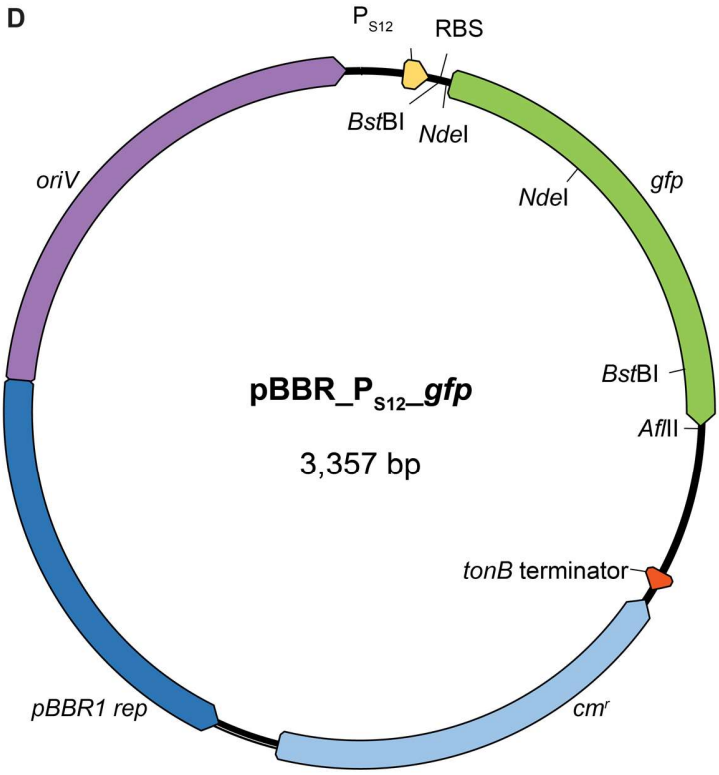

A
Flanking region A
~ 600 - 1000 nt
Flanking region B
~ 600 - 1000 nt
1st PCR
wild-type gene
2nd PCR
antibiotic resistance
Triple PCR product
3rd PCR
antibiotic resistance
B
AC_fw
A
A_rv
B_fw
B
BD_rv
antibiotic resistance
D_fw
D
BD_rv
AC_fw
C
C_rv
wild-type gene
D_fw
I
C_rv


C
Δmtal1
M1WT
A
B
C
D
I
pheS
M
3000 bp
1000 bp
500 bp


D
P S12
RBS
BstBI
NdeI
oriV
NdeI
gfp
BstBI
AflII
pBBR_P S12_gfp
3,357 bp
tonB terminator
pBBR1 rep
cmr

