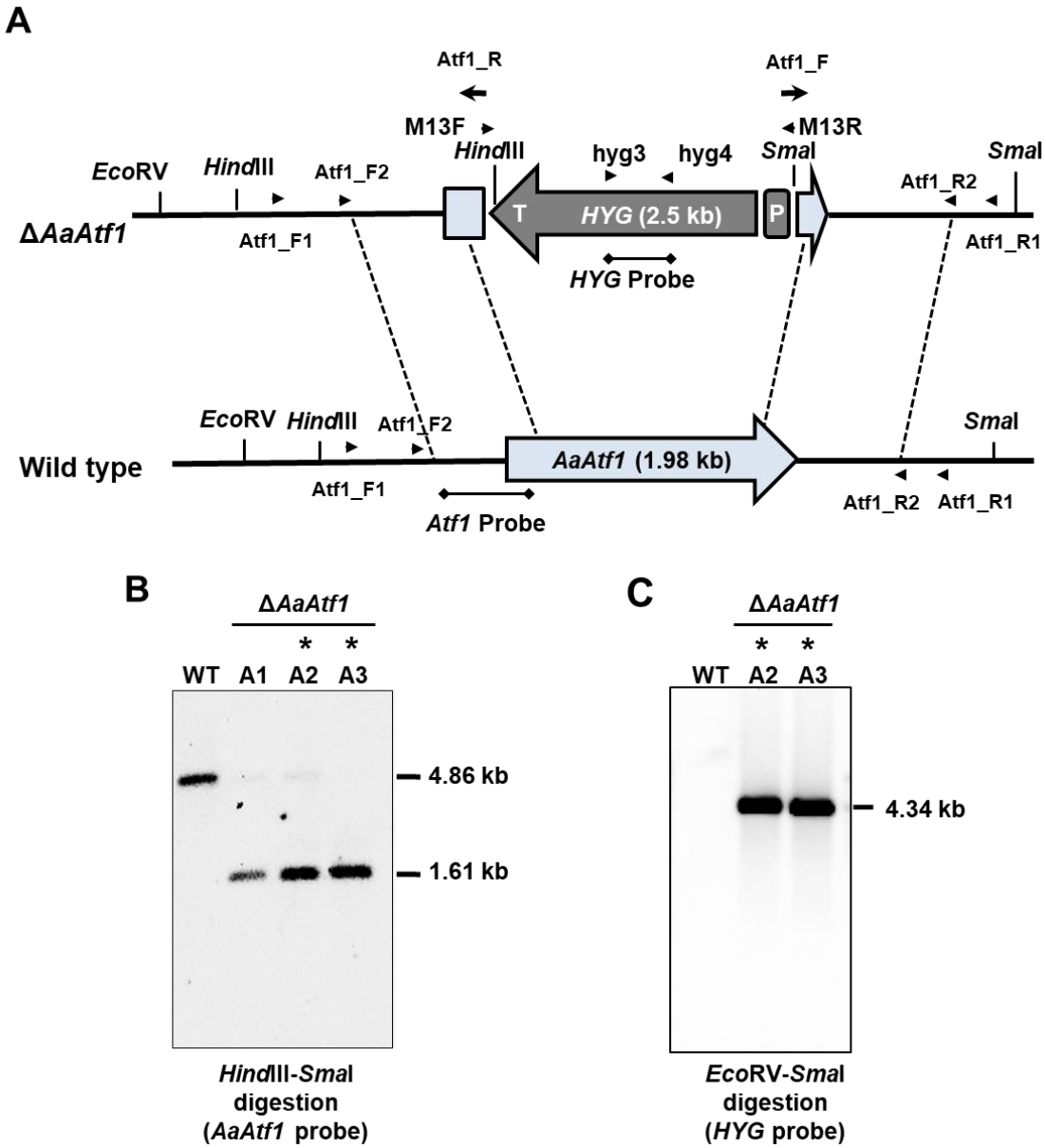

Figure S1. Deletion of the *AaAtf1* gene using a split marker approach in the tangerine pathotype of *A. alternata*. (**A**) Schematic depiction of gene disruption of *AaAtf1* via homologous recombination using truncated but overlapping DNA fragments of a hygromycin phosphotransferase-coding gene (*HYG*) under control by the *Aspergillus nidulans trpC* promoter (P) and terminator (T). Split marker fragments were amplified with the primers as indicated. (**B**)(**C**) Southern-blot analysis of genomic DNA prepared from wild-type (WT), three putative *AaAtf1* mutants (A1, A2, and A3). Fungal DNA was cleaved with *Hind*III and *Sma*I (**B**), or *Eco*RV and *Sma*I (**C**), electrophoresed, blotted to a nylon membrane and hybridized with an *AaAtf1* (**B**) or a *HYG* (**C**) probe. The Δ*AaAtf1* mutants (A2 and A3) used in this research are indicated by asterisks.

