Supporting Information

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SI Materials and Methods

***H. capsulatum* Insertional Mutagenesis.** *H. capsulatum* strain G217B was transformed with the *Agrobacterium tumefaciens* strain LBA1100 [the kind gift of Bruce Klein (University of Wisconsin, Madison) with permission from Paul Hooykas (Leiden University, Leiden, The Netherlands)] carrying the plasmid pRH5b (1) or pBTS4 (2). The coculturing of *H. capsulatum* with *A. tumefaciens* was done as previously described (2), and transformants were selected on HMM agarose plates supplemented with 200 μg/ml hygromycin B and 200 μg/ml cefotaxime. Appoximately 40,000 insertion mutants were screened visually at 37°C to identify a total of 15 filamentous strains. The locations of the insertions were mapped by digesting mutant genomic DNA with Hind*III* and performing inverse PCR (3). The primers OAS907 (5′-gtaagcgcccactccacatc-3′) and OAS908 (5′-gttgcgcagcctgaatggcg-3′) were used to amplify the left border of mutants generated with either plasmid. To map the right border of mutants generated with pBTS4, the primers OAS750 (5′-ggctccttcaacgttgcggt-3′) and OAS910 (5′-gcttccggctcgtatgttgtg-3′) were used. To map the right border of mutants generated with pRH5b, the primers OAS1322 (5′-gggcgacacggaaatgttgaatactc-3′) and OAS750 were used. Two mutants had insertions in the ORF of *RYP2* (M9 and M15), and one mutant had a 20-kb deletion at the site of insertion that contained the *RYP3* gene as well as two other predicted genes (F14) (Fig. S1).

Genomic DNA Preparation and Southern Analysis. Genomic DNA from *H. capsulatum* wild-type and mutant cells was isolated and subjected to Southern analysis as previously described (1).

RNA Interference Against *RYP2* and *RYP3*. *Integrated RNAi constructs.* *H. capsulatum* strain G217B was cocultured with *A. tumefaciens* strain LBA1100 containing either the vector control pVN69 (1), pRH15, or pRH17 (Fig. S2*A*). All plasmids are derived from pFANTAi4 (4). pRH15 contains a 487-bp hairpin repeat of the large exon in the *RYP2* coding sequence (Fig. S2*B*) generated by PCR amplification of *H. capsulatum* G217B genomic DNA with OAS1482 (5′-ggggacaagtttgtacaaaaaagcaggctcacattgaatcgagcggaacca-3′) and OAS1483 (5′-ggggaccactttgtacaagaaagctgggtagattcgcccatctgtgacat-3′). pRH17 contains a 498-bp hairpin repeat targeting one of the long exons in the *RYP3* coding sequence (Fig. S2*B*) which was PCR-amplified using OAS1484 (5′-ggggacaagtttgtacaaaaaagcaggctcacgacacctcattctacgtcc-3′) and OAS1485 (5′-ggggaccactttgtacaagaaagctgggtaaacgagccttcagtgcgaac-3′). Integrated RNAi strains were used to perform the experiments described in Figs. 3, 5, and 6.