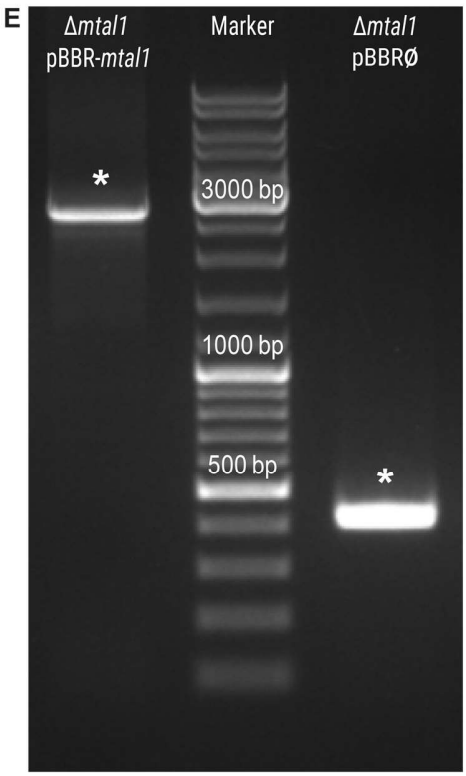

E
Δmtal1
pBBR-mtal1
Marker
Δmtal1
pBBRØ
3000 bp
1000 bp
500 bp

Figure S1. Generation and confirmation of *Mycetohabitans rhizoxinica* transcription-activator like effector (MTAL) knock out and complemented strains (related to STAR methods).
(A) Schematic representation of the construction of knockout vectors.
(B) Schematic representation of the confirmation of the successful gene inactivation. The PCR products of the knockout strain correspond to amplicons A and B; whereas products C and D are amplified from the wild type. The knockout strains were checked for the absence of contaminating *M. rhizoxinica* wild type (M1WT) by amplifying an internal fragment (amplicon I) form the wild type gene of interest. The removal of the knockout vector was confirmed by amplification of the counter selection marker gene *pheS* (amplicons pheS).
(C) The *mtal1* gene was deleted to generate *M. rhizoxinica* Δ*mtal1*. PCR products were obtained from genomic wild type and Δ*mtal1* DNA using control primers listed in Data S6A. Bands corresponding to the expected size are indicated by asterisks (*).
(D) Schematic of the vector used to generate complementation plasmids.
(E) Confirmation of complemented *M. rhizoxinica* Δ*mtal1* (Δ*mtal1* pBBR-*mtal1*) by colony PCR. *M. rhizoxinica* Δ*mtal1* was also transformed with an empty control plasmid as negative control generating *M. rhizoxinica* Δ*mtal1* pBBR∅. PCR products were amplified using control primers listed in Data S6B. Bands corresponding to the expected size are indicated by asterisks (*).