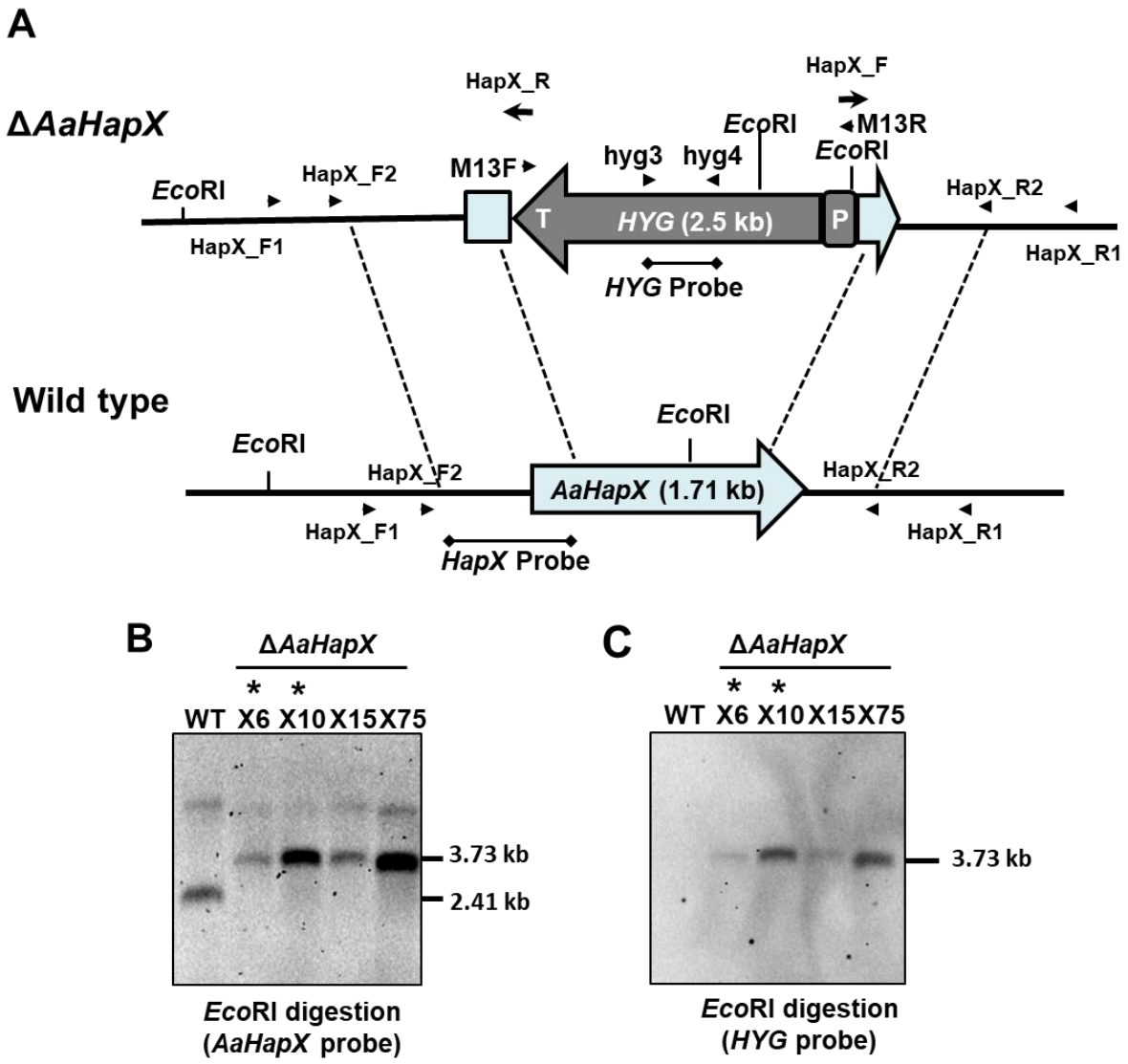

Figure S2. Deletion of the *AaHapX* gene using a split marker approach in the tangerine pathotype of *A. alternata*. (**A**) Schematic depiction of gene disruption of *AaHapX* via homologous recombination using truncated but overlapping DNA fragments of a hygromycin phosphotransferase-coding gene (*HYG*) under control by the *Aspergillus nidulans trpC* promoter (P) and terminator (T). Split marker fragments were amplified with the primers as indicated. (**B**)(**C**) Southern-blot analysis of genomic DNA prepared from wild-type (WT) and four putative Δ*AaHapX* mutants (X6, X10, X15, and X75). Fungal DNA was cleaved with *Eco*R1, electrophoresed, blotted to a nylon membrane, and hybridized with an *AaHapX* (**B**) or a *HYG* (**C**) probe. The Δ*AaHapX* mutants (X6 and X10) used in this research are indicated by asterisks.