Episomal RNAi constructs. *H. capsulatum* strain G217B*ura5*Δ was transformed by electroporation as described previously (5), with the vector control pCR186 [a kind gift of Chad Rappleye (Ohio State University) derived from pCR138 (6)], pRH27, or pRH29 (Fig. S2*A*). pRH27 contains the same 487 bp of the *RYP2* coding sequence as pRH15. The hairpin repeat segments were generated by PCR amplification of *H. capsulatum* G217B genomic DNA with tailed primers OAS1507 (5′-ggcgcgcccattgaatcgagcggaacca-3′) and OAS1508 (5′-ctcgaggattcgcccatctgtgacat-3′) and tailed primers OAS1509 (5′-actagtcattgaatcgagcggaacca-3′) and OAS1510 (5′-accggtgattcgcccatgtgtgacat-3′) followed by the insertion of each piece into the AscI-XhoI sites and the SpeI-AgeI sites of the pCR186 backbone, respectively. pRH29 was constructed in a similar manner, with the 498bp of *RYP3*, as in pRH17, as the hairpin repeat segments. The fragments were PCR amplified by the AscI and XhoI tailed primers OAS1499 (5′-ggcgcgcccgacacccattctacgtcc-3′) and OAS1500 (5′-ctcgagaacgagccttcagtgcgaac-3′) and SpeI and AgeI tailed primers OAS1501 (5′-actagtcgacacctcattctacgtcc-3′) and OAS1502 (5′-accggtaacgagccttcagtgcgaac-3′). Transformants were selected on HMM agarose plates grown at 37°C for 2 weeks. Episomal RNAi strains were used to perform the experiments shown in Fig. 1. To screen for cells that lost the episomal RNAi plasmids, these strains were grown on HMM agarose plates supplemented with 100 μg/ml uracil and incubated at 37°C with 5% CO_2 for 2 weeks. Colonies were patched onto HMM agarose plates in the absence of uracil supplementation to identify cells that no longer contained the *URA5* marker present on the episomal plasmid. Genomic DNA was obtained and screened by PCR using primers that recognize the episomal plasmid to confirm that plasmid sequences were not present [PCR was performed with OAS249 (5′-ggcgattaagttgg-3′) and OAS1488 (5′-cacatgaagcagcacgactt-3′)].

Microscopy. All images were obtained by using a Zeiss Axiovert 200 microscope with either a DIC or Phase objective.

RNA Preparation and Northern Analysis. Total RNA from *H. capsulatum* wild-type and mutant cells was isolated and subjected to Northern analysis as previously described (1). Probes were amplified from G217B genomic DNA with the following primers: *RYP2* – OAS1964 (5′-ccctccacctattatccaac-3′) and OAS1976 (5′-agaactgtacgggcttcctt-3′); *RYP3* – OAS1977 (5′-gaacttcgtcaacgttggca-3′) and OAS1978 (5′-gaaaaaggtgaaggaatgac-3′); and *GAPDH* – OAS1120 (5′-accaacaggcctacatgctc-3′) and OAS1121 (5′-tactgctcgctgttgattgc-3′).

Mapping of *RYP2, RYP3,* and *VEA1* Transcripts. Total RNA was isolated from *H. capsulatum* G217B and treated with DNase*I* (Promega). To identify the 5′ and 3′ ends of the *VEA1*, *RYP2*, and *RYP3* transcripts, the FirstChoice RLM-RACE Kit (Ambion) was used per the manufacturer's instructions. For 5′ RACE, the following primers were used: *RYP2* – OAS1970 (5′-accttgaaagccatgtggac-3′) and OAS1971 (5′-aacgacttcatctctcgagc-3′); *RYP3* – OAS1972 (5′-tatgcggtttgcgggtatcc-3′) and OAS1973 (5′-gacgtagaatgaggtgtcga-3′); and *VEA1* – OAS1974 (5′-cttgggctccttcattcct-3′) and OAS1975 (5′-cctgtcaggacaggccatga-3′). For 3′ RACE, the following primers were used: *RYP2* – OAS1958 (5′-aaggctatcctactgctgga-3′) and OAS1959 (5′-atgtcacagatgggcgaatc-3′); *RYP3* – OAS1960 (5′-tcaatgttggttcggcacct-3′) and OAS1961 (5′-gacagtcacatggctccaaa-3′); and *VEA1* – OAS1962 (5′-tacgaccaatcgaagtcctc-3′) and OAS1963 (5′-cacctccagcagcaatagca-3′). The transcript coding sequences were amplified from reverse transcribed cDNA using the following primer pairs: *RYP2* – OAS1964 (5′-ccctccacctattatccaac-3′) and OAS1965 (5′-gattcgcccatctgtgacat-3′); *RYP3* – OAS1966 (5′-ccatggatcacctccagcat-3′) and OAS1967 (5′-tttggagccatgtgactgtc-3′); and *VEA1* – OAS1968 (5′-gagactgagcataccatgtc-3′) and OAS1969 (5′-tgctattgctgctggaggtg-3′). All amplified products were cloned by using the TOPO-TA system (Stratagene) and sequenced with M13-forward and M13-reverse primers.