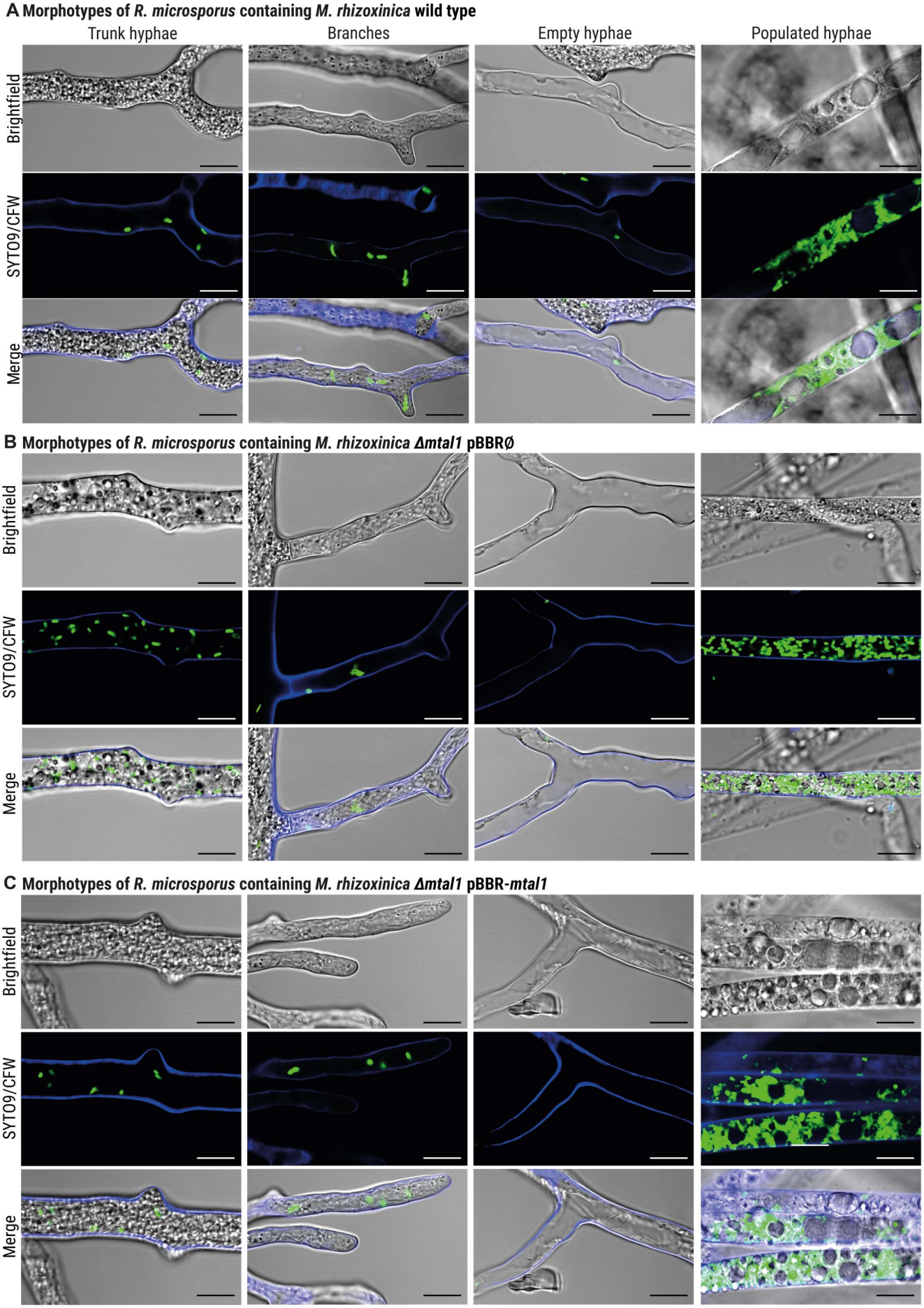

A Morphotypes of R. microsporus containing M. rhizoxinica wild type
Trunk hyphae
Branches
Empty hyphae
Populated hyphae
Brightfield
SYTO9/CFW
Merge
B Morphotypes of R. microsporus containing M. rhizoxinica Δmtal1 pBBRØ
Brightfield
SYTO9/CFW
Merge
C Morphotypes of R. microsporus containing M. rhizoxinica Δmtal1 pBBR-mtal1
Brightfield
SYTO9/CFW
Merge

Figure S2. Phenotypic observations of *Rhizopus microsporus* co-cultivated with SYTO9-stained *Mycetohabitans rhizoxinica* strains in bacterial-fungal interaction (BFI) devices (related to Figure 2C).
(A) Microscopic images of *R. microsporus* (stained with calcofluor white) co-cultivated with *M. rhizoxinica* wild type depicting four types of hyphae. Scale bars: 10 µm.
(B) Microscopic images of *R. microsporus* (stained with calcofluor white) co-cultivated with *M. rhizoxinica* Δ*mtal1* control (Δ*mtal1* pBBRØ) depicting four types of hyphae. Scale bars: 10 µm.
(C) Microscopic images of *R. microsporus* (stained with calcofluor white) co-cultivated with complemented *M. rhizoxinica* Δ*mtal1* (*M. rhizoxinica* Δ*mtal1* pBBR-*mtal1*) depicting four types of hyphae. Scale bars: 10 µm.

