Appendix

Fungal phytochrome chromophore biosynthesis at mitochondria

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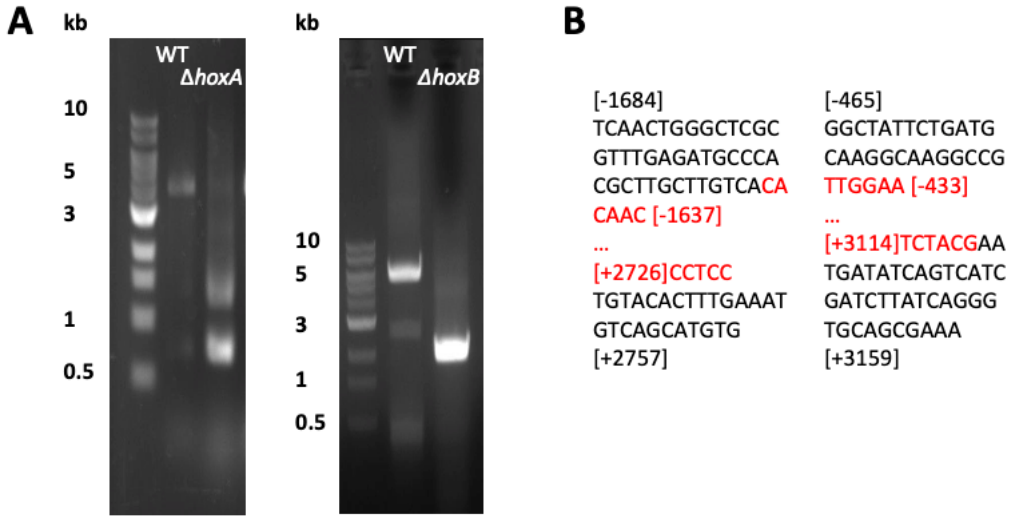
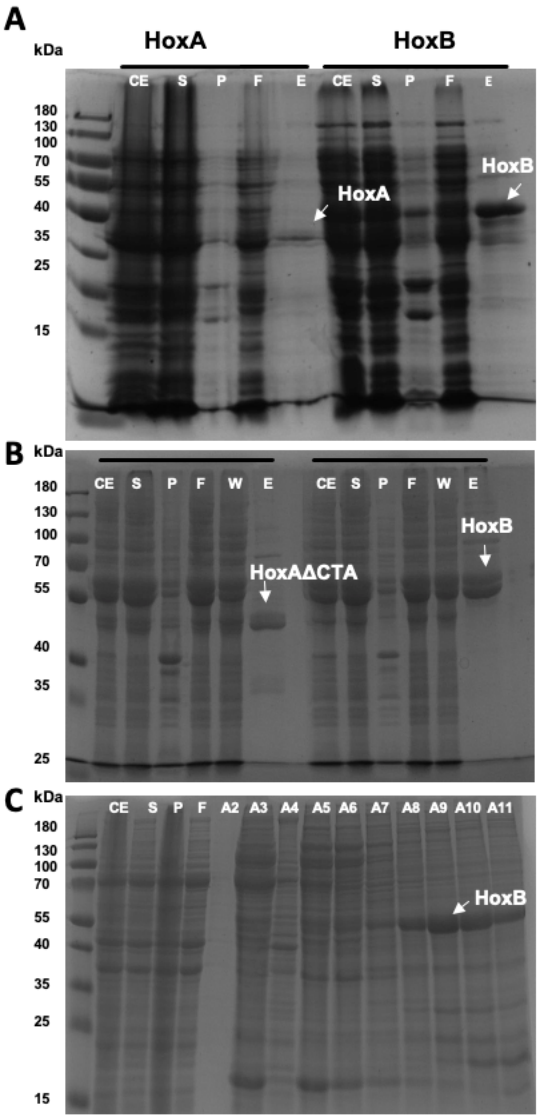
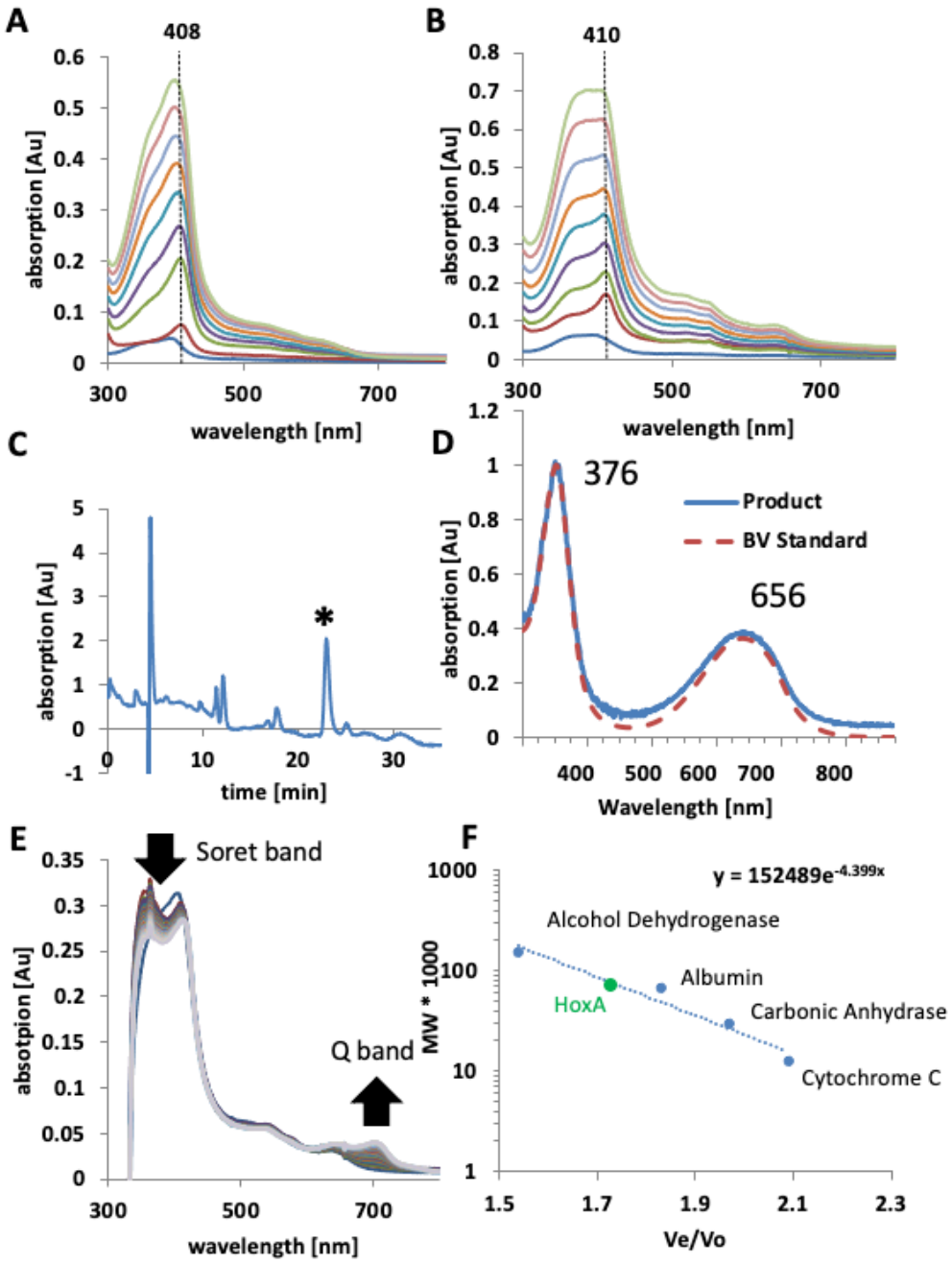
Appendix Figures