Quantitative RTPCR (qRTPCR). Total RNA from *H. capsulatum* G217B and control, *RYP2*, and *RYP3* RNAi strains was isolated and subjected to qRTPCR as previously described (5). The transcripts were amplified with the following primers: *RYP2* – OAS1942 (5′-cggctcgagagatgaagtcgtt-3′) and OAS1943 (5′-aagtgtacgggcttccttccg-3′); *RYP3* – OAS1944 (5′-ccaaaggccaagat-

ggagaagg-3′) and OAS1945 (5′-ggaaatgagaggaagggggaaaga-3′); *RYP1* – OAS1057 (5′-acccttgcagcttacaacct-3′) and OAS1058 (5′-tccgtccatcgcttaatacc-3′); and *TEF1* – OAS1687 (5′-tcgatgccattgacgccattgaac-3′) and OAS1689 (5′-gagaacttgcaagcaagttgggca-3′). All intensity signals from the *RYP* genes were normalized to the *TEF1* expression levels.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation was performed as previously described (1). PCR primers were designed at ≈500-bp intervals across the 6-kb intergenic region immediately upstream of the *RYP2* ORF (Table S2). PCR primers were designed at ≈150-bp intervals across the 1.2-kb intergenic region immediately upstream of the *RYP3* ORF (Table S2). *TEF1* was used as the reference ORF (Table S2).

Spore Viability. ***Spore harvest.*** Spores were obtained from the control, *RYP2*, and *RYP3* RNAi strains by first growing a dense culture in HMM liquid for 2 weeks at either room temperature (for the control) or 37°C (for the RNAi mutants). To initiate spore formation using standard conditions, 1 ml of culture was inoculated onto 15 cm Sabouraud agar plates. The plates were allowed to grow in a closed container with minimal exposure to light at room temperature for 4 weeks. Plates were flooded with PBS, and spores were harvested by gentle agitation with a glass rod. The spore suspensions were filtered through glass wool to minimize contaminating filaments and collected by centrifugation at 2,000 × *g* for 10 min at 4°C. Spores were stored at 4°C. ***Viability testing.*** Spores from the control, *RYP2*, and *RYP3* RNAi strains were stained with lactophenol blue and quantified immediately after harvesting. BHI agar plates supplemented with 10% sheep's blood were inoculated with spores at regular intervals over the course of 7 days, and incubated at 30°C. The percent viability was calculated as a ratio of the number of CFUs growing on the BHI plates to the number of spores inoculated initially. The percent viability of the each strain was normalized to the viability of the control strain on Day 0.

Host–Cell Interactions. ***Macrophage infections.*** Murine bone marrow-derived macrophages (BMDMs) were isolated and differentiated as described previously (5). The macrophages were seeded into wells of 24-well tissue culture plates containing coverslips for use in staining. The BMDMs were infected with spores from the control, *RYP2*, or *RYP3* RNAi strains at an MOI of 0.1. The infected macrophage plates were spun for 5 min at 500 × *g* at room temperature. After 2 h incubation, the macrophages were washed twice with BMM (5) and then incubated in fresh BMM. At 48 and 72 h postinfection, the BMM was aspirated and the macrophages were fixed in 3.7% formaldehyde for 1 min. The macrophages were washed twice with water and stored in PBS at 4°C.

Infection Staining. The *H. capsulatum* cells and macrophage monolayer were stained with Periodic Acid-Schiff base (PAS) (Sigma). The cells were incubated with periodic acid for 5 min and then washed 5 times with water. Schiff base was added to the cells and incubated for 5–10 min. Cells were then washed for 5 min under continuously running water. The macrophage nuclei were visualized by staining with methyl green (Vector Labs) for 5 min and then washed for 1 min under running water. The coverslips were removed from the wells and mounted onto glass slides with Permount (Fisher).