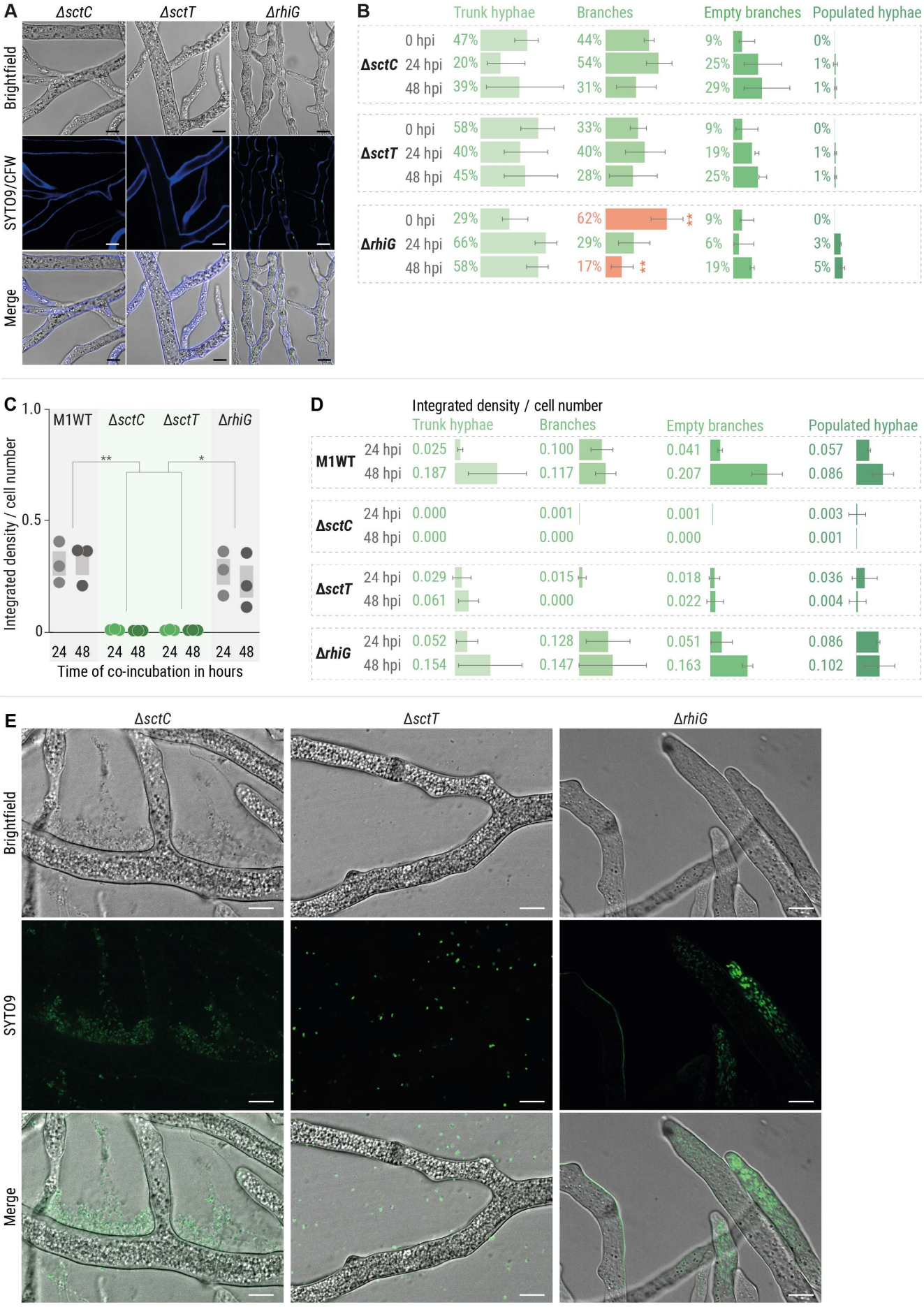

A
ΔsctC
ΔsctT
ΔrhiG
Brightfield
SYTO9/CFW
Merge
B
Trunk hyphae
Branches
Empty branches
Populated hyphae
ΔsctC
0 hpi 47% 44% 9% 0%
24 hpi 20% 54% 25% 1%
48 hpi 39% 31% 29% 1%
ΔsctT
0 hpi 58% 33% 9% 0%
24 hpi 40% 40% 19% 1%
48 hpi 45% 28% 25% 1%
ΔrhiG
0 hpi 29% 62% ** 9% 0%
24 hpi 66% 29% 6% 3%
48 hpi 58% 17% * 19% 5%
C
M1WT
ΔsctC
ΔsctT
ΔrhiG
Integrated density / cell number
1.0
0.5
0
**
*
24 48 24 48 24 48 24 48
Time of co-incubation in hours
D
Integrated density / cell number
Trunk hyphae
Branches
Empty branches
Populated hyphae
M1WT
24 hpi 0.025 0.100 0.041 0.057
48 hpi 0.187 0.117 0.207 0.086
ΔsctC
24 hpi 0.000 0.001 0.001 0.003
48 hpi 0.000 0.000 0.000 0.001
ΔsctT
24 hpi 0.029 0.015 0.018 0.036
48 hpi 0.061 0.000 0.022 0.004
ΔrhiG
24 hpi 0.052 0.128 0.051 0.086
48 hpi 0.154 0.147 0.163 0.102
E
ΔsctC
ΔsctT
ΔrhiG
Brightfield
SYTO9
Merge

Figure S3. Phenotypic observation and quantification of *Rhizopus microsporus* co-cultivated with *Mycetohabitans rhizoxinica* knockout strains in bacterial-fungal interaction (BFI) devices (related to Figures 2C and 2D, Figures 3A and 3B).

(A) Microscopic images of *R. microsporus* (stained with calcofluor white) co-cultivated with *M. rhizoxinica* type 3 secretion system knockout strains (*M. rhizoxinica* Δ*sctC* or *M. rhizoxinica* Δ*sctT*), or a rhizoxin-deficient strain (*M. rhizoxinica* Δ*rhiG*). Bacterial strains were stained with SYTO9 prior to co-incubation. Scale bars: 10 µm.