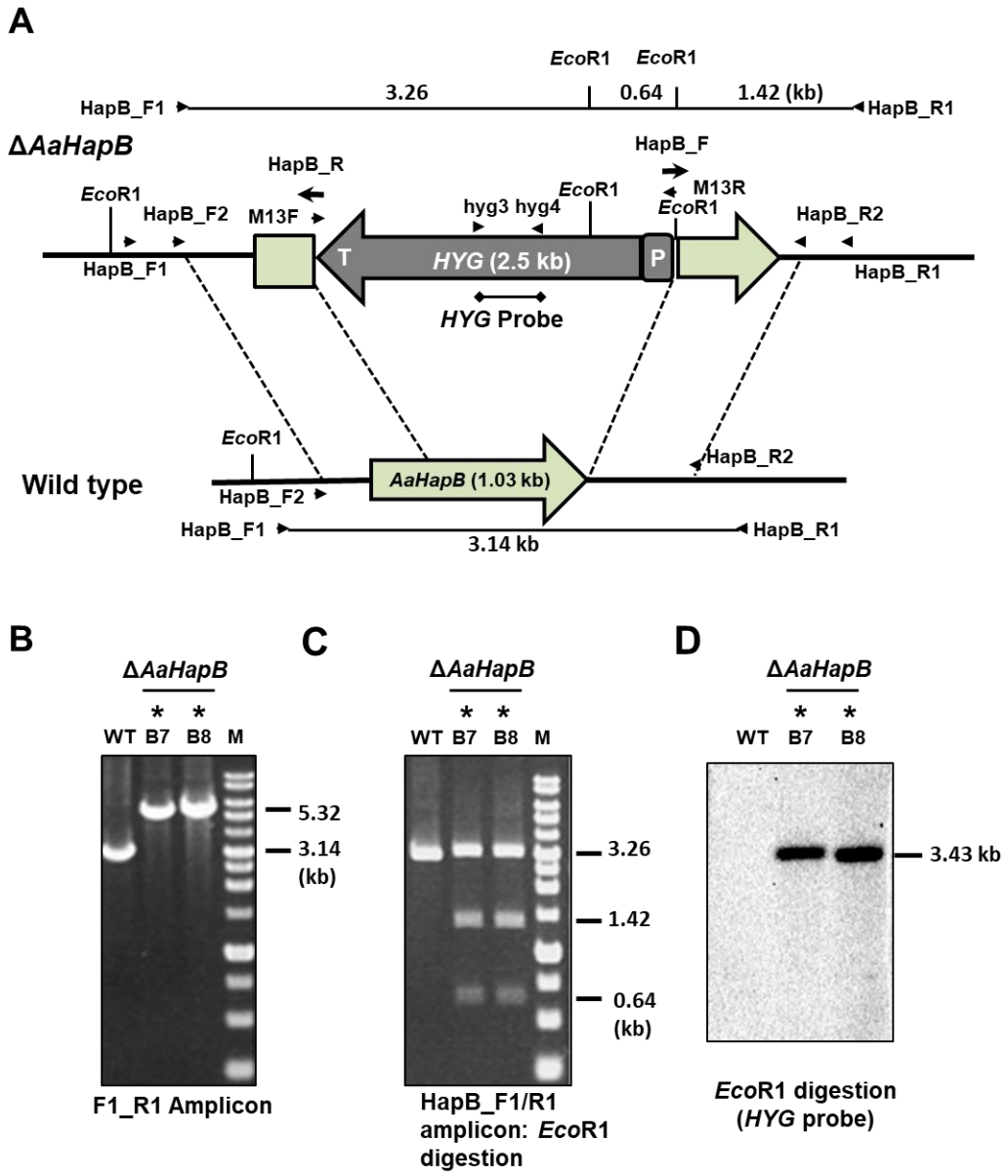

Figure S3. Deletion of the *AaHapB* gene using a split marker approach in the tangerine pathotype of *A. alternata*. (**A**) Schematic depiction of gene disruption of *AaHapB* via homologous recombination using truncated but overlapping DNA fragments of a hygromycin phosphotransferase-coding gene (*HYG*) under control by the *Aspergillus nidulans trpC* promoter (P) and terminator (T). Split marker fragments were amplified with the primers as indicated. (**B**) Gel image showing DNA fragments amplified from wild-type (WT) and two putative Δ*AaHapB* mutants (B7 and B8) with the primers HapB_F1/HapB_R1. (**C**) Restriction enzyme length polymorphism patterns of the F1/R1 amplicons after *Eco*R1 digestion. (**D**) Southern-blot analysis of genomic DNA prepared from wild-type (WT) and two transformants (B7 and B8). Fungal DNA was cleaved with *Eco*RI, electrophoresed, blotted to a nylon membrane, and hybridized with a *HYG* probe. The Δ*AaHapB* mutants (B7 and B8) used in this research are indicated by asterisks.

