

Supplemental Material

Transcriptomic profile of *Penicillium digitatum* reveals novel aspects of the mode of action of the antifungal protein AfpB

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Supplemental Table S1. List of organisms and their genome assembly obtained from NCBI used to improve the functional annotation of *P. digitatum* CECT 20796 (PHI26).

Species	Assembly	GenBank/RefSeq assembly accession	PMID	Ref
<i>Penicillium chrysogenum</i>	ASM71027v1	GCA_000710275.1	25059858	(1)
<i>Penicillium expansum</i>	ASM76974v1	GCF_000769745.1	25338147	(2)
<i>Aspergillus fumigatus</i>	ASM265v1	GCF_000002655.1	16372009	(3)
<i>Aspergillus nidulans</i>	ASM14920v2	GCF_000149205.2	16372000	(4)
<i>Aspergillus niger</i>	ASM285v2	GCF_000002855.3	18318841	(5)
<i>Botrytis cinerea</i>	ASM14353v4	GCF_000143535.2	26913498	(6)
<i>Fusarium oxysporum</i>	FO_FOSC_3_a_V1	GCF_000271745.1	-	-
<i>Magnaporthe oryzae</i>	MG8	GCF_000002495.2	15846337	(7)
<i>Neurospora crassa</i>	NC12	GCF_000182925.2	12712197	(8)
<i>Penicillium digitatum</i>	ASM1676781v1	GCA_016767815.1	-	-
<i>Penicillium italicum</i>	ASM76976v1	GCA_000769765.1	25338147	(2)

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Supplemental Table S2. RNA-Seq stats for Experiment I and II sample reads.

		Processed reads	Mapped reads	% mapped reads	Reads mapped to transcripts	% mapped reads to transcripts
Experiment I	A1	29787538	26047801	87.45	19724825	75.73
	A2	25236596	22242410	88.14	16593847	74.60
	A3	26478870	22823236	86.2	17091531	74.89
	A4	27120849	24097558	88.85	18416676	76.43
	B1	28076281	23652476	84.24	17910005	75.72
	B2	26853161	23480270	87.44	17571617	74.84
	B3	35095222	31115749	88.66	23827223	76.58
	B4	29341169	26799911	91.34	20406194	76.14
Experiment II	1A	33258488	31016180	93.3	20568280	66.31
	1B	28980317	27147855	93.7	19610962	72.24
	1C	22740015	20935103	92.1	14900386	71.17
	2A	24555108	22915209	93.3	16619102	72.52
	2B	40613625	37288336	91.8	25716069	68.97
	2C	36282399	34070114	93.9	23941690	70.27
	3A	33138400	30729838	92.7	21970201	71.49
	3B	28944386	26744483	92.4	19316593	72.23
	3C	33298233	31177915	93.6	21894138	70.22
	4A	34958519	32854821	94	26070138	79.35
	4B	27054052	25329369	93.6	20128189	79.47
	4C	25110404	23478951	93.5	18378926	78.28
	5A	31941085	29944200	93.7	22441156	74.94
	5B	21798004	23915586	92.4	15620764	65.32
	5C	25739493	23915586	92.9	18270262	76.39

Supplemental Table S3. FungalBraid (FB) elements used and FB vectors generated in this study.

Code	Genetic Element(s)	FB Plasmid	Reference
FB012	<i>P_{trpC}::hph::T_{tub}</i>	pUPD2	(1)
FB013	<i>P_{gpdA}::HSVtk::T_{tub}</i>	pUPD2	(1)
FB087	PDIG_14850 5' flanking sequence	pUPD2	This work
FB088	PDIG_14850 3' flanking sequence	pUPD2	This work
FB089	PDIG_81760 5' flanking sequence	pUPD2	This work
FB090	PDIG_81760 3' flanking sequence	pUPD2	This work
FB091	FB013::FB089::FB012::FB090	pDGB α 2	This work
FB092	FB013::FB087::FB012::FB088	pDGB α 2	This work
FB168	PDIG_14840 5' flanking sequence	pUPD2	This work
FB169	PDIG_14840 3' flanking sequence	pUPD2	This work
FB171	FB013::FB168::FB012::FB169	pDGB α 2	This work
FB172	FB013::FB168::FB012::FB088	pDGB α 2	This work

