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Supplementary Information for

A TAL effector-like protein of an endofungal bacterium increases the stress tolerance and alters the transcriptome of the host

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Materials and Methods:

Bacterial and fungal growth:

R. microsporus was grown on half-strength potato dextrose agar (½PDA) at 28°C unless otherwise noted. *Mycetohabitans* spp. were grown at 28°C in liquid or on solid Lysogeny Broth (LB) amended with 1% glycerol and 30 µg/mL ampicillin, with 10 µg/mL gentamycin or 25 µg/mL kanamycin as needed. For cloning purposes, *E. coli* was grown at 37°C on LB amended with 10 µg/mL gentamycin, 25 µg/mL kanamycin, or 10 µg/mL chloramphenicol as needed. Chemically competent *E. coli* cells were used for transformation, while transformation of *Mycetohabitans* spp. was done by electroporation using a standard protocol for *P. syringae.*

To isolate bacteria, one to two-day-old *R. microsporus* cultures were scraped from a plate and ground with a plastic pestle in a microcentrifuge tube. LB was added to the tube and then twice filtered through 0.2 µM filters (Whatman) to remove fungal debris. The filtered liquid was plated with sterile beads and incubated until colonies appeared (5 or more days). To cure the fungus, *R. microsporus* was successively cultured on liquid and solid media amended with 20 µg/mL ciprofloxacin until no sporulation occurred. Reinfection was carried out by cocultivation of bacterial and fungal partners on solid media as described by Lastovetsky*, et al.* (1) until sporulation was seen.

Cloning of *btl* genes and derivatives:

All plasmids used in this study are listed in Table S5, including a series of *btl19-13* Gateway™ entry vectors that were created for use in this study. All primers used in this study as listed in Table S6. *btl19-13* was amplified with Q5 High-Fidelity 2X Master Mix (NEB) from *Mycetohabitans* sp. B13 genomic DNA (primers 1990/1991) and cloned into SacI-digested pHM1 (2) with NEBuilder HiFi DNA Assembly Master Mix (NEB) to create pSC31 by Gibson Assembly. The entry vector pENTR4 (Thermo Fisher Scientific) and pSC31 were digested with KpnI and EcoRI and the respective linearized vector and insert bands were gel purified with the Monarch DNA Gel Extraction Kit (NEB), ligated with Quick Ligase (NEB), and transformed into *E. coli* DH5α. The resulting Gateway™ entry vector plasmid, pMEC0, contained *btl19-13* with the native promoter (600 bp), start, and stop codon. Other entry vectors containing derivatives with and without the promoter, start codon, stop codon, or first 45 amino acids were all made with the Q5 Site-Directed Mutagenesis Kit (NEB) and relevant primers (Table S6) and named sequentially as they were created (Table S5). A plasmid encoding a Btl19-13 derivative with the "RIRK" motif in the C-terminus (aa 692-695) mutated to alanines was similarly created by mutagenesis (primers 2247/2248). For the Btl19-13:mCherry fusion, mCherry was PCR-amplified from pGEM-mCherry with primers 2371/2947 that contained SacI restriction enzyme cut sites. The PCR product and pMEC6 were digested with SacI (NEB) and ligated. The resulting pMEC6-mCherry plasmid was transformed into *E. coli* DH5α and encodes Btl19-13:mCherry driven by its native promoter. Similar to *btl19-13*, *btl18-14* with a 338 bp promoter was amplified by PCR (primers 3188/3189) from *Mycetohabitans sp.* B14 genomic DNA, digested with BtgI and EcoRI, gel purified, and cloned directly into the BtgI and EcoRI-digested entry vector pENTR4 to make pMEC13.

Two expression vectors were modified to be Gateway™ destination vectors for expression in *Mycetohabitans* sp. B13. Gateway™ destination vector cassettes (Thermo Fisher Scientific) were ligated into pBBR1-MCS5 (3) digested with SacI-HF and XhoI and into pCPP6260 (4) digested with SacI-HF and PvuI-HF (NEB) to create pMEF1 and pMEF2, respectively. The resulting plasmids were transformed into *E. coli* DB3.1. LR Clonase II (Thermo Fisher Scientific) was used to recombine pMEC6-mCherry and pMEF2 to create pMEF6, the expression vector for Btl19-13:mCherry. The *btl19-13* complementation plasmid, pBtl19-13, is the product of an LR reaction between pMEC0 and pMEF1. Similarly, pMEC13 was recombined with pMEF1 to make the plasmid pBtl18-14 containing *btl18-14*.

Microscopy of *R. microsporus* for *btl19-13* expression:

The plasmid pMEF6 was transformed into *Mycetohabitans* sp. B13 before reinfection into its native *R. microsporus* host. Glycerol stocked spore cultures were suspended in ½ PDB and pipetted onto ½ PDA pads on microscope slides. These were incubated overnight and then stained with DAPI-Fluoromount-G® (SouthernBiotech). Slides were imaged on a DeltaVision imaging workstation (Applied Precision). Images were deconvolved with softWoRx software (Applied Precision), and final images were edited with Fiji (5).

Cya translocation assay and immunoblot:

Derivatives of *btl19-13* were transferred to pCPP5371 (6) by LR Clonase II (Thermo Fisher Scientific) and transformed into *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 or the *Pst* DC3000 *hrcQ-U* mutant (T3SS-) (7). The Cya translocation assay was modified from the protocol described by Chakravarthy, Huot and Kvitko (8). Strains of *Pst* were grown on King's B medium for 2 days at 28°C then suspended in 10 mM $MgCl_2$ to an OD_{600} of 0.5. The youngest fully expanded leaves of 5-week-old *N. benthamiana* plants were infiltrated with a blunt syringe, each strain in triplicate on three separate plants. For each infiltration site, 1 cm^2 of infiltrated leaf tissue was harvested 6 hours post infiltration and frozen in liquid nitrogen. Copper BBs (Crosman) were used to grind the tissue by vortexing before 300 µL of 0.1 M HCl was added. Samples were diluted 1:50 to fall within the range of the assay and processed with the Direct cAMP ELISA Kit (Enzo) according to manufacturer's instructions. Total protein content was measured with the Bio-Rad Protein Assay method.

