Supporting Information

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SI Text

Feeding Experiments. ***Pure protoplasts.*** Sterigmatocystin (ST; 20 μg/mL) was added (fed) to 1 mL of a pure protoplast fraction (10^6 protoplasts) and this mixture was incubated overnight at 30 °C. Aflatoxin content was measured by ELISA before and after incubation and normalized to total protein in the protoplast fraction. Control protoplasts were incubated under the same conditions without added ST. Protoplasts prepared from *A. parasiticus* AFS10 (derived from SU-1, *aflR* disruption, no aflatoxin enzymes or aflatoxin accumulate) and LW1432 (*omtA* disruption) were also included as controls.

To measure aflatoxin content, protoplasts were pelleted at 1,000 $\times$ *g*, the supernatant was discarded, and the pellet was suspended with 500 μL of chloroform and shaken 20 times to extract aflatoxins. The suspension was centrifuged at 10,000 $\times$ *g*; the lower organic layer was removed and dried to evaporate chloroform; and the aflatoxin residue was dissolved in 70% methanol.

Protoplasts prepared from strain SU-1 were incubated with and without added ST, and the vesicle-vacuole fraction was purified as described. After this organelle fraction was removed from the gradient, we combined the remaining gradient contents (designated the nonvacuole-vesicle fraction). Aflatoxins in the vesicle-vacuole and the nonvacuole-vacuole fractions were extracted with chloroform (500 μL chloroform per 100 μL of volume fraction), and the chloroform evaporated. The extracts were dissolved in 70% methanol, the aflatoxin measured by ELISA, and the data normalized to the total protein in the appropriate fraction.

Vesicle-vacuole and nonvesicle-vacuole fractions. The vesicle-vacuole and nonvesicle-vacuole fractions prepared from 1 mL of pure protoplasts (containing $\approx 10^6$ protoplasts) were incubated in parallel, with or without added ST (20 μg/mL) at 30 °C overnight. Aflatoxin (measured by ELISA) in each fraction was normalized to total protein in the appropriate fraction.

Identification, Cloning, and Disruption of *A. parasiticus vb1*. We identified gene sequence (92.m03481) via strong identity (70%) to yeast *ypt7* using a published *A. flavus* genome database (www.aspergillusflavus.org/). 92.m03481 was used to identify cosmid C31 (harbors *vb1*) by PCR screening of an *A. parasiticus* cosmid library (5′-TCGGCG CGGATTTCCTTAC-3′, forward, 5′- GGCTTCCTTGGCACTGGTTTC-3′, reverse). We amplified a 5-Kb fragment in cosmid C31 (5′-AGTTAACCTTAG-GAAATCAGATG-3′, forward, and 5′-AAGCAGAGGGAA TATTGGTC-3′, reverse), excised a 4.4-Kb XbaI/XmaI fragment from the PCR product, and subcloned it into the XbaI/XmaI region of pUC19 (New England Biolabs) to generate plasmid pC4k. A 6.3-Kb HindIII fragment containing the selectable marker *niaD* (obtained from plasmid pSL82) (6, 7) was blunt ended using the Klenow fragment of DNA PolI and inserted into a ClaI site in pC4K (also blunt ended by Klenow enzyme) to construct the *vb1* disruption plasmid pVB. Transformation of *A. parasiticus* NR-1 protoplasts was performed as described previously (6, 7). Strains carrying the *vb1* gene disruption (AC5, AC7, and AC11) were confirmed by Southern blot analysis as described previously (6, 7). Strain AC34 carried wild-type *niaD* that replaced the mutated *niaD* in NR-1.

Nucleotide sequence analysis was conducted using an automated nucleotide sequencer (ABI robotic catalyst and 373A DNA sequencer) at the Plant Research Laboratory, Michigan State University. The nucleotide sequence accession number for *vb1* is AY52045.

Counting Vesicles and Statistical Analysis. To count vesicles and vacuoles, mycelial fragments (width ranging from 4 μm to 6 μm) were analyzed in 10 fields; the number of vesicles and vacuoles per 50-μm mycelial length was then calculated. Data were obtained from 2 biological replicates. For statistical analysis (determination of 2-tailed *P* value by unpaired *t* test), vesicle counts were normalized to vacuole counts.

For statistical analysis, we used GraphPad Instat software. For densitometry measurements, Odyssey software was used to compare the intensity of the protein bands in Western blot, and Adobe photoshop was used to compare the PCR products in gene-expression (RT-PCR) experiments.