References:

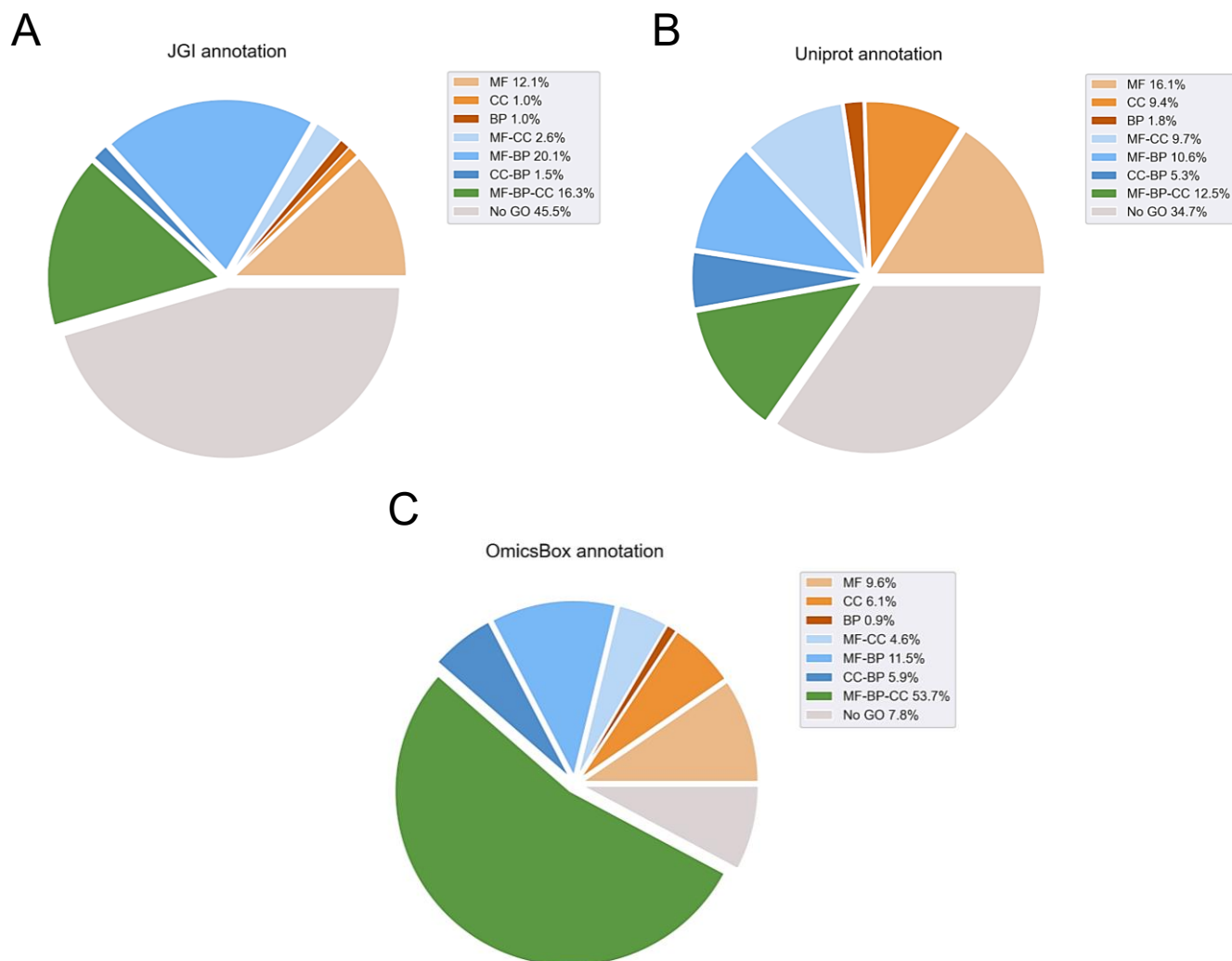
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Supplemental Table S4. Primers used in this study.