For the corresponding immunoblot, ~1.5 cm^2 infiltrated tissue samples were frozen and ground. Protein was extracted in 150 µL of buffer [125 mM Tris pH 8.5, 1% SDS, 10% glycerol] with a fungal protease inhibitor (Millipore Sigma) and resolved by 4-20% Tris Glycine SDS-PAGE (Bio-Rad). Proteins were transferred to a membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Cya fusion proteins were detected with a 1:300 diluted primary anti-Cya (3D1) mouse monoclonal IgG antibody (Santa Cruz Biotechnology) and a 1:4000 diluted secondary HRP-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific). Clarity ECL substrate (Bio-Rad) was used for visualization.

Microscopy of yeast for Btl19-13 localization:

Entry vectors with *btl19-13* gene derivatives and previously cloned *Xanthomonas oryzae* pv*. oryzicola* effector Tal1c were recombined into pAG426GAL-eGFP-ccdB (AddGene: #14323; deposited by Susan Lindquist) with LR Clonase II (Thermo Fisher Scientific). For Btl19-13 truncations, the products of this reaction were digested with PspXI and SalI to remove the majority of the Btl19-13 coding region, then self-ligated to contain a little over 2 repeats from the DNA binding domain and the C-terminus (aa 599-714). These constructs were transformed into *Saccharomyces cerevisiae* InvSc1 (Invitrogen) and selected on yeast synthetic uracil drop-out (ura-) medium (Sigma). Individual colonies were used to inoculate cultures of liquid ura- medium with 2% glucose and incubated overnight at 28°C with shaking. Cells were spun down and resuspended in ura- medium containing 0.5 % glucose then allowed to grow for 6 hours. Sterilized 50% galactose was added to the culture to a final concentration of 2%, and the culture was grown overnight. NucBlue Live ReadyProbes Reagent (Thermo Fisher Scientific) was added to the yeast cells and the cells incubated for at least an hour before imaging by LSM 710 confocal microscope (Zeiss). Final images were edited with Fiji (5).

Electrophoretic mobility shift assay:

The coding sequence of Btl19-13 was codon optimized by GenScript for expression in *E. coli* and delivered in pUC57. The coding sequence was cut from this vector with NotI and BsrGI (NEB) and moved into an expression vector that adds an N-terminal 6xHis-tag to make pFR178. *E. coli* Rosetta pLysS (DE3) (Novagen) cells were transformed with pFR178. Proteins were expressed and purified as described by Rinaldi, Doyle, Stoddard and Bogdanove (9).

Electrophoretic mobility shift assays were done as previously described using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) with slight modifications (9). Duplexed oligonucleotides were ordered from Integrated DNA Technologies with a biotin label on the 5' end of the reverse strand. The forward sequences were: binding element (BE) 5'-CGTGGCGTCGTCCACGATTCCGGATTGGACCGACGGCGTC-3' and frame-shifted binding element (BE^{FS}) 5'-CGTGGCGTCGTCCACGATTCGGATTGGACCGACGGCGTC-3'. Labeled DNA and purified protein were incubated at room temperature for an hour in binding buffer [10 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/µl Poly-dIdC, 0.05% NP-40, 0.4 mM EDTA and 0.1 mg/mL BSA]. Binding reactions were mixed with sample buffer and run on a 1.3% agarose gel at 110 V for ~45 min at 4°C. DNA and DNA-protein complexes were transferred to a positively charged Nylon membrane (Sigma-Aldrich) at 100 V for 30 min at 4°C in 0.5×TBE buffer. The DNA was cross-linked to the nylon membrane by exposure to UV for 12 min in a ChemiDoc imaging system (Bio-Rad). DNA was detected by chemiluminescence following the manufacturer's protocol for the EMSA kit.

GUS assay:

A designer TAL effector (dT19-13) was assembled using the Golden Gate TALEN and TAL Effector Kit (AddGene: Kit #1000000024; (10)) and additional modules created in-house for ND, N*, and NS repeats. The RVD sequence of dT19-13 was designed to resemble Btl19-13 as closely as possible (Figure S3) with the available RVD modules: ND ND NI ND NN NI NG NG N* ND NN NN NS N* NG NN NN NI N*. dT19-13 was assembled into a backbone plasmid (pTAL1) encoding the *Xanthomonas oryzae* pv*. oryzicola* effector Tal1c N- and C-termini to create pMEC15. LR Clonase II (Thermo Fisher Scientific) was used to recombine the regions encoding dT19-13 from pMEC15, Btl19-13 from pMEC3, and Tal1c from pCS468 into the binary vector pGWB5, to create pMEC15G, pMEC3G, and pMECtal1cG, respectively. These plasmids were transformed into *Agrobacterium tumefaciens* (*At*) GV3101.

The previously assembled binary vector pCS752 has the pepper Bs3 promoter driving GUS expression with a unique AscI cut site just upstream of the native AvrBs3 binding element (11). The plasmid was digested with AscI (NEB) and dephosphorylated with Antarctic Phosphatase (NEB). Oligonucleotides (primers 3390-3393) were ordered from IDT, annealed, phosphorylated, and ligated into the AscI cut site. Two vectors were made: pMEC-GUS1 has the Btl19-13 binding element (BE: 5'-CCACGATTCCGGATTGGAC-3') and pMEC-GUS2 has a scrambled element (sBE: 5'-GTGCACTCGTTAGCCAGAC-3'). Each binding element was preceded by a T residue to be compatible with TAL effectors, though not required for Btl protein binding (12, 13). The constructs were sequenced confirmed and transformed into *At* GV3101.