Homology Analysis. The protein sequences used to generate the phylogenetic tree and alignments are as follows. Hc: *H. capsulatum* Vea1 EU543494, Ryp2 EU543495, Ryp3 EU543496; Bd: *B. dermatitidis* Vea1, Ryp2, and Ryp3 were identified by TBLASTN search of the Hc protein sequences against the *B. dermatitidis* genome assembly version 3.0 (http://genome.wustl.edu/); Pb: *P. brasiliensis* Vea1, Ryp2, and Ryp3 were identified by TBLASTN search of the Hc protein sequences against the version 1 of the Pb03 genome assembly (www.broad.mit.edu/annotation/fgi/); Ci: *C. immitis* Vea1 CIMG_06878.2, Ryp2 CIMG_01530.2, Ryp3 CIMG_09962.2 (www.broad.mit.edu/annotation/fgi/); An: *A. nidulans* VeA AF109316, VosA DQ856465, VelB EF540815; Af: *A. fumigatus* VeA XM747526, VosA EF544392, VelB Afu1g01970 (www.broad.mit.edu/annotation/fgi/); Mg: *M. grisea* VeA MGG08556.4, VosA MGG00617.4, VelB MGG01620.4 (www.broad.mit.edu/annotation/fgi/); Nc: *N. crassa* VE-1 NCU01731.2, VosA NCU05964.3, VelB NCU02775.3 (www.broad.mit.edu/annotation/fgi/); Fv: *F. verticillioides* VeA DQ274059, VelB FVEG_01498.3 (www.broad.mit.edu/annotation/fgi/); and Ac: *A. chrysogenum* VeA AM410093. The sequences were aligned with clustalX and the phylogenetic tree was generated using NJ Plot (7).

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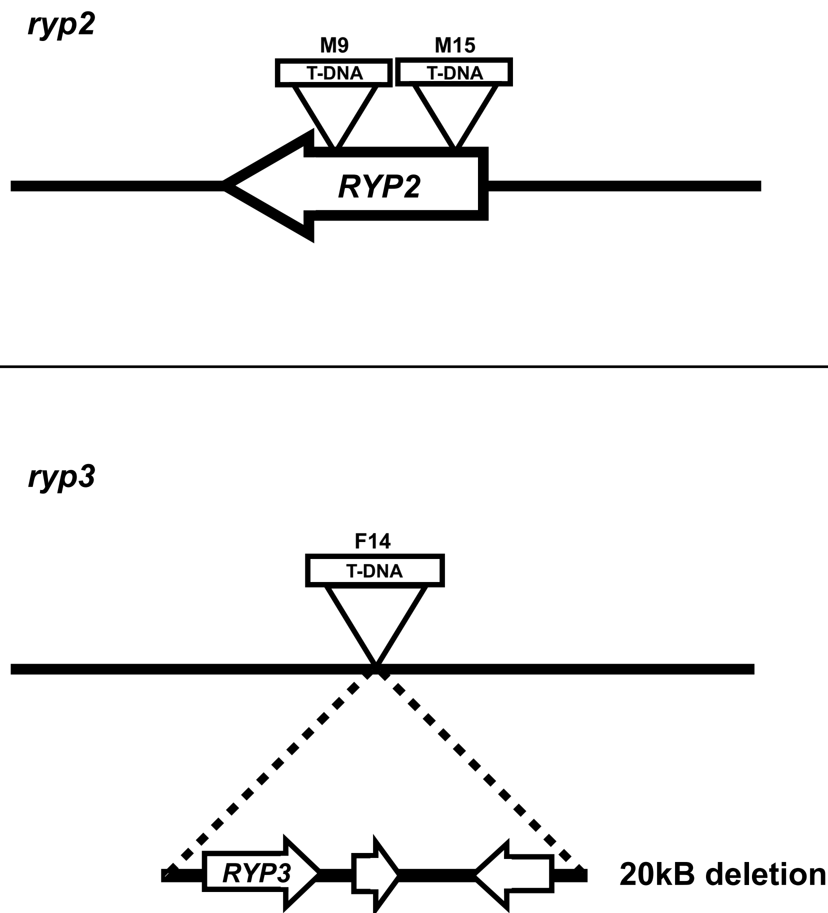

Fig. S1. Schematic representation depicting the position of the T-DNA insertion events in the *ryp2* and *ryp3* mutants obtained from the screen. Two independent T-DNA insertions mutants, M9 and M15, were located within the *RYP2* ORF. F14 contained a 20-kb deletion that eliminated the *RYP3* ORF as well as two unrelated genes, one of which represents the remnant of a retrotransposon.