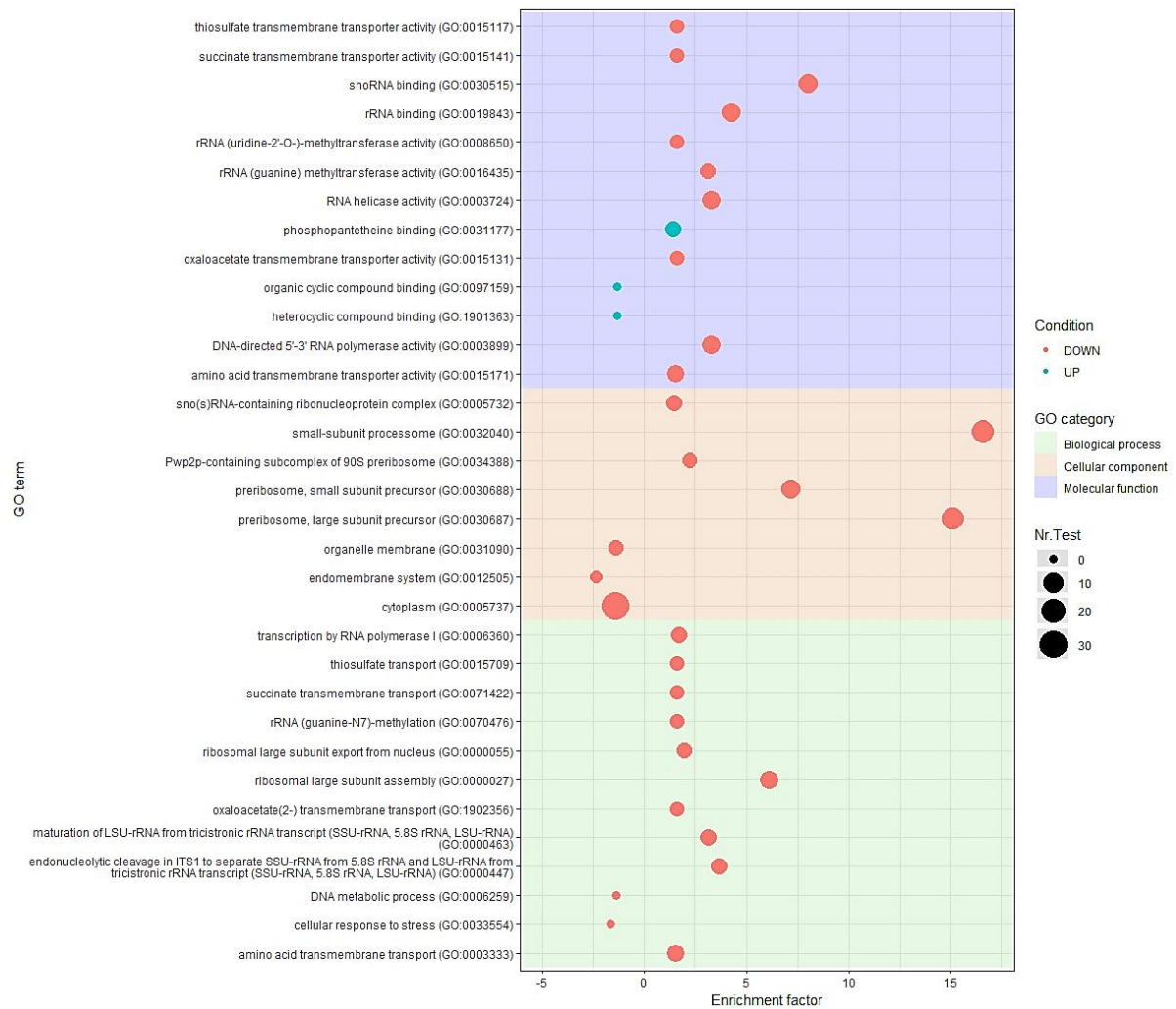
Name	F/R	Sequence 5'→3'	Origin	Use	Reference
OJM85	F	AGCGGTGACAAGTACGTTCC	<i>β-tub</i>	qPCR	(1)
OJM86	R	ACCCTTGCCCCAGTTGTTAC	<i>β-tub</i>	qPCR	(1)
OJM151	F	TGGGGCAGAGGGAAGTTGAG	<i>L18a</i>	qPCR	(2)
OJM152	R	ACCGACGCTGTTGAGGCTCT	<i>L18a</i>	qPCR	(2)
OJM232	R	GTTTGCCAGTGATACACATGGG	<i>hph</i>	PCR	(3)
OJM334	F	CGACTTCAGGAAGGGGTGTA	<i>18S rRNA</i>	qPCR	(4)
OJM335	R	CTTGATGTGGTAGCCGTT	<i>18S rRNA</i>	qPCR	(4)
OJM477	R	CGCCATGTAGTGATTGACCG	<i>hph</i>	PCR	This work
OJM604	F	CATATCCAAGCCATTCGTCTCC	<i>PDIG_81760</i>	PCR	This work
OJM605	R	GGTCTTACACTTCTGAATTCC	<i>PDIG_81760</i>	PCR	This work
OJM611	R	CGTTTGCGGAGGTCTACTCCC	<i>PDIG_14850</i>	PCR	This work
OJM612	F	CAGTATTCGGTTCTTAGCGC	<i>PDIG_14850</i>	qPCR/PCR	This work
OJM613	R	GCCTGGAGGAATCTGGTAGG	<i>PDIG_14850</i>	qPCR	This work
OJM614	F	ATGAAGTCTCAAACCGTCCTC	<i>PDIG_81760</i>	qPCR/Seq	This work
OJM615	R	TCAATTGGAGCGGCCATGAC	<i>PDIG_81760</i>	qPCR/PCR/Seq	This work
OJM616	F	CGATCCTCCATCTCTTTCCCGC	<i>PDIG_81760</i>	Seq	This work
OJM617	R	GCTGGATCTATACAAACTCC	<i>PDIG_81760</i>	Seq	This work
OJM626	F	TACTTGCCTGTGCTGATCCT	<i>PDIG_14840</i>	PCR	This work
OJM627	R	CTTCATCATTACAGCACCGCG	<i>PDIG_14840</i>	PCR	This work
OJM648	F	GACGGCACGCCAATGATTGTG	<i>PDIG_14840</i>	qPCR	This work
OJM649	R	GTCGCCAATTCTCTCCACAGC	<i>PDIG_14840</i>	qPCR	This work