Strains of *At* were used to inoculate liquid cultures and left to grow shaking overnight at 28°C. The next morning, cultures were resuspended in 10 mM $MgCl_2$ with 150 µM of acetosyringone and mixed so each strain was at an OD_{600} of 0.3 in the final mixture. Following a 2-hour incubation, cultures were infiltrated into fully expanded leaves of 5-week-old *N. benthamiana* plants with a needleless syringe after scoring the leaf with a needle. Two leaf punches, each 1 cm in diameter, were harvested 48 hours post infiltration, frozen in liquid nitrogen, and ground with glass beads by vortexing. The ground tissue was suspended in 200 µL of GUS Extraction Buffer [50 mM sodium phosphate (pH 7), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton-X100, 0.1% SDS] then vortexed and centrifuged to pellet debris.

The supernatant was used in a Bio-Rad Protein Assay to determine total protein concentration and a MUG assay (Sigma-Aldrich) to determine GUS activity.

Creating a *btl19-13* mutant:

The 1.6 kb upstream and 1.3 kb downstream flanking regions of the *btl19-13* coding sequence were amplified by PCR from *Mycetohabitans* sp. B13 genomic DNA, with primers 1720/1721 and 1724/1725 respectively, and TOPO® cloned into pCR8 (Thermo Fisher Scientific). The construct containing the upstream region was digested with EcoRI-HF and MfeI (NEB), the construct with the downstream region was digested with EcoRI-HF and SalI-HF (NEB), and these were gel purified, ligated, and PCR amplified with OneTaq (NEB) to fuse them before being TOPO® cloned into pCR8. SalI-HF, EcoRI-HF, and XhoI were used to digest the resulting pCR8 vector and EcoRI-HF and SalI-HF were used to digest pK18mobsacB (14), a broad host range vector for use in disrupting genes by sucrose counterselection. The *btl19-13* flanking-region fusion fragment and pK18mobsacB were ligated to create pSC29. *Mycetohabitans* sp. B13 was transformed with pSC29 via electroporation with an overnight recovery period before plating on selective medium. Single colonies were then plated on media amended with 5% sucrose to counterselect for pK18mobsacB loss via double crossover and recover a marker-less mutant, B13Δ*btl19-13*. For genetic complementation and rescue tests, resepctively, pBtl19-13 and pBtl18-14 were each transformed into B13Δ*btl19-13*.

R. microsporus* sporulation assays*:

Single sporangia from sporulating cultures were plated onto fresh ½ PDA and sealed with micropore paper tape (3M). The plates were kept at room temperature on a laboratory bench. There were 7-10 biological replicates, each from a separate infection event. After 22 days, spores were harvested by scraping the mycelial mat off of the plate surface and vortexing for 2 minutes in 0.00125% Tween-20. Spores were filtered through a sterile cotton swab in a 5 mL serological pipette and quantified twice for two technical replicates using a TC20 Automated Cell Counter (Bio-Rad). Data were analyzed with ANOVA in R (v3.5.3).

For mating plates, the compatible isolates of *R. microsporus*, ATCC 52813 and 52814, were both cured and infected with B13 or B13Δ*btl19-13*. Drops of glycerol stocked spore suspensions were placed on opposite sides of a ½ PDA plate and allowed to grow together. Plates were photographed after zygospores started to appear on day 5 or later. Spores were imaged with a Canon EOS attached to an Olympus SZX12.

***R. microsporus* growth assays:**

R. microsporus was plated from glycerol stocked spore suspension onto ½ PDA. Two days later, 2 mm x 2 mm patches of mycelia were used to inoculate the center of new plates: water agar, ½ PDA + 500 mM sodium chloride, ½ PDA + 3 mM hydrogen peroxide, or ½ PDA + 0.005% sodium dodecyl sulfate. Each day until growth reached the edge of the plate, colony diameters were measured on 2 axes and the average measurement was recorded. For each repeat, there were 10 plates of fungi infected with each bacterial genotype. The experiment was repeated twice for each condition and set of genotypes. Data from each repeat were analyzed with ANOVA in R, and each repeat showed the same trend.

RNAseq:

Spore suspensions from a single glycerol stock were plated onto ½ PDA and incubated for 24 hours. Half of the vegetative growth from three plates was pooled for each biological replicate. Three biological replicates were done for each of the three bacterial genotypes. RNA extractions were done with RNeasy Plant Mini Kit (Qiagen) according to manufacturer's protocol, except for sample homogenization, which was carried out as follows. Tissue was directly placed into a 1.5 mL tube containing 450 uL of RLT buffer (subsidized with DTT as per

kit instructions) and two Copper BBs (Crosman). Tubes were vortexed for 10 sec before incubating at 55°C for 5 min, then vortexted again. This slurry was used as the input for the kit. RNA quality and quantity was assessed by agarose gel, NanoDrop (Thermo Fisher Scientific), and Qubit Fluorometer (Invitrogen) before being sent to Novogene for eukaryotic library preparation and paired-end (2 x 150 bp) Illumina sequencing. Each sample library yielded >20 million reads (>6 Gb). RNA-seq analysis was carried out with Trimmomatic for quality control and trimming adapter sequences followed by HISAT2 (15) to align the reads to the *R. microsporus* ATCC 52813 genome assembly Rhimi1_1 (16). StringTie (17) was used to generate gene counts which were fed into DESeq2 (18) for expression analysis. Genes were considered differentially expressed for an adjusted *p* value <0.05 and a log2foldchange>|1|. Binding element (BE) prediction was done with TALE-NT 2.0 on the 500 bp promoter regions of the differentially expressed genes, with the option of a T or C in the -1 position (19).