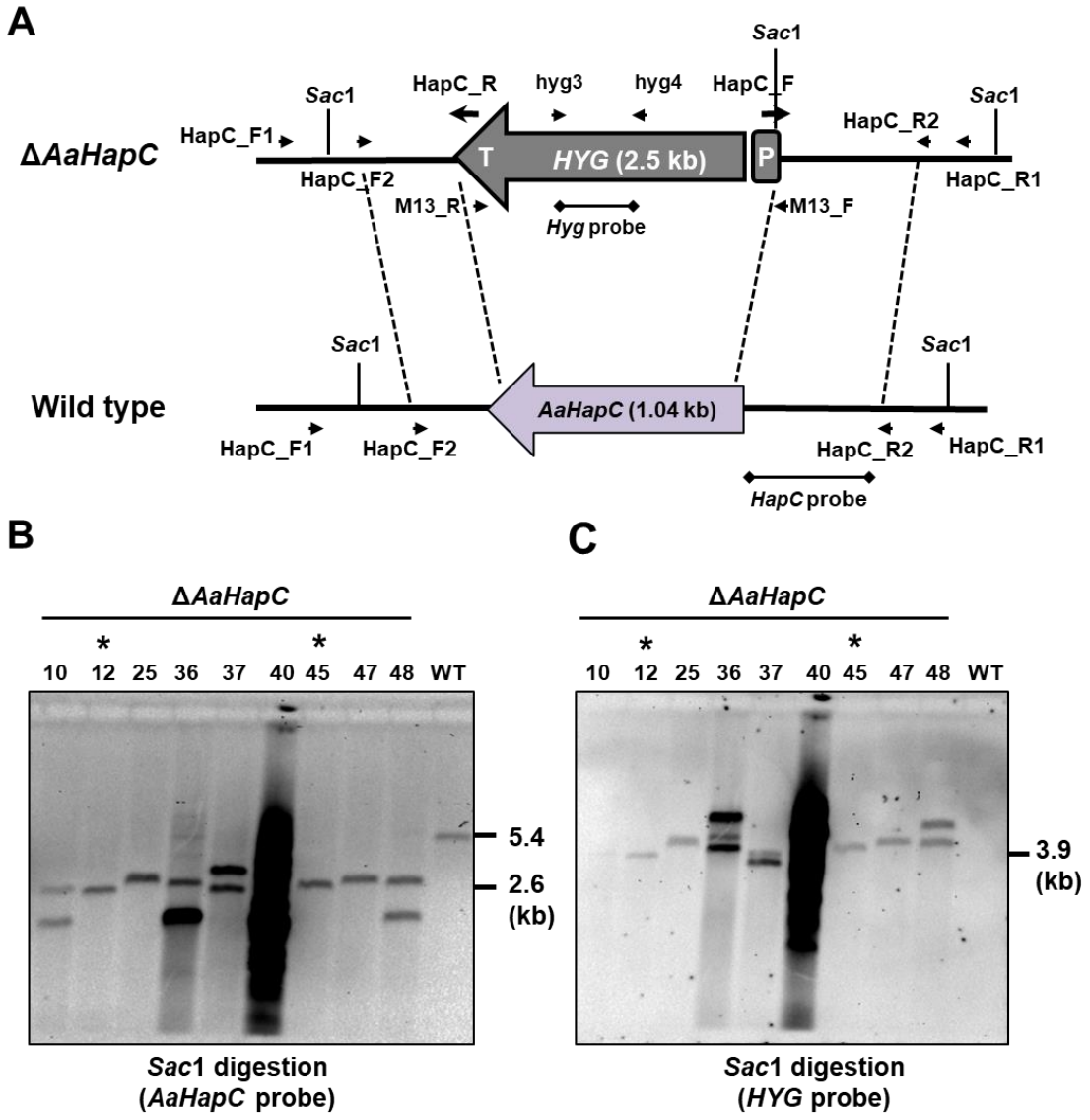

Figure S4. Deletion of the *AaHapC* gene using a split marker approach in the tangerine pathotype of *A. alternata*. (**A**) Schematic depiction of gene disruption of *AaHapC* via homologous recombination using truncated but overlapping DNA fragments of a hygromycin phosphotransferase-coding gene (*HYG*) under control by the *Aspergillus nidulans trpC* promoter and terminator. Split marker fragments were amplified with the primers as indicated. (**B**)(**C**) Southern-blot analysis of genomic DNA prepared from wild-type (WT) and nine transformants (10, 12, 25, 36, 37, 40, 45, 47, and 48). Fungal DNA was cleaved with *Sac*I, electrophoresed, blotted to a nylon membrane, and hybridized with an *AaHapB* (**B**) or a *HYG* (**C**) probe. The Δ*AaHapC* mutants (C12 and C45) used in this research are indicated by asterisks.