References:

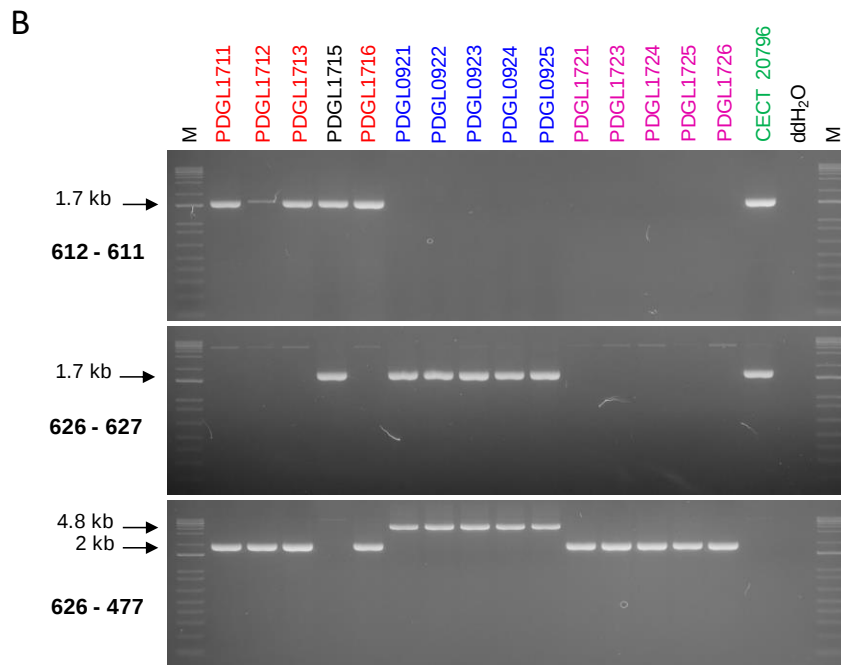
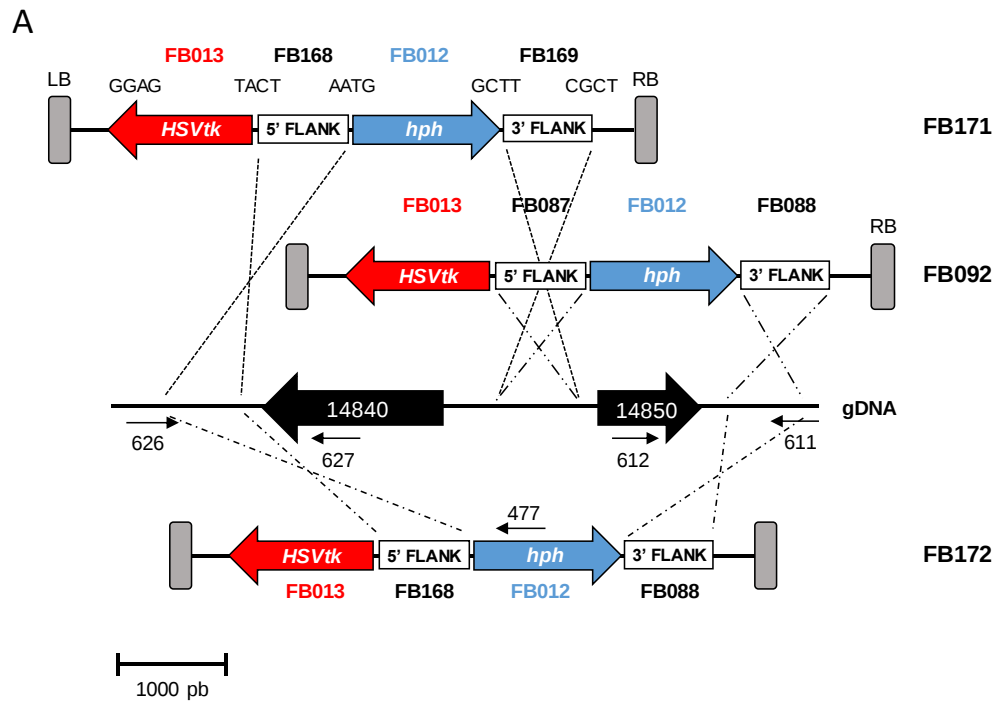
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Supplemental Fig. S1: Comparison of *P. digitatum* CECT 20796 functional annotation between different public databases and the OmicsBox annotation. GO term categories referring to molecular function (MF), cellular component (CC) and biological process (BP) were used to analyze differences between the functional annotation present in public databases such as JGI database (A), Uniprot database (B) and OmicsBox annotation result (C). Proteins were grouped into one single GO term category or different categories which present a combination of two, three or none of the above mentioned GO term categories.