Genomic DNA preparation and Southern blots:

Three milliliters of turbid liquid cultures of bacteria were used in the MasterPure™ Gram Positive DNA Purification Kit (Lucigen) according to manufacturer's instructions. For strains that were newly isolated, we tested to see if they were *Mycetohabitans spp*. by PCR using previously developed 23S primers that are specific for *Burkholderia* and related species: GlomGiGF and LSU483r (20). PCR fragments were then gel-purified and sequenced to confirm. When we could not successfully extract bacteria from a fungal isolate with no known symbiont, we extracted fungal genomic DNA using the E.Z.N.A. Fungal Mini Kit (Omega) and carried out the same PCR to verify that no bacteria were present.

Southern blot analysis was done using the Amersham ECL Direct Labeling and Detection Kit (GE Healthcare Life Sciences). About 2.5 µg of genomic DNA was digested overnight with the restriction enzyme AatII (NEB), then run for 18 hours at 35 V on a 1% agarose gel at 4˚C. The gel was post-stained in a 3X GelRed solution to visualize equal loading and digest efficiency. The DNA was depurinated with a 10-minute wash in 0.25 M HCl and washed in denaturation buffer [250 µM NaOH, 1.5 mM NaCl] before being transferred to an Amersham Hybond N+ nylon membrane Kit (GE Healthcare Life Sciences) overnight. The blot was crosslinked and then hybridized at 60˚C overnight with a 2.1 kb probe of *btl19-13* amplified from pMEC0. The membrane was washed twice for ten minutes at 60˚C in Primary Wash Buffer [2 M Urea, 0.1% SDS, 50 mM Sodium Phosphate pH 7, 150 mM NaCl, 0.1 mM $MgCl_2$, 2% Blocking Reagent supplied with kit] and twice for five minutes in Secondary Wash Buffer [1 M Tris base, 2 M NaCl]. It was then incubated with Amersham CDP Star Detection Reagent Kit (GE Healthcare Life Sciences) for 4 minutes and visualized on a ChemiDoc imaging system (Bio-Rad) under signal accumulation mode with the hi-resolution blot sensing program.

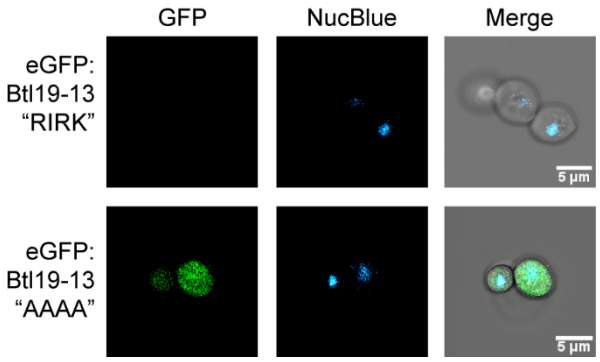

Figure S1. Expression of full-length Btl19-13 in yeast cells. Representative confocal micrographs of *Saccharomyces cerevisiae* transformed to express eGFP:Btl19-13 or eGFP:Btl19-13 ("RIRK" to "AAAA"; aa 692-695) and stained with NucBlue to indicate nuclei. Protein expression was induced with galactose 24 hours prior to imaging.

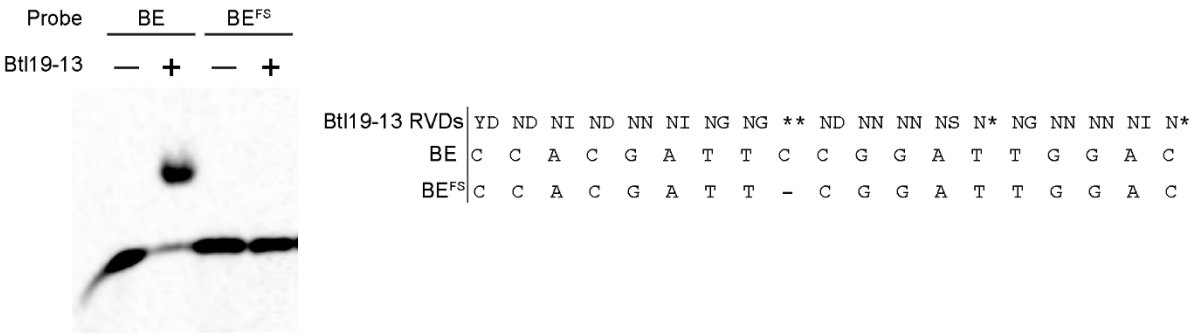

Figure S2. Binding of Btl19-13 to two predicted binding elements. Electrophoretic mobility shift assay of Btl19-13 with BE, the binding element that is predicted to be the best following the TAL effector RVD code, or BE^{FS}, the same binding element with a frameshift where the ** RVD would bind. For each binding reaction, 150 pM of DNA probe was added. The addition of 100 nM protein is indicated with a plus (+) or minus (-). To the right are the sequences of BE and BE^{FS} as they correspond to the RVDs of Btl19-13. An asterisk (*) indicates a missing amino acid in the RVD based on alignment with other repeats.

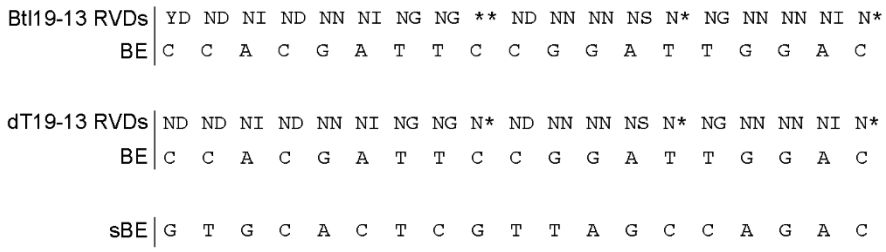

Figure S3. Alignment of Btl19-13 and dT19-13 RVDs with the binding element (BE) and scrambled binding element (sBE) used in the GUS assay. An asterisk (*) indicates a missing amino acid in the RVD based on alignment with other repeats.