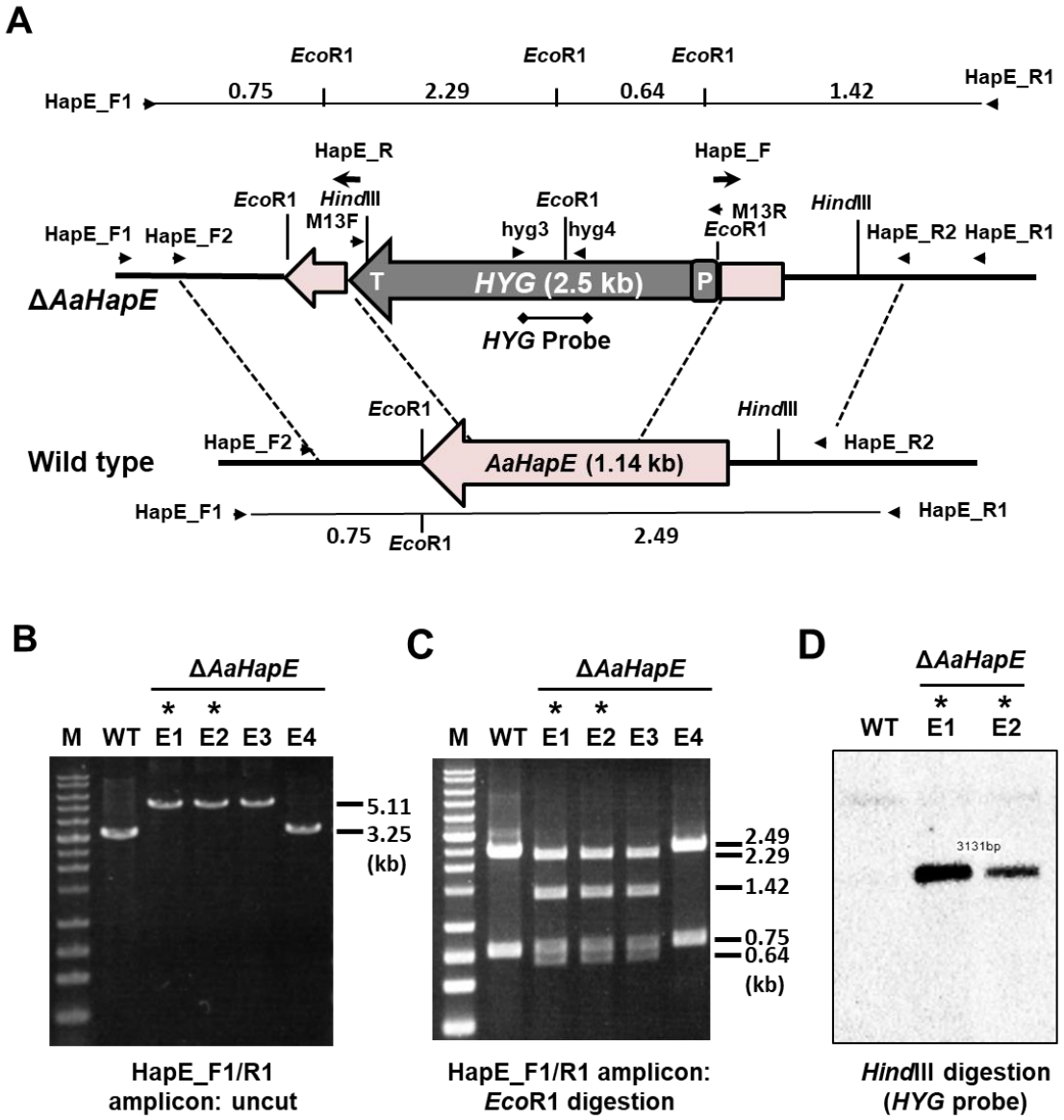

Figure S5. Deletion of the *AaHapE* gene using a split marker approach in the tangerine pathotype of *A. alternata*. (**A**) Schematic depiction of gene disruption of *AaHapE* via homologous recombination using truncated but overlapping DNA fragments of a hygromycin phosphotransferase-coding gene (*HYG*) under control by the *Aspergillus nidulans trpC* promoter (P) and terminator (T). Split marker fragments were amplified with the primers as indicated. (**B**) Gel image showing DNA fragments amplified from wild-type (WT), four transformants (E1, E2, E3, and E4) with the primers HapE_F1/HapE_R1. (**C**) Restriction enzyme length polymorphism patterns of the F1/R1 amplicons after *Eco*R1 digestion. (**D**) Southern-blot analysis of genomic DNA prepared from wild-type (WT) and two transformants (E1 and E2). Fungal DNA was cleaved with *Hind*III, electrophoresed, blotted to a nylon membrane, and hybridized with a *HYG* probe. The Δ*AaHapE* mutants (E1 and E2) used in this research are indicated by asterisks.

Atf1

Distance

		1	2	3	4	5	6	7	8
Aspergillus fumigatus Af293	1		0.21	0.31	0.61	0.61	0.70	0.66	0.64
Aspergillus niger ATCC 1015	2	73.94		0.30	0.65	0.65	0.71	0.65	0.65
Penicillium digitatum PHI26	3	62.81	65.74		0.59	0.60	0.61	0.66	0.64
Fusarium oxysporum f. sp. *lycopersici* 4287	4	43.31	44.54	44.17		0.07	0.36	0.70	0.68
Fusarium graminearum PH-1	5	43.23	44.64	44.25	92.61		0.39	0.69	0.67
Magnaporthe oryzae 70-15	6	39.60	41.68	43.72	64.05	62.52		0.78	0.77
Alternaria alternata EV-MIL-31	7	40.13	41.71	39.80	41.02	41.46	37.42		0.00
Alternaria alternata SRC1IrK2f	8	40.87	43.22	40.41	41.81	42.25	38.40	90.81	

Identity (%)

Conserved Motif

Aft1_OSA: Aft1 osmotic stress response (OSM) domain

Aft1_HRA domain

Aft1_HRR domain

bZIP_ATF2 domain

Dimer interface

Coiled coil DNA binding domain

Name	*p*-value	Motif Locations
Aspergillus fumigatus Af293	6.08e-231	
Aspergillus niger ATCC 1015	1.11e-230	
Penicillium digitatum PHI26	4.10e-223	
Fusarium oxysporum f. sp. *lycopersici* 4287	7.86e-220	
Fusarium graminearum PH-1	1.80e-224	
Magnaporthe oryzae 70-15	4.70e-198	
Alternaria alternata EV-MIL-31	9.45e-219	
Alternaria alternata SRC1IrK2f	8.12e-219	

Figure S6. Pairwise comparison and alignment of conserved motifs of the AaAtf1 sequence with fungal Atf1 orthologs. A pairwise comparison of the sequence was performed using CLC Genomic Workbench 9.5.1 to calculate genetic distance using the jukes-cantor model and percent identity. Conserved domains were predicted by NCBI and the MEME Suite server.

HapX

Distance

		1	2	3	4	5	6	7	8
Aspergillus fumigatus Af293	1		0.21	0.35	0.88	0.84	0.86	1.26	1.26
Aspergillus niger ATCC 1015	2	76.85		0.33	0.86	0.80	0.89	1.20	1.20
Penicillium digitatum PHI26	3	65.00	65.70		0.90	0.86	0.91	1.32	1.32
Fusarium oxysporum f. sp. *lycopersici* 4287	4	24.41	25.28	24.61		0.13	0.68	1.22	1.22
Fusarium graminearum PH-1	5	30.97	32.25	31.38	72.06		0.68	1.23	1.23
Magnaporthe oryzae 70-15	6	29.10	28.23	28.76	37.99	45.26		1.34	1.35
Alternaria alternata EV-MIL-31	7	17.73	18.38	17.55	15.96	19.43	17.52		0.00
Alternaria alternata SRC1IrK2f	8	17.73	18.38	17.55	15.96	19.43	17.39	99.81	

Identity (%)

Conserved Motif

Hap4-like CCAAT-binding complex binding domain (CBC-BD)

bZIP_YAP: Basic leucine zipper (bZIP) domain of Yeast Activated Protein (YAP)

Coiled coil DNA binding domain

Dimer interface

CCR A $CX_2CX_{4-5}CXC$ B $CGXCX_5CXC$ C $CGFCX_{5-6}CXC$ D CX_2CX_9C E $CCX_{7-8}CC$

Name	*p*-value	Motif Locations
Aspergillus fumigatus Af293	2.53e-222	
Aspergillus niger ATCC 1015	6.98e-224	
Alternaria alternata EV-MIL-31	7.42e-179	
Alternaria alternata SRC1IrK2f	7.42e-179	
Fusarium oxysporum f. sp. *lycopersici* 4287	1.47e-138	
Fusarium graminearum PH-1	7.18e-171	
Magnaporthe oryzae 70-15	8.37e-146	
Penicillium digitatum PHI26	3.44e-207	

Figure S7. Pairwise comparison and alignment of conserved motifs of the AaHapX sequence with fungal HapX orthologs. A pairwise comparison of the sequence was performed using CLC Genomic Workbench 9.5.1 to calculate genetic distance using the jukes-cantor model and percent identity. Conserved domains containing cysteine (C) residues were predicted by NCBI and the MEME Suite server.