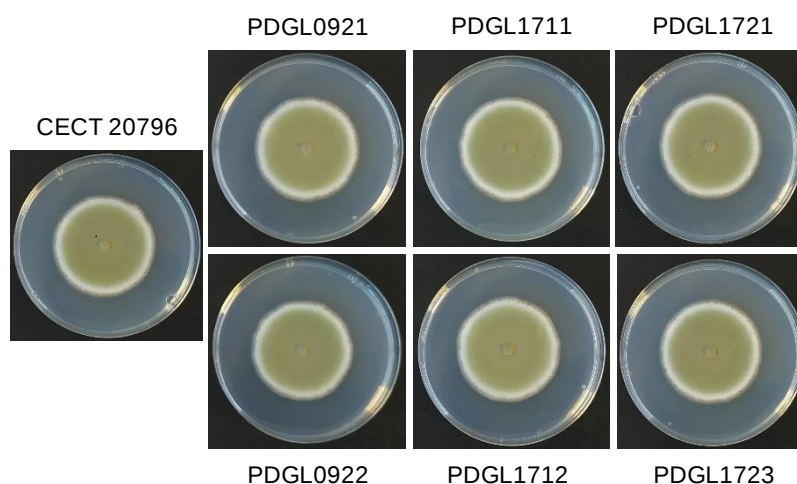


Supplemental Fig. S2. Gene Ontology (GO) enrichment analysis for the differentially expressed genes (DEGs) in the $\Delta afpB$ mutant (PDMG122). The y-axis indicates the GO term and the x-axis shows the enrichment factor calculated as $-\log_{10}(\text{FDR})$ for over-represented categories and $\log_{10}(\text{FDR})$ for under-represented categories ($\text{FDR} < 0.05$). The bubble size represents the number of DEGs detected for each GO term. The color background differentiates the GO category and the bubble colors distinguish up-regulated (blue) and down-regulated (red) genes.

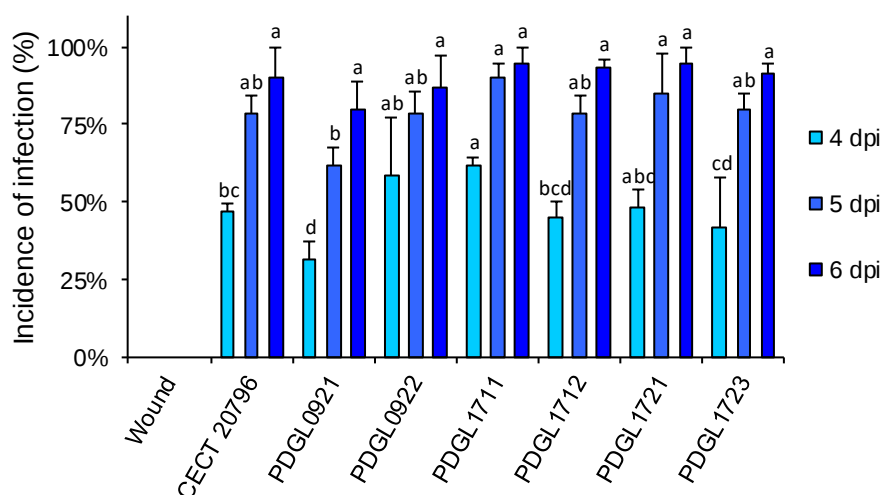


Supplemental Fig. S3. Generation of *P. digitatum* PDIG_14840 and PDIG_14850 single and double deletion strains and confirmation by PCR. (A) Schematic representation of PDIG_14840 and PDIG_14850 genes in the parental strain CECT 20796 and the FB constructs used for single (FB092 and FB171) and double (FB172) deletion. All primers used for PCR analysis are localized in the figure. (B) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. The PDIG_14850 deletion mutants (PDGL0921 to PDGL0925) and the double deletion mutants (PDGL1721 to PDGL1726) did not show any amplicon with primers 612/611, while PDIG_14840 deletion strains (PDGL1711 to PDGL1714, and PDGL1716), the ectopic transformant PDGL1715 and the parental strain showed an amplicon of 1.7 kb (first panel). The PDIG_14840 deletion mutants and the double deletion mutants showed no amplicon with primers 626/627, while the PDIG_14850 deletion mutants, the ectopic transformant and the parental strain showed an amplicon of 1.7 kb (second panel). All the positive transformants showed an amplicon with primers 626/477 indicating the presence of the hygromycin marker (third panel).