(B) The fungal mycelium area (as a percentage) of each type of hyphae was measured over a 48-hour time period of co-incubation in BFI devices. At time point 0, cultures of *M. rhizoxinica* Δ*sctC*, *M. rhizoxinica* Δ*sctT*, or *M. rhizoxinica* Δ*rhiG* were stained with SYTO9, individually added to the inlet, and co-incubated with apo-symbiotic *R. microsporus*. Images were taken at the time of infection (0 hours post infection; hpi), as well as 24 and 48 hpi. N = 3 biological replicates ± SEM (Data S1E – S1H).

(C) Apo-symbiotic *R. microsporus* was co-incubated with SYTO9-stained *M. rhizoxinica* wild type (M1WT), T3SS-deficient *M. rhizoxinica* (*M. rhizoxinica* Δ*sctC* or *M. rhizoxinica* Δ*sctT*), or rhizoxin-deficient *M. rhizoxinica* (*M. rhizoxinica* Δ*rhiG*) for 48 hours. Following fluorescence microscopy at 485/498 nm, the integrated density per bacterial cell number was calculated for both measurements (24 and 48 hours post infection; hpi) using Fiji. Dots represent three independent replicates (N = 3 biological replicates) ± SEM (grey bars). One-way ANOVA with Tukey's multiple comparison test (**p<0.05*, ***p<0.002*, SI Appendix, Data S2E – S2H).

(D) The integrated density per cell number was measured for each individual type of hyphae following reinfection with *M. rhizoxinica* wild type (M1WT), *M. rhizoxinica* Δ*sctC*, *M. rhizoxinica* Δ*sctT*, or *M. rhizoxinica* Δ*rhiG*. N = 3 biological replicates ± SEM. One-way ANOVA with Tukey's multiple comparison test (Data S3E – S3H).

(E) Fluorescence microscopy images of *R. microsporus* co-incubated with *M. rhizoxinica* Δ*sctC*, *M. rhizoxinica* Δ*sctT*, or *M. rhizoxinica* Δ*rhiG*. T3SS-deficient *M. rhizoxinica* are unable to reinfect *R. microsporus*, while fungal reinfection by rhizoxin-deficient *M. rhizoxinica* is similar to wild-type *M. rhizoxinica*. Scale bars: 10 µm.