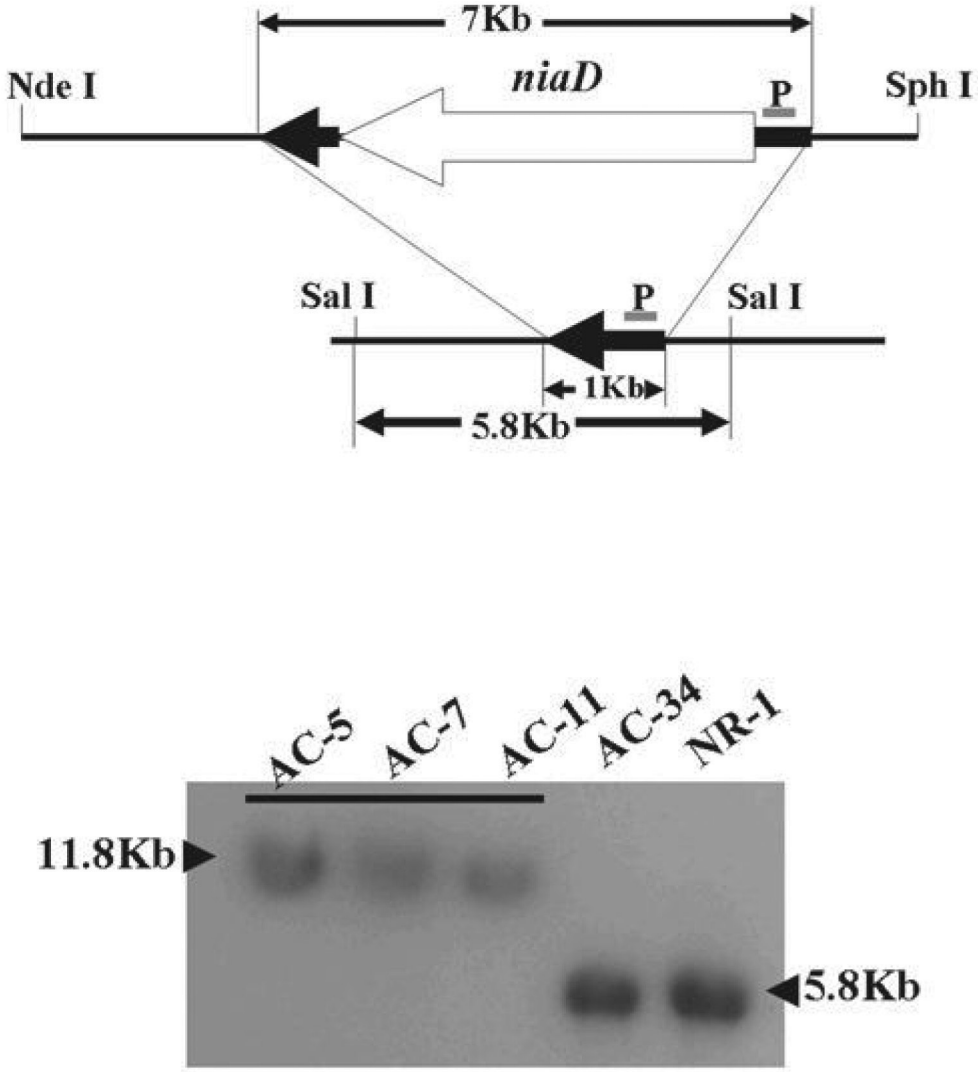

Fig. S1. Disruption of *A. parasiticus vb1*. (*Upper*) Schematic depicting integration of the SphI/NdeI linear fragment (10 kB) leading to replacement of wild-type *vb1* by the disruption construct carrying Δ*vb1*::*niaD*. (*Lower*) Southern blot analysis to confirm disruption of *vb1* in *A. parasiticus* strains AC-5, AC-7, and AC-11; P designates a 0.3-Kb *vb1* probe. Disruption of *vb1* was confirmed by the presence of an 11.8-Kb SalI fragment. A 5.8-Kb SalI fragment confirmed the presence of wild-type *vb1*.