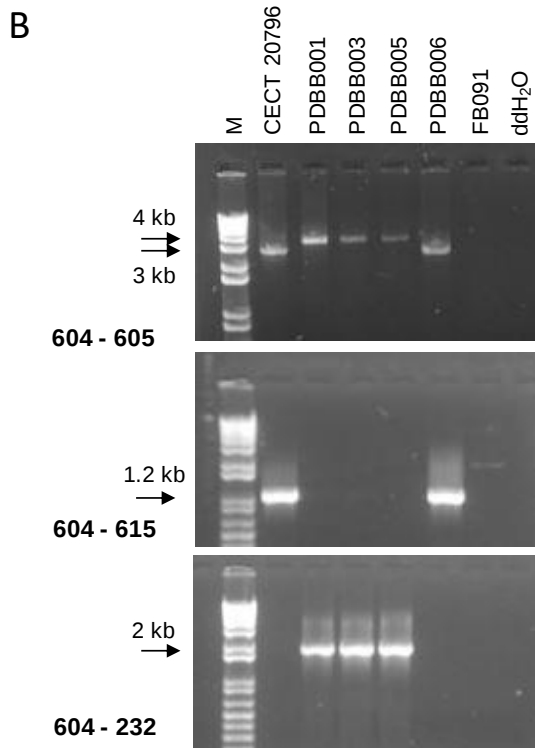
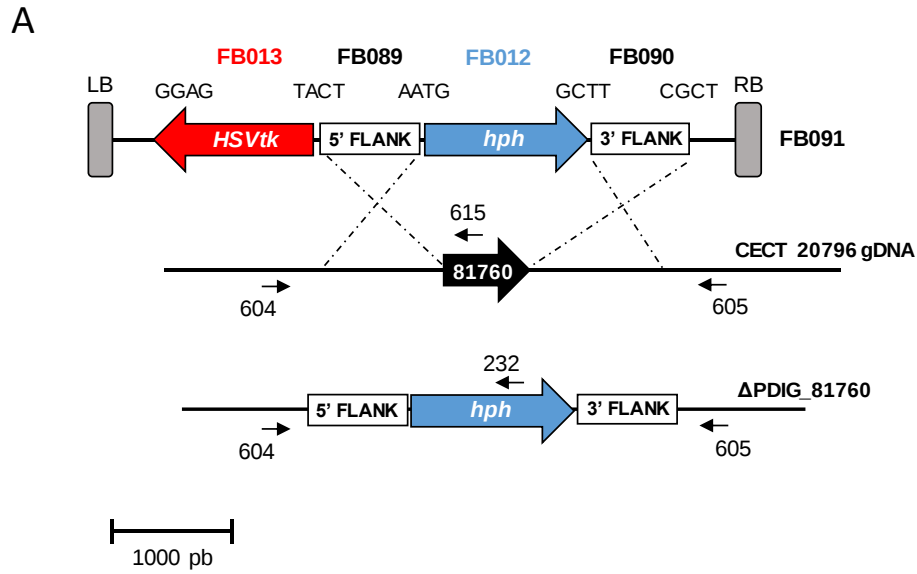
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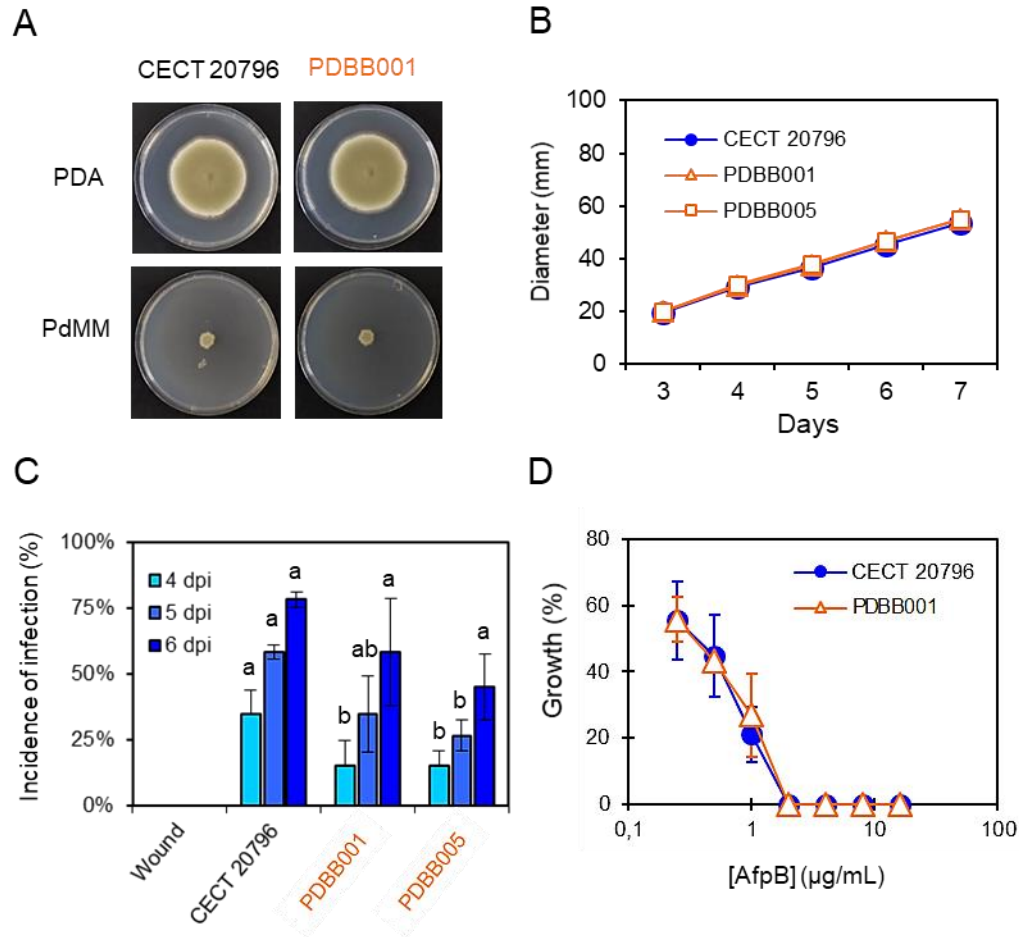
B