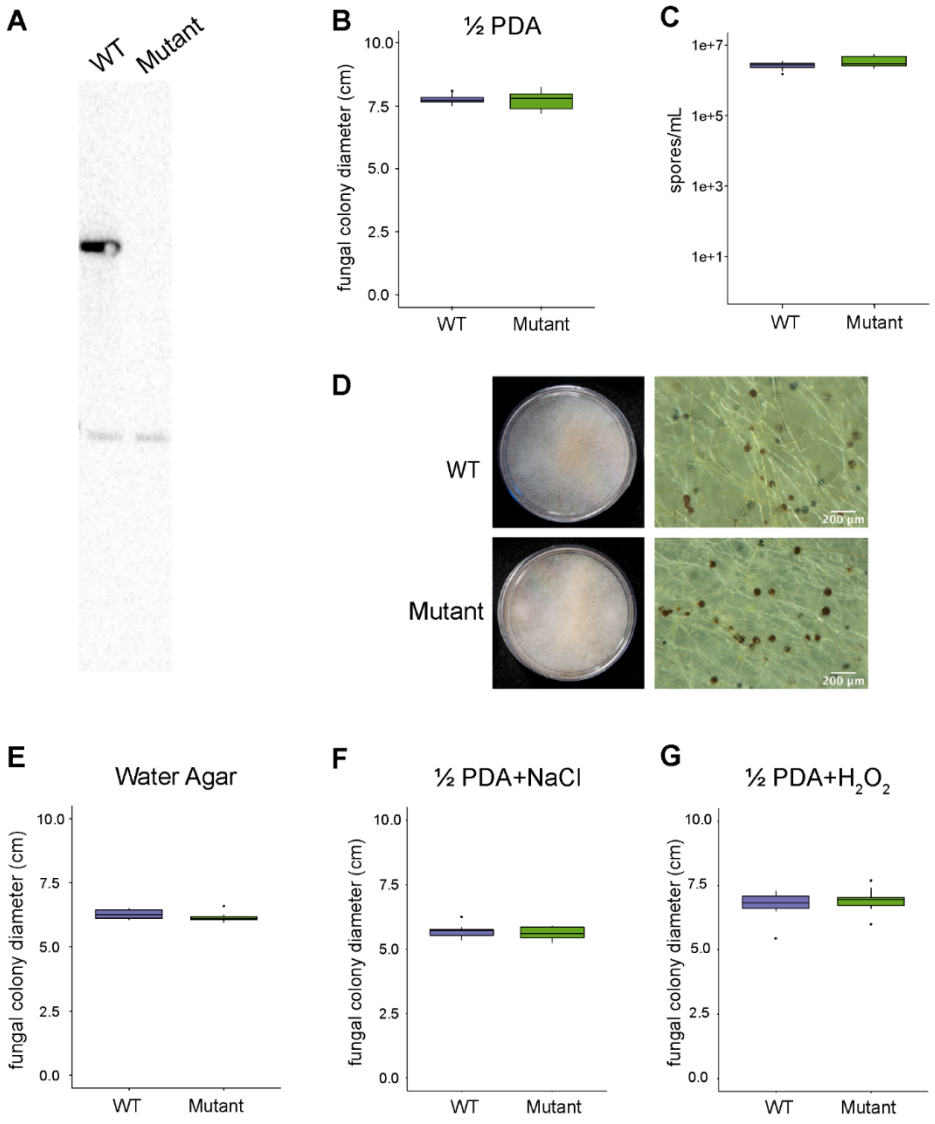

Figure S4. Sporulation and growth of *R. microsporus* infected with wildtype B13 (WT) or a *btl19-13* mutant. (A) Southern blot of bacterial genomic DNA confirming the *btl19-13* deletion in the mutant strain, B13Δ*btl19-13*; the fragment of a second *btl* gene remains. **(B)** Growth of *R. microsporus* on half-strength potato dextrose agar (½ PDA), after four days. Data represent 7-10 biological replicates, each started with a sporangium from a different infection plate. **(C)** Spores were counted from the same plates in (B) after 21 days. **(D)** Photos of sporulating mating plates. Spore suspensions of *R. microsporus* ATCC 52814 and ATCC 52813 infected with B13 or B13Δ*btl19-13* were placed on opposite sides of the plate and allowed to grow together for 5 days at 28°C. **(E-G)** Fungal colony diameter of *R. microsporus* after growing for 3 days on water-agar, 5 days on ½ PDA with 500 mM sodium chloride, or 3 days on ½ PDA with 3 mM hydrogen peroxide at 28°C. Data represent one repeat of 10 biological replicates, each started with a 2 mm x 2 mm plug from a two-day old plate started from glycerol stocked spores. These experiments were repeated twice with similar results. All data were analyzed by ANOVA with a post-hoc Tukey's test ($p < 0.05$).