Fig. S2. Diagrams of the constructs used to produce the RNAi strains used in this study. (*A*) Schematics of the episomal RNAi plasmids pRH15 and pRH17 based on pCR186. pRH27 and pRH29 are the integrating RNAi plasmids based on the pFANTAi4 backbone. See *Materials and Methods* for details. (*B*) Diagram of the 5′ to 3′ exon (rectangle) and intron (line) structure of *RYP2* and *RYP3*. Gray bar indicates the ≈500-bp sequence used for RNAi.

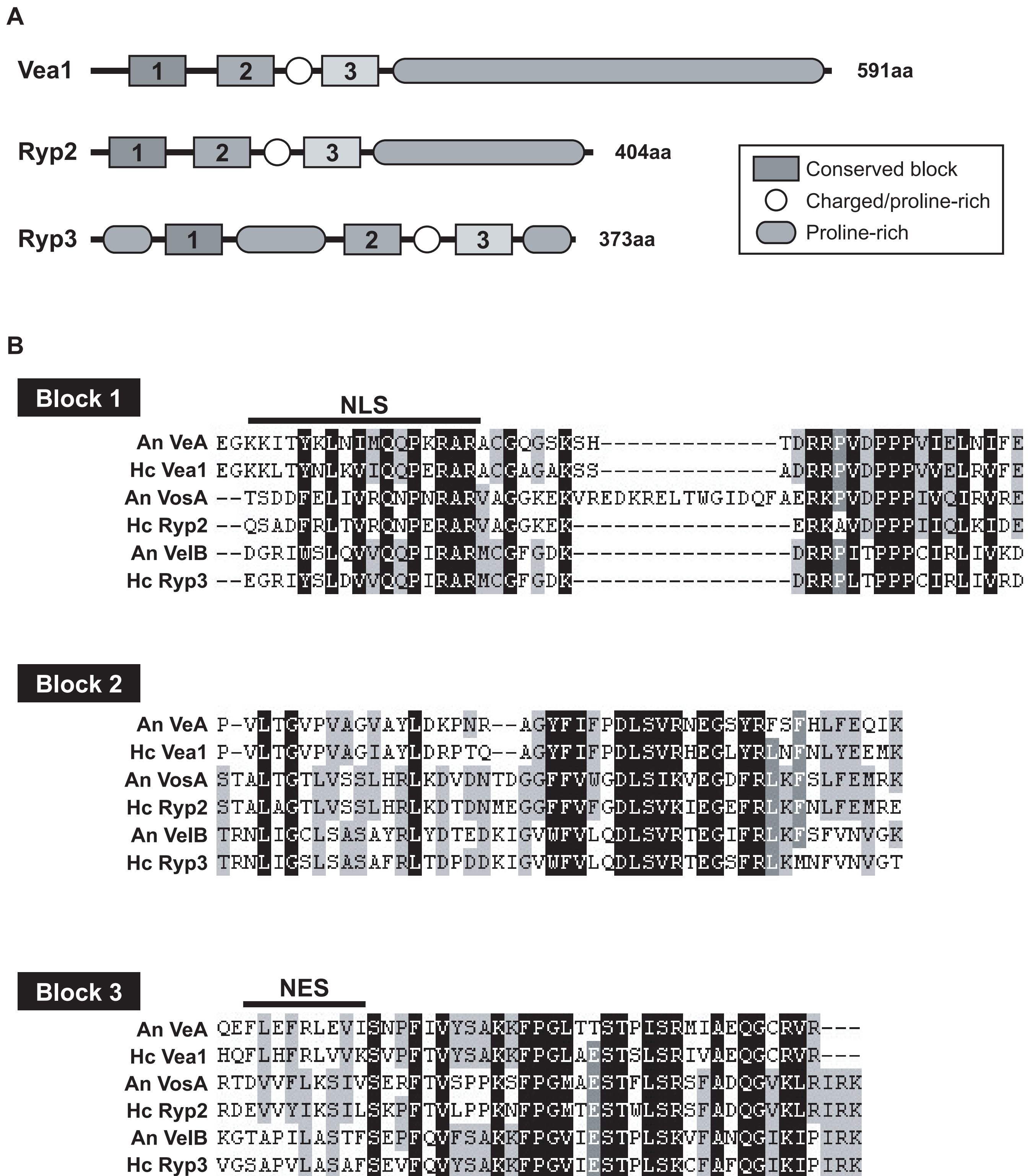

Fig. S3. Ryp2, Ryp3, and VeA are highly conserved. (*A*) Diagram representing the protein domain structure of the VeA family members in *H. capsulatum*. Blocks 1, 2, and 3 are shown in more detail in (*B*). (*B*) Alignments of the highly conserved regions of the VeA family members in *H. capsulatum* and *A. nidulans*. Putative NLS and NES in *A. nidulans* VeA are indicated (8, 9).