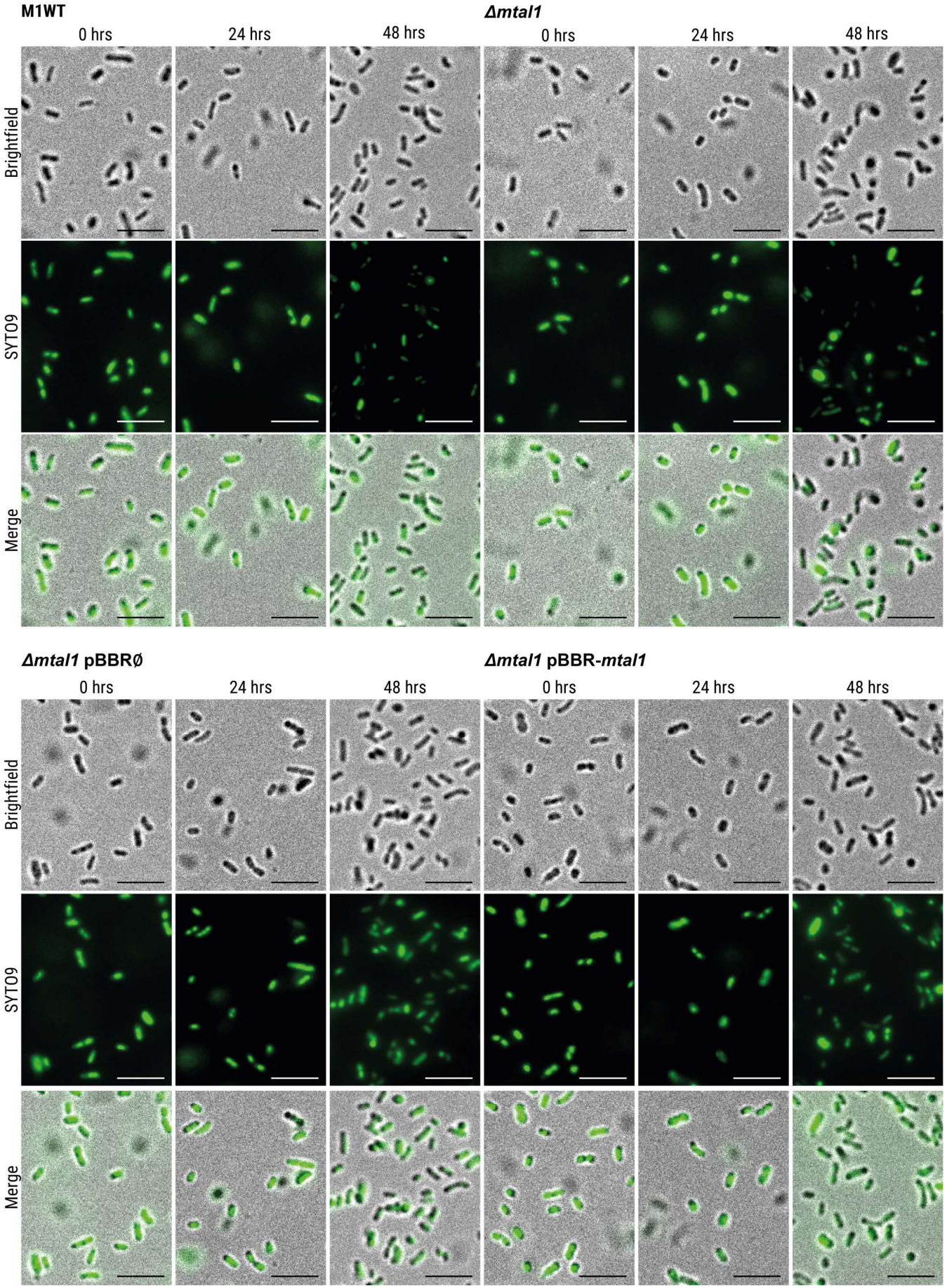

M1WT
Δmtal1
0 hrs
24 hrs
48 hrs
0 hrs
24 hrs
48 hrs
Brightfield
SYTO9
Merge
Δmtal1 pBBRØ
Δmtal1 pBBR-mtal1
0 hrs
24 hrs
48 hrs
0 hrs
24 hrs
48 hrs
Brightfield
SYTO9
Merge

Figure S4. Imaging of axenic SYTO9-stained *Mycetohabitans rhizoxinica* cells over 48 hours of growth (related to Figure 3A). To test whether SYTO9-stained bacteria remain detectably stained, axenic *M. rhizoxinica* wild type, *M. rhizoxinica* Δ*mtal1, M. rhizoxinica* Δ*mtal1* pBBR∅ or *M. rhizoxinica* Δ*mtal1* pBBR-*mtal1* were imaged after 0, 24, and 48 hrs of incubation. Scale bars: 5 µm.