HapB

Distance

		1	2	3	4	5	6	7	8
Aspergillus fumigatus Af293	1		0.12	0.31	0.68	0.68	0.62	0.63	0.67
Aspergillus niger ATCC 1015	2	68.02		0.25	0.60	0.60	0.53	0.53	0.57
Penicillium digitatum PHI26	3	71.62	59.73		0.69	0.69	0.63	0.63	0.70
Alternaria alternata EV-MIL-31	4	44.62	36.41	43.92		0.00	0.65	0.71	0.72
Alternaria alternata SRC1IrK2f	5	44.62	36.41	43.92	100.00		0.65	0.71	0.72
Fusarium oxysporum f. sp. *lycopersici* 4287	6	41.58	36.74	41.33	39.67	39.67		0.13	0.41
Fusarium graminearum PH-1	7	41.84	37.19	42.13	38.32	38.32	86.64		0.38
Magnaporthe oryzae 70-15	8	41.22	35.34	40.16	38.74	38.74	61.71	63.01	

Identity (%)

Conserved Motif

CBFB_NFYA: CCAAT-binding domain, transcription factor subunit B (CBF-BNF-YA)

Name	*p*-value	Motif Locations
Aspergillus fumigatus Af293	1.58e-244	
Aspergillus niger ATCC 1015	5.90e-204	
Penicillium digitatum PHI26	3.97e-231	
Alternaria alternata EV-MIL-31	2.68e-188	
Alternaria alternata SRC1IrK2f	2.68e-188	
Fusarium oxysporum f. sp. *lycopersici* 4287	2.77e-180	
Fusarium graminearum PH-1	8.08e-179	
Magnaporthe oryzae 70-15	2.11e-135	

Figure S8. Pairwise comparison and alignment of conserved motifs of the AaHapB sequence with fungal HapB orthologs. A pairwise comparison of the sequence was performed using CLC Genomic Workbench 9.5.1 to calculate genetic distance using the jukes-cantor model and percent identity. Conserved domains were predicted by NCBI and the MEME Suite server.

HapC

Distance

		1	2	3	4	5	6	7	8
Aspergillus fumigatus Af293	1		0.16	0.26	0.54	0.47	0.44	0.45	0.38
Aspergillus niger ATCC 1015	2	79.82		0.26	0.50	0.44	0.45	0.46	0.39
Penicillium digitatum PHI26	3	61.00	63.75		0.53	0.48	0.48	0.50	0.42
Fusarium oxysporum f. sp. *lycopersici* 4287	4	39.93	40.82	35.03		0.14	0.35	0.46	0.41
Fusarium graminearum PH-1	5	51.06	56.00	48.00	63.71		0.34	0.39	0.34
Magnaporthe oryzae 70-15	6	53.22	56.50	48.59	49.60	67.31		0.41	0.34
Alternaria alternata EV-MIL-31	7	50.22	51.79	44.94	38.76	54.63	53.00		0.12
Alternaria alternata SRC1IrK2f	8	44.54	46.40	39.68	35.89	49.29	49.06	72.87	

Identity (%)

Conserved Motif

CBFD_NFYB_HMF: Histone-like transcription factor (CBF/NF-Y), H4 superfamily

Name	*p*-value	Motif Locations
Aspergillus fumigatus Af293	3.57e-153	
Aspergillus niger ATCC 1015	1.32e-167	
Penicillium digitatum PHI26	4.73e-157	
Fusarium oxysporum f. sp. *lycopersici* 4287	2.99e-116	
Fusarium graminearum PH-1	5.79e-153	
Magnaporthe oryzae 70-15	4.26e-153	
Alternaria alternata EV-MIL-31	4.61e-132	
Alternaria alternata SRC1IrK2f	1.47e-137	

Figure S9. Pairwise comparison and alignment of conserved motifs of the AaHapC sequence with fungal HapC orthologs. A pairwise comparison of the sequence was performed using CLC Genomic Workbench 9.5.1 to calculate genetic distance using the jukes-cantor model and percent identity. Conserved domains were predicted by NCBI and the MEME Suite server.

HapE

Distance

		1	2	3	4	5	6	7
Fusarium oxysporum f. sp. *lycopersici* 4287	1		0.21	0.45	0.47	0.43	0.60	0.60
Fusarium graminearum PH-1	2	79.14		0.37	0.44	0.41	0.54	0.54
Magnaporthe oryzae 70-15	3	57.89	63.29		0.46	0.49	0.54	0.54
Aspergillus fumigatus Af293	4	56.10	57.04	57.39		0.09	0.48	0.48
Aspergillus niger ATCC 1015	5	57.34	57.59	56.27	86.50		0.46	0.46
Alternaria alternata EV-MIL-31	6	45.79	49.22	47.98	50.46	50.62		0.00
Alternaria alternata SRC1IrK2f	7	45.79	49.22	47.98	50.46	50.62	100.00	

Identity (%)