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Table S1. Relative gene expression by qRTPCR

Sample	*RYP2*		*RYP3*		*RYP1*	
	Relative expression	SD	Relative expression	SD	Relative expression	SD
RT control filaments	1	0.341	1	0.414	1	0.353
ryp2–1	0.268	0.061	0.330	0.145	1.532	0.680
ryp2–2	0.280	0.064	0.563	0.255	2.012	0.736
ryp3–1	0.513	0.135	0.214	0.159	5.351	1.606
ryp3–2	0.165	0.050	0.219	0.100	0.791	0.408
ryp1	2.274	1.365	0.344	0.163	nd	nd
37°C control yeast	36.979	11.010	3.301	1.089	23.244	6.320
ryp2–1	0.107	0.040	0.164	0.090	0.752	0.366
ryp2–2	0.054	0.018	0.121	0.048	0.322	0.149
ryp3–1	0.066	0.022	0.049	0.025	0.178	0.088
ryp3–2	0.028	0.008	0.079	0.034	0.424	0.194
ryp1	0.468	0.206	0.229	0.112	nd	nd

nd, not determined; SD, standard deviation.

Table S2. Primers used for ChIP analysis

RYP2		*RYP3*		*TEF1*	
Primer	Sequence 5′ to 3′	Primer	Sequence 5′ to 3′	Primer	Sequence 5′ to 3′
OAS1738	tttggttcgtggggtaattt	OAS1852	tcaacttcaccgtgtcctacc	OAS1768	catcaagcccggtatggtc
OAS1739	agtacacagagttgggcttgc	OAS1853	acagcgttcctgaccttgtt	OAS1769	agggtaaccagcctggagtt
OAS1740	tccaagatctgtgaagcaggt	OAS1854	catcacagcagccgcatc		
OAS1741	caatgagatcccacagtcca	OAS1855	gccggatgaaacaaaacaac		
OAS1742	agcgatcatgtaaagggaaca	OAS1856	tccaaacctcccagactttc		
OAS1743	cgctaaatacttctggctggt	OAS1857	caagtacaggccctttcgtc		
OAS1744	cctcggctggaaagtaacaa	OAS1858	aggcccgttttcttcttgag		
OAS1745	gcaatggatgacaaacgaaa	OAS1859	aaagagaaagagaaacacctgacg		
OAS1746	agatggaaaattgggaatcg	OAS1860	agatctgtgccgtgcaagt		
OAS1747	ttccaaatatcaagtcgacagc	OAS1861	gtgcaaaacgggagaaaaag		
OAS1748	acaatgggcccgttttatta	OAS1862	gatatatccattcgttgcagttg		
OAS1749	ctcatgaggtcattaacattcctaga	OAS1863	tgatatacccctatccgaggaa		
OAS1750	tgatgcaccgataggtttga	OAS1864	gcgttcaccggatatgattt		
OAS1751	ttgcacctactagcgccttt	OAS1865	tatcctgcgttcttcgcttc		
OAS1752	agcacaagagcgaaaaccat	OAS1866	gtccggacaaatcaggtgag		
OAS1753	atccaactcaagtcgtcgaaa	OAS1867	tcccccgaaaagatatggat		
OAS1754	cattgtgcttacggctttca	OAS1868	gatggttaacggctttattatttgtt		
OAS1755	agagccattcgaagttgagg	OAS1869	tctaagtgtcaaatcacacaacacc		
OAS1756	gtacgacgcccttgtttctc				
OAS1757	tctcaagctctccggtgatt				
OAS2088	cataggtcgaatttgggaaca				
OAS2089	ggatcgaggaaagtggaatg				
OAS2090	tggcttcttagataggacctgct				
OAS2091	ggttaacgaggctggttcata				