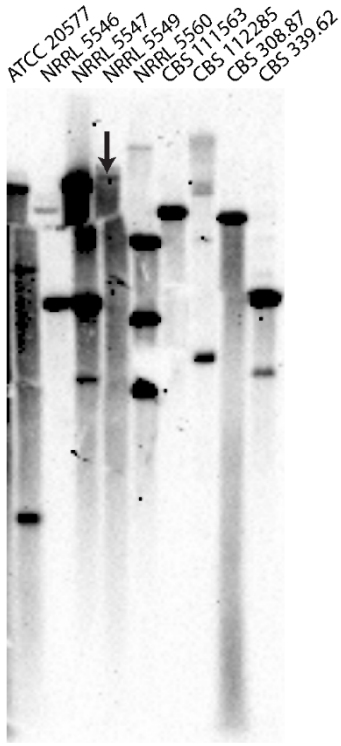

Figure S5. Southern blot from Figure 4B following longer exposure. Southern blot of genomic DNA prepared from nine *Mycetohabitans* spp., probed with *btl19-13* amplified from B13. Strains are identified by the culture collection accession number of their fungal hosts. An arrow indicates the band that was not clearly visible in the shorter exposure in Figure 4B.

Table S1. Information about Btl proteins encoded in three genomes of *Mycetohabitans* spp.

Btl Protein[a]	Repeats	Strain	Gene ID	Plasmid or Chromosome	HrpB Box (bp upstream)	NLS	Repeat Variable Diresidue (RVD) Sequence
Btl28-1	28	B1	RBRH_01770	Plasmid 2 (173 kb)	-239 to -216	RIRK	YD NG ** NN NG ** NN NI NS NI NS NI NN NG ** NK NA NI NS NI NI NI ND NS NG KG NT NG
Btl21-1	21	B1	RBRH_01844	Plasmid 1 (822 kb)	-230 to -207	RIRK	YD NI NI NN NT NN NI NI NN ND NI NT NR NS ND NN NG NG NS N*
Btl7-1	7	B1	RBRH_01778	Plasmid 2 (173 kb)	-219 to -196	QIRK	HD NG NI NG NG NG NN
Btl19-13	19	B13	SAMN04487768_2744	Chromosome	-239 to -216	RIRK	YD ND NI ND NN NI NG NG ** ND NN NN NS N* NG NN NN NI N*
Btl18-14	18	B14	SAMN04487769_0211	Plasmid	-239 to -216	RIRK	YD NG NI NI NG ** ND ND NI NS NN NS N* NG NN NN NI N*
Btl15-14c	15	B14	SAMN04487769_1929	Chromosome	-239 to -216	RIRK	YD ND NI NI NG ** ND NN NN NS N* NG NN NI N*
Btl15-14p	15	B14	SAMN04487769_0204	Plasmid	-219 to -196	RIRK	YD NG NG NG NG NI NI NI NI NI NI S* NN NN NI

[a] See manuscript text for description of nomenclature. 1, bacterial isolate from ATCC 62417; 13, bacterial isolate from ATCC 52813; 14; bacterial isolate from ATCC 52814

Table S2. Predicted promoter regulatory elements for *btl* genes.

btl gene[a]	Predicted HrpB Box	Predicted HrpB Box Location (bp upstream of start codon)	Predicted -10 Element
btl28-1	TTCGctcagtaggtgaagcgTTCG	-239 to -216	caaagt
btl21-1	TTCGttcagcacgcaatgcgTTCG	-230 to -207	caaagt
btl7-1	TTCGccggctcggcggtgcgTTCG	-219 to -196	caaggt
btl19-13	TTCGcccagtaggtgaagtgTTCG	-239 to -216	caaagt
btl18-14	TTCGcccagtaggtgaagtgTTCG	-239 to -216	caaagt
btl15-14c	TTCGcccagtaggtgaagcgTTCG	-239 to -216	caaagt
btl15-14p	TTCGccggatcggtggcgcgTTCG	-219 to -196	caaggt

[a] Nomenclature as in Table S1.

Table S3. Genes that were differentially expressed in *R. microsporus* infected with wildtype B13 (WT) and complement, B13∆*btl19-13*(pBtl19-13) (Comp), compared to the mutant, B13∆*btl19-13* (Mut).

Gene ID	Gene Description	log2FC WT vs Mut	log2FC Comp Vs Mut	BE Ratio Prediction[a]	BE Start Position
RHIMIDRAFT_298141	*hypothetical protein*	7.69	7.96	2.31	219
RHIMIDRAFT_253996	*hypothetical protein*	5.94	6.56	2.82	275
RHIMIDRAFT_266868	*hypothetical protein*	5.52	6.37	-	-
RHIMIDRAFT_232418	*DUF1960-domain-containing protein*	3.76	5.23	2.97	91
RHIMIDRAFT_313464	*hypothetical protein*	2.85	3.05	2.92	218
RHIMIDRAFT_267780	*hypothetical protein*	2.05	2.07	2.89	88
RHIMIDRAFT_283293	*hypothetical protein*	1.65	1.92	2.71	172
RHIMIDRAFT_206793	*4-dihydromethyltrisporate dehydrogenase*	2.30	1.79	2.87	297
RHIMIDRAFT_208941	*DNA clamp loader*	1.06	1.27	2.44	143
RHIMIDRAFT_275463	*hypothetical protein*	-1.32	-1.06	2.25	324
RHIMIDRAFT_119095	*hypothetical protein*	-1.06	-1.33	2.75	37
RHIMIDRAFT_279123	*hypothetical protein*	-2.22	-1.62	2.70	203
RHIMIDRAFT_232860	*hypothetical protein*	-3.60	-3.11	2.88	297
RHIMIDRAFT_264374	*hypothetical protein*	-5.40	-3.48	2.69	218
RHIMIDRAFT_274095	*hypothetical protein*	-5.05	-3.89	2.32	424

[a] Best binding element (BE) for BTL19-13 predicted in the 500 bp promoter region of this gene by TALE-NT 2.0 (19); lower scores are better.

Table S4. Isolates of *R. microsporus* and *Mycetohabitans* spp. used or identified in this study.