Conserved Motif

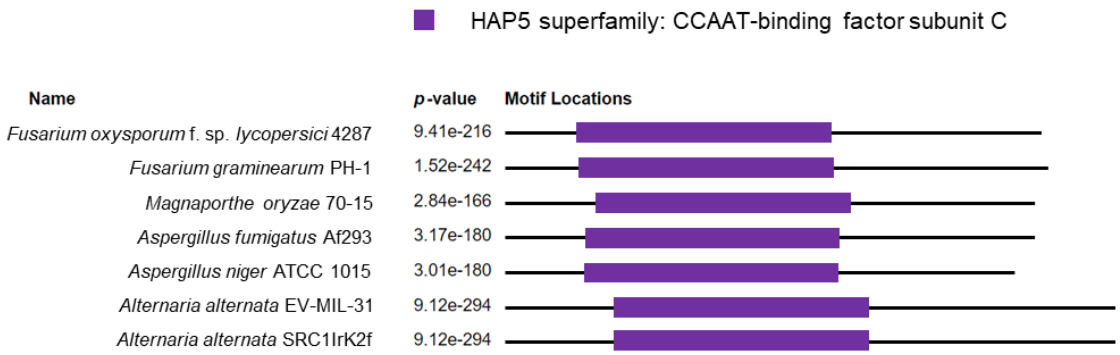

Figure S10. Pairwise comparison and alignment of conserved motifs of the AaHapE sequence with fungal HapE orthologs. A pairwise comparison of the sequence was performed using CLC Genomic Workbench 9.5.1 to calculate genetic distance using the jukes-cantor model and percent identity. Conserved domains were predicted by NCBI and the MEME Suite server.

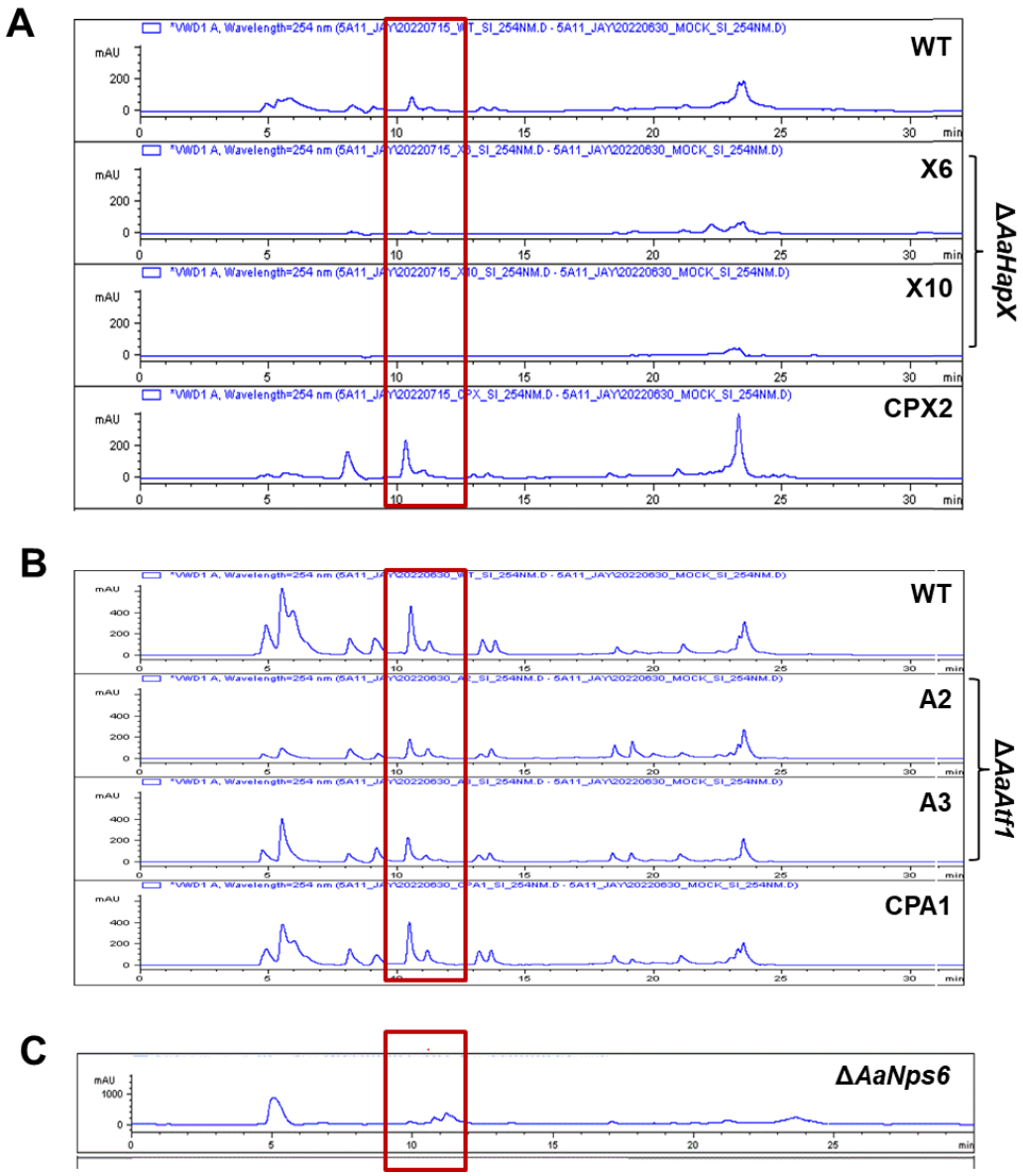

Figure S11. HPLC analyses of siderophores isolated from the strains of *A. alternata*. (**A**) WT: wild type, Δ*AaHapX*: the *AaHapX* deletion mutants (X6 and X10), and CPX2: the *AaHapX* complementation strain. (**B**) Δ*AaAtf1*: the *AaAtf1* deletion mutants (A2 and A3) and CPA1: the *AaAtf1* complementation strain. (**C**) Δ*AaNps6*: the *AaNps6* deletion mutant defective for a nonribosomal peptide synthetase involved in the biosynthesis of siderophores. Siderophores (indicated by red rectangle boxes) were separated in a reversed-phase Supelco Ascentis C-18 column (Sigma-Aldrich) using a mobile phase consisting of a linear gradient of 0.2% formic acid in water and 0.2% formic acid in methanol (from 75:25 to 55:45 in 10 min, 25:75 in 5 min, and 0:100 for final 5 min).