Supplemental Fig. S4. Phenotypic characterization of *P. digitatum* deletion strains for the acetoin biosynthesis pathway-related genes. (A) Representative images of growth on PDA plates of *P. digitatum* CECT 20796 (PHI26), the deletion strains for PDIG_14850 (PDGL0921 and PDGL0922) PDIG_14840 (PDGL1711 and PDGL1712) and the double deletion strains (PDGL1721 and PDGL1723). (B) Infection of orange fruits cv Navel by *P. digitatum* CECT 20796 and the deletion strains. Bars show the mean values of the percentage of infected wounds and standard deviation (SD) of three replicates of five oranges at 4, 5 and 6 dpi. Letters show significant differences among the treatments at each independent day (one-way ANOVA and Tukey's HSD test, $p < 0.05$).



Supplemental Fig. S6. Generation of *P. digitatum* PDIG_81760 single deletion strains and confirmation by PCR. (A) Schematic representation of PDIG_81760 in the parental strain CECT 20796 and the FB construct (FB091) used for single deletion. All primers used for PCR analysis are localized in the figure. (B) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. The PDIG_81760 deletion mutants (PDBB001, PDBB003 and PDBB005) showed different amplicon size compared with CECT 20796 or PDBB006 ectopic strain with external primers 604/605, indicating hygromycin cassette replacement (first panel). The PDIG_81760 deletion mutants showed no amplicon with primers 604/615, where 615 was an internal primer from PDIG_81760 gene. Only CECT 20796 parental strain and PDBB006 ectopic strain showed an amplicon of expected size with these primer pair (second panel). All the positive transformants showed an amplicon with primers 604/232 indicating the presence of the hygromycin resistance cassette (third panel). Note that PDIG_81760 is in reverse orientation in the CECT 20796 genome.



Supplemental Fig. S7. Phenotypic characterization of *P. digitatum* PDIG_81760 deletion strains (PDBB001 and PDBB005) and infection assays on citrus fruits. (A) Representative images of growth on PDA and PdMM plates of *P. digitatum* parental strain CECT 20796 and PDBB001. (B) Colony diameter of PDBB001 and PDBB005 transformant strains from 3 to 7 days of growth at 25 °C in solid PDA medium, compared to parental CECT 20796 strain. (C) Infection of orange fruits cv Lane Late by three different strains. Bars show the mean values of the percentage of infected wounds and standard deviation (SD) of three replicates of five oranges at 4, 5 and 6 days post infection (dpi). Letters show significant differences among the different strains at each independent day (one-way ANOVA and Tukey's HSD test, $p < 0.05$). (D) Dose-response curves showing the antifungal activity of AfpB against *P. digitatum* wild-type strain CECT 20796 and the deletion mutant strain PDBB001.