Fungal Accession	Taxon	Origin	Bacterial Species	Strain Name(s)	Bacterial Presence Determined
ATCC 62417	*Rhizopus microsporus* van Tieghem	Rice seedlings, Japan	*Mycetohabitans rhizoxinica*	HKI 0454/B1	Partida-Martinez and Hertweck (21)
ATCC 20577	*Rhizopus sp.* strain F-1360	Soil, Japan	*Mycetohabitans sp.*	HKI 0512/B2	Lackner*, et al.* (22)
NRRL 13479; CBS 699.68; ATCC 52813	*Rhizopus microsporus* van Tieghem	Soil, Ukraine	*Mycetohabitans endofungorum*	HKI 0402/ B4/B13	Lackner*, et al.* (22) Dolatabadi*, et al.* (23);
NRRL 13478; CBS 700.68; ATCC 52814	*Rhizopus microsporus* van Tieghem	Forest soil, Georgia	*Mycetohabitans endofungorum*	HKI 0403/ B7/B14	Lackner*, et al.* (22) Dolatabadi*, et al.* (23);
CBS 261.28; ATCC 52811	*Rhizopus microsporus* van Tieghem	Unspecified, USA	*Mycetohabitans rhizoxinica*	HKI 0513/B6	Lackner*, et al.* (22) Dolatabadi*, et al.* (23);
CBS 262.28; ATCC 52812	*Rhizopus microsporus* var Chinensis	Unspecified, USA	*Mycetohabitans sp.*	-	This study
NRRL 5546	*Rhizopus microsporus*	Soil, Brazil	*Mycetohabitans sp.*	-	This study
NRRL 5547	*Rhizopus microsporus*	Soil, Philippines	*Mycetohabitans sp.*	-	This study
NRRL 5549; ATCC 46352	*Rhizopus microsporus*	Rabbit Dung, Wisconsin, USA	*Mycetohabitans sp.*	-	This study
NRRL 5560	*Rhizopus microsporus*	Corn, USA	*Mycetohabitans sp.*	-	This study
CBS 112285	*Rhizopus microsporus* Tieghem	Ground nuts, Mozambique	*Mycetohabitans endofungorum*	HKI 0456/B5	Lackner*, et al.* (22)
CBS 111563	*Rhizopus microsporus* Tieghem	Sufu starter culture, Vietnam	*Mycetohabitans endofungorum*	HKI 0455/B3	Lackner*, et al.* (22) Dolatabadi*, et al.* (23);
CBS 339.61	*Rhizopus microsporus var. oligosporus* (Saito) Schipper & Stalpers	Tempeh, Indonesia	*Mycetohabitans rhizoxinica*	-	Dolatabadi*, et al.* (23);
NRRL 28628; CBS 308.87	*Rhizopus microsporus* van Tieghem	Necrotic tissue from a man's hand, Australia	*Mycetohabitans sp.*	HKI 0404/B8	Lackner*, et al.* (22)

Table S4 cont.

Accession	Taxon	Origin	Bacterial Species	Strain Name	Bacterial Presence Determined
CBS 113206	*Rhizopus microsporus* var. *tuberosus*	Koji beer, China	None	NA	This study
NRRL 1508	*Rhizopus microsporus*	Soil, USA	None	NA	This study

Table S5. Plasmids used in this study.

Plasmid	Description	Source
pK18mobsacB	Broad host range marker-less deletion vector	Schafer*, et al.* (14)
pSC29	pK18mobsacB with *btl19-13* flanking regions	This study
pHM1	Broad host range cosmid vector	Hopkins, White, Choi, Guo and Leach (2)
pSC31	pHM1 containing *btl19-13* with native promoter	This study
pMEC0	pENTR4-Btl19-13 with native promoter, native start codon, and native stop codon in pENTR4 (Invitrogen)	This study
pMEC1	pENTR4-Btl19-13 without native promoter or stop codon, but with start codon	This study
pMEC2	pENTR4-Btl19-13 without native promoter or start codon, but with stop codon	This study
pMEC3	pENTR4-Btl19-13 without native promoter but with start and stop codon	This study
pMEC5	pMEC2 with RIRK NLS mutated to AAAA (aa 692-695)	This study
pMEC6	pMEC0 with no stop codon; *btl19-13* with native promoter and native start codon	This study
pMEC6-mCherry	*btl19-13* with native promoter, native start codon, and a C-terminal mCherry fusion	This study
pMEC7	pMEC3 with N-terminal 45 aa truncation	This study
pMEC8	pMEC3 with only first 45 aa	This study
pMEC13	*btl18-14* with native promoter, native start codon, and native stop codon	This study
pBBR1-MCS5	Broad host range expression vector with multiple cloning site	Obranić, Babić and Maravić-Vlahoviček (3)
pMEF1	pBBR1-MCS5 with a Gateway Destination vector cassette added at the MCS	This study
pBtl19-13	pMEF1 containing *btl19-13* with native promoter from pMEC0	This study
pBtl18-14	pMEF1 containing *btl18-14* with native promoter from pMEC13	This study
pCPP6260	NptII promoter driving EYFP expression	Worley (4)
pMEF2	pCPP6260 with a Gateway Destination vector cassette	This study
pMEF6	pMEF2 encoding Btl19:13:mCherry with its native promoter	This study
pCPP5371	Gateway destination vector for Cya fusion proteins	Oh, Kvitko, Morello and Collmer (6)
pCPP5388	pCPP5371 expressing AvrPto:Cya	Lam*, et al.* (24)
pMEC1C	pCPP5371 expressing Btl19-13:Cya	This study
pMEC7C	pCPP5371 expressing Btl19-13[45:]:Cya	This study
pMEC8C	pCPP5371 expressing Btl19-13[1:45]:Cya	This study
pAG426GAL-EGFP-ccdb	Gateway destination vector for yeast expression; GAL1 promoter driving N-terminal eGFP tagged expression	Addgene plasmid # 14323 deposited by Susan Lindquist
pCS468	A Gateway entry vector containing Tal1c cloned from *Xanthomonas oryzae* pv. *oryzicola*	C. Schmidt
pYeGFP-TAL1C	pAG426GAL-eGFP-tal1c	This study
pMEC2Y	pAG426GAL-eGFP-Btl19-13	This study
pMEC5Y	pAG426GAL-eGFP-Btl19-13 NLS mutant; RIRK mutated to AAAA (aa 692-695)	This study

Table S5 cont.