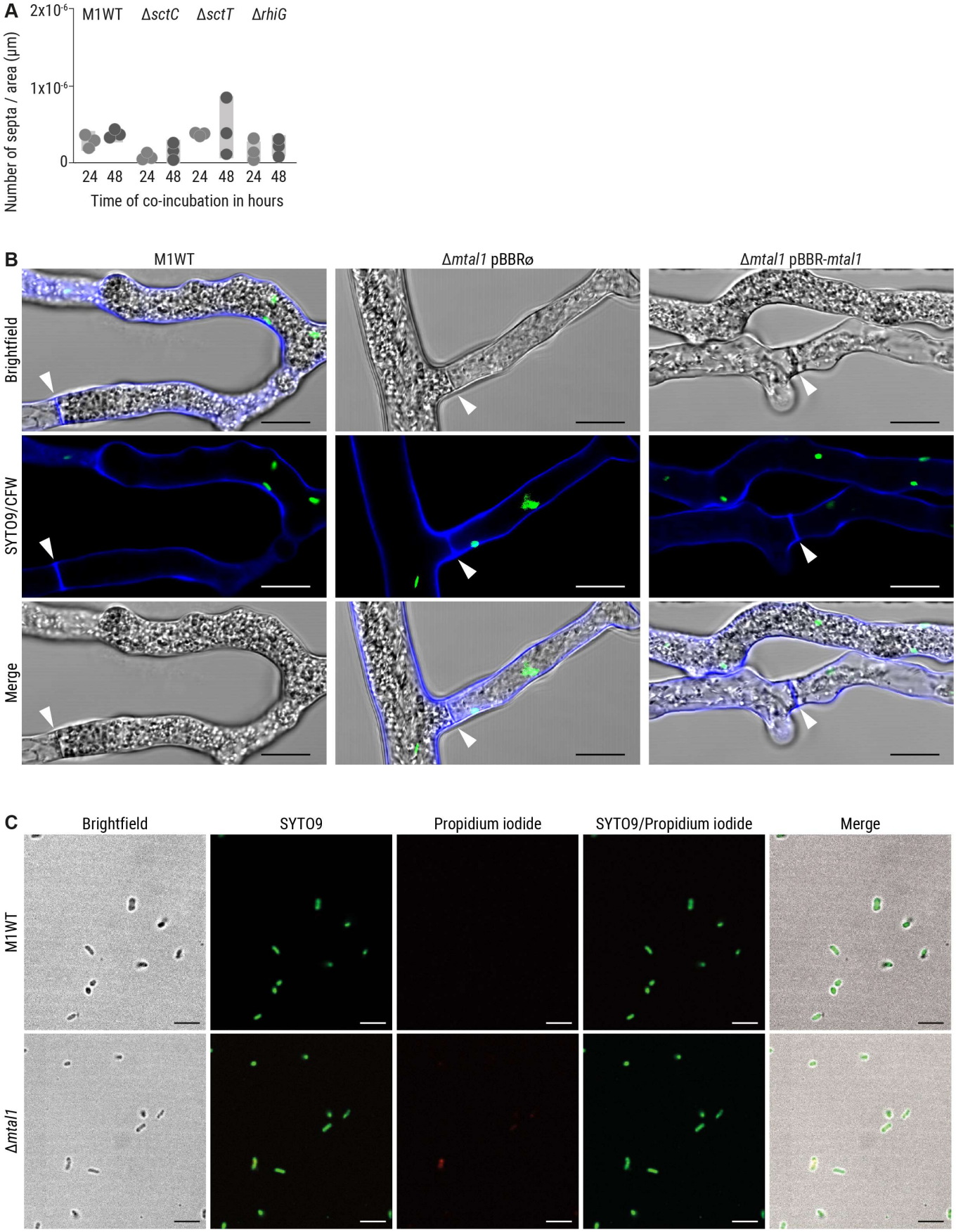

A
2x10-6
1x10-6
0
M1WT
ΔsctC
ΔsctT
ΔrhiG
Number of septa / area (µm)
24 48 24 48 24 48 24 48
Time of co-incubation in hours
B
M1WT
Δmtal1 pBBRø
Δmtal1 pBBR-mtal1
Brightfield
SYTO9/CFW
Merge
C
Brightfield
SYTO9
Propidium iodide
SYTO9/Propidium iodide
Merge
M1WT
Δmtal1

Figure S5. Septa formation in *Rhizopus microsporus* following co-incubation with *Mycetohabitans rhizoxinica* strains (related to Figure 4B and Figure 5A).
(A) The number of septa per area (in µm) was calculated for *R. microsporus* containing either *M. rhizoxinica* wild-type (M1WT), T3SS-deficient *M. rhizoxinica* (*M. rhizoxinica* Δ*sctC* or *M. rhizoxinica* Δ*sctT*), or rhizoxin-deficient *M. rhizoxinica* (*M. rhizoxinica* Δ*rhiG*). Dots represent three independent replicates (N = 3 biological replicates ± SEM). One-way ANOVA with Tukey's multiple comparison test (Data S4E – S4H).
(B) Fluorescence microscopy images showing the formation of septa (blue) in *R. microsporus* following co-incubation with *M. rhizoxinica* wild-type (M1WT), *M. rhizoxinica* Δ*mtal1* pBBRØ, or *M. rhizoxinica* Δ*mtal1* pBBR-*mtal1* (green). Scale bars: 10 µm.
(C) The majority of axenic wild-type *Mycetohabitans rhizoxinica* (M1WT) and *Mycetohabitans* transcription-activator like effector 1 (MTAL1) mutant strains are alive. M1WT and MTAL1 mutant strains (*Δmtal1*) were visualized with LIVE/DEAD BacLight fluorescent dyes. Green fluorescence (SYTO9) indicates that bacteria are alive, while red fluorescence (propidium iodide) is indicative of dead bacteria. Scale bars: 5 µm.