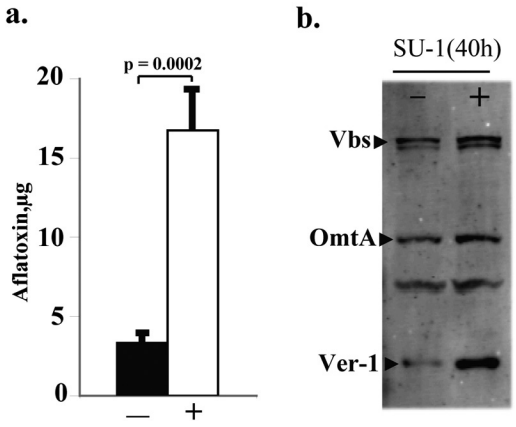

Fig. S2. Effect of Sortin3 treatment on SU-1. *A. parasiticus* SU-1 was grown in YES medium for 40 h and samples prepared for aflatoxin (*A*) and Western blot (*B*) analyses (see *Methods*). (*A*) Comparison of aflatoxin accumulation in the growth medium in the presence (+) or absence (−) of Sortin3. Aflatoxin was normalized to dry weight (see *Methods*). (*B*) Comparison of accumulation of aflatoxin enzymes Ver-1, Vbs, and OmtA in the presence (+) or absence (−) of Sortin3.

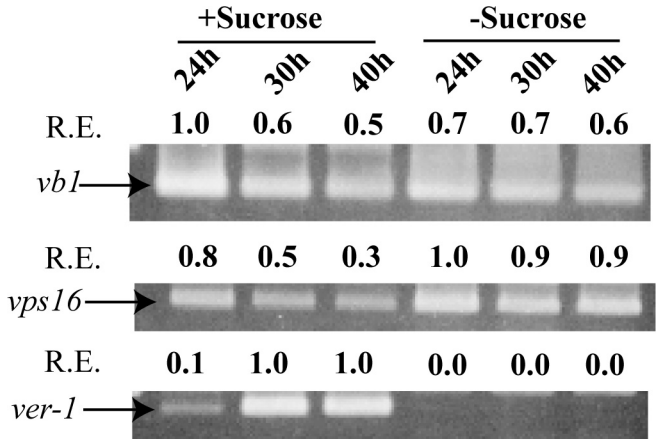

Fig. S3. Transcript levels (relative expression, RE) for *vb1*, *vps16*, and *ver-1* in SU-1 grown in aflatoxin-inducing (+sucrose) and -noninducing (−sucrose) media. *A. parasiticus* was grown in YES (+sucrose) or YEP (−sucrose) for 24, 30, or 40 h and samples prepared for RT-PCR analysis of transcript levels (RE). See *Methods* and Table 2 for description of growth conditions, RT-PCR analysis, and description of RE.

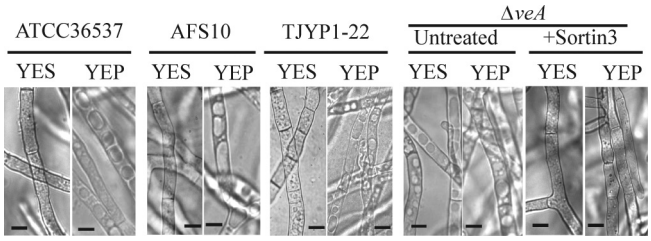

Fig. S4. Comparison of vesicle-vacuole morphology in *A. parasiticus* mutant strains carrying defects in aflatoxin synthesis. *A. parasiticus* strains ATCC36537, AFS10, TJYP1–22, and Δ*veA* were grown for 40 h in YES or YEP media and vesicle-vacuole morphology analyzed by light microscopy (see *Methods* and Fig. 1). Images were acquired under bright field. (Scale bar: 5 μm.)

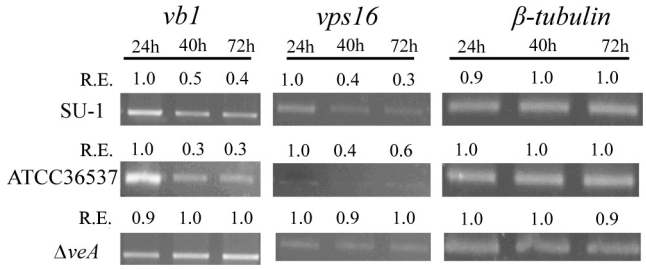

Fig. S5. Comparison of *vb1*and *vps16* transcript levels (RE) in *A. parasiticus* grown in aflatoxin-inducing (YES) medium. Fungal strains (SU-1, ATCC36537, and Δ*veA*) were grown for 24, 40, or 72 h in YES medium and RE calculated for *vb1*, *vps16*, and *ver-1*. See *Methods* and Table 2 for description of growth conditions, RT-PCR analysis, and description of RE.