Plasmid	Description	Source
pMEC5Y	pAG426GAL-eGFP-Btl19-13 NLS mutant; RIRK mutated to AAAA (aa 692-695)	This study
pMEC2YC	pAG426GAL-eGFP-Btl19-13[599-714]	This study
pMEC5YC	pAG426GAL-eGFP-Btl19-13[599-714] NLS mutant; RIRK mutated to AAAA (aa 692-695)	This study
pFR178	Expression of codon-optimized Btl19-13 with a N-terminal 6xHis tag under lac induction	This study
pTAL1	A gateway entry vector with the Tal1c N- and C- termini; created as part of the kit to make designer TAL effectors	Cermak*, et al.* (10)
pMEC15	Gateway entry vector with dT19-13; pTAL1 with the RVD sequence most closely related Btl19-13 assembled in between the Tal1c N- and C- termini	This study
pGWB5	Binary Gateway Destination vector with a 35S promoter	Nakagawa*, et al.* (25)
pCS752	Binary vector with 343 bp Bs3 promoter from pepper driving GUS expression	Doyle*, et al.* (11)
pGWB5-AvrBs3	pGWB5 expressing AvrBs3 cloned from *Xanthomonas euvesicatoria*	Cernadas*, et al.* (26)
pMEC3G	pGWB5 expressing Btl19-13 from pMEC3	This study
pMEC15G	pGWB5 expressing dT19-13 from pMEC15	This study
pMECtal1cG	pGWB5 expressing Tal1c from pCS468	This study
pMEC-GUS1	pCS752 with a Btl19-13 binding element inserted into the AscI cut site	This study
pMEC-GUS2	pCS752 with a scrambled binding element inserted into the AscI cut site	This study

Table S6. Oligonucleotides used for cloning in this study.

Number	Sequence	Purpose
1720	AATCGAATTCGTTGTGTAGATGATCTTGCGA	Forward primer, with EcoRI site, for Btl19-13 KO, Flank A
1721	AAACAATTGGGCATGGTTGGTAAGCTGAGCCCAAAC	Reverse primer, with MfeI site, for Btl19-13 KO, Flank A
1724	AAACAATTGCCCGTTGCCGTGCGCGTGAGTAAG	Forward primer, with MfeI site, for Btl19-13 KO, Flank B
1725	TAGGTCGACGCGAGCTTCTTGATTTC	Reverse primer, with SalI site, for Btl19-13 KO, Flank B
1990	CCAGTGAATTCGAGCTTCACTGGCCTTCAATGCTG	Reverse primer to amplify Btl19-13 for pSC31
1991	CCCGGGTACCGAGCTGTTGGCTCCTTTGCCAATAG	Forward primer for amplify Btl19-13 for pSC31
2247	GCAGCGATGGTGGCCTCTTTGCTAGG	Forward primer to change “RIRK” to “AAAA” in Btl19-13
2248	TGCTGCACCAGCTCCTCCTCGGCG	Reverse primer to change “RIRK” to “AAAA” in Btl19-13
2307	ATGCCGGCGACGTTTATG	Forward primer to delete Btl19-13 promoter
2308	AGCTCGGTACCGGATCC	Reverse primer to delete Btl19-13 promoter
2309	CCGGCGACGTTTATGCATC	Forward primer to delete Btl19-13 promoter and start codon
2310	AGCTCGAATTCGCGGCCG	Forward primer to delete stop codon in Btl19-13
2311	CTGGCCTTCAATGCTGGTAAC	Reverse primer to delete stop codon in Btl19-13
2371	ATAAATGAGCTCCTACTTGTACAGCTCGTCCA	mCherry amplification with SacI cut site (reverse)
2405	CTTGCTCAAGTACTAAGTCG	Forward primer for Q5 mutagenesis to delete first 45 amino acids in Btl19-13
2406	CATAGCTCGGTACCGGAT	Reverse primer for Q5 mutagenesis to delete first 45 amino acids in Btl19-13
2407	AGCTCGAATTCGCGGCCG	Forward primer for Q5 mutagenesis to delete everything but the first 45 amino acids in Btl19-13
2408	CTCATCGTGCTGATTTGAGATAAACGC	Reverse primer for Q5 mutagenesis to delete everything but the first 45 amino acids in Btl19-13
2947	AAATAAAGAGCTCAGTGAGCAAGGGCGAGGAG	mCherry amplification with SacI cut site (forward)
3188	ATTACCATGGGCTGTCAAGACTTCGAGCCA	Forward primer, with BtgI site, for cloning Btl18-14 with its promoter
3189	ATATGAATTCTCACGGGCCTTCAATGCT	Reverse primer, with EcoRI site, for cloning Btl18-14 with its promoter
3390	CGCGTCCACGATTCCGGATTGGAC	Btl19-13 BE for inserting in pCS752 (forward)
3391	CGCGGTCCAATCCGGAATCGTGGA	Btl19-13 BE for inserting in pCS752 (reverse)
3392	CGCGTGTGCACTCGTTAGCCAGAC	Scrambled Btl19-13 BE for inserting in pCS752 (forward)
3393	GTCTGGCTAACGAGTGCACACGCG	Scrambled Btl19-13 BE for inserting in pCS752 (reverse)

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