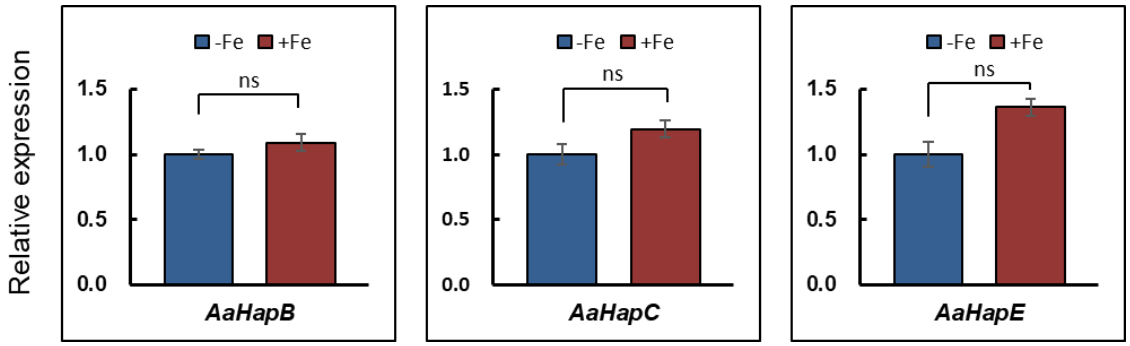

Figure S12. qRT-PCR analyses. No significant difference in the expression of *AaHapB*, *AaHapC*, and *AaHapE* in the wild type of *A. alternata* in response to iron. Fungal mycelium grown in PDB for 2 days was harvested, washed with sterile water, shifted to a minimal medium (MM) amended with or without 0.2 mM $FeCl_3$, and incubated for an additional 24 h. The relative expression level from three independent reactions was calculated by a comparative Ct method (*ΔΔCT*) in relation to the expression of the fungal β-tubulin-coding gene. In each assay, the expression level in the wild type grown in MM was set to 1, and subject to statistical analysis of Student's t-test ($p < 0.01$). All experiments were repeated at least two times, showing similar trends.

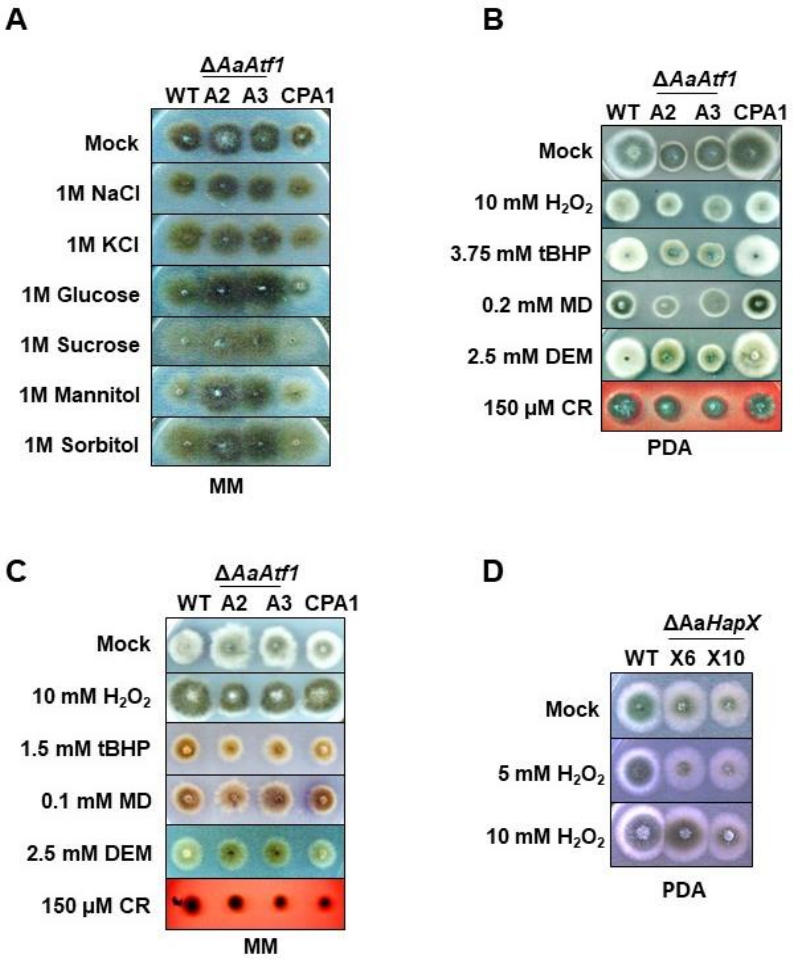

Figure S13. Sensitivity tests. Fungal strains were grown on MM or PDA amended with or without a chemical at 28°C for 3 to 7 days. Abbreviations: tBHP, *tert*-butyl-hydroperoxide; MD, menadione; DEM, diethyl maleate; and CR, Congo red.