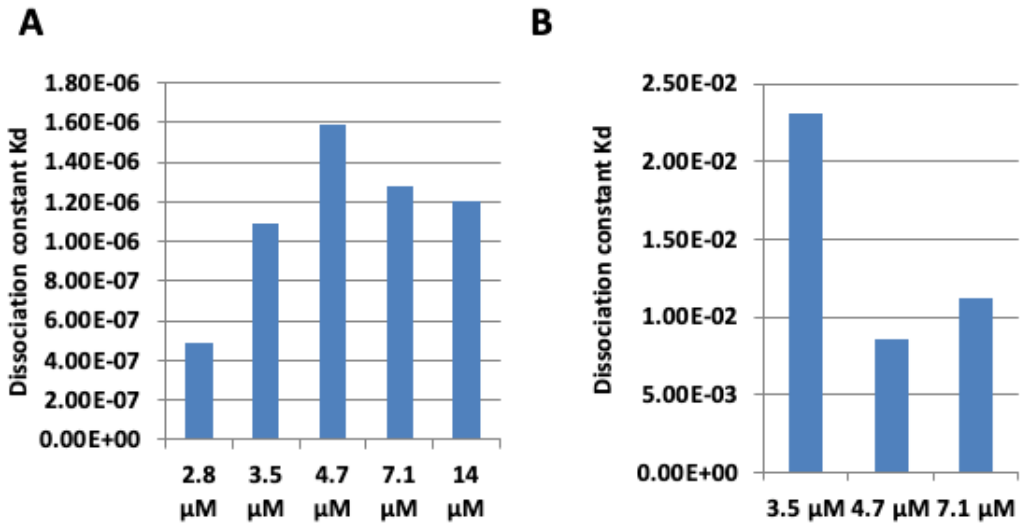
Appendix Fig. S1: Deletion of the heme oxygenase genes. A, Both genes were deleted using CRISPR/Cas9. We analyzed 20 transformants for each gene. PCR analysis of wild type and genomic DNA of the deletion mutants was performed to confirm the deletion event. The *hoxA* gene locus was amplified with HoxA_check_for and HoxA_check_rev and resulted in a 4.5 kb band. The same primer combination resulted in a 0.75 kb band in the deletion mutant. In the case of *hoxB,* the primer combination was: HoxB_check_for and HoxB_check rev. The band obtained with wild type was 5.5 kb and the one with the mutant DNA was 1.75 kb. **B,** The PCR fragments of the deletion mutants obtained in **A** were sequenced. The sequences show the deletion event for *hoxA* (left) and *hoxB* (right). The numbers refer to the distance from the start codon. The red sequences are the direct border sequences of the deletion.



Appendix Fig. S2: HO expression in *E. coli* and in *P. pastoris*. A, HoxA and HoxB were expressed in *E. coli* for 20h at 15°C. Purification was performed using the Strep-tag system. HoxA remained largely in the insoluble pellet, while HoxB was soluble. HoxB was inactive in biochemical assays. **B,** HoxA and HoxB were purified like in **a**, but the C-terminal anchor (CTA) was deleted from HoxA resulting in a soluble form (HoxAΔCTA). **A, B,** Protein fractions were separated in an SDS PAGE analysis. Fractions are: Crude extract (CE), supernatant 1 (S), pellet (P), flow through (F), eluate (E). **C,** HoxB was expressed in *P. pastoris* for 2.5 days at 30°C. The SDS PAGE shows the fractions of the purification as in **A, B** and in addition factions A2-A11 from the Mono S cation exchanger.



Appendix Fig. S3: Biochemical analyses of HoxA and HoxB. A, A spectrum of a solution with 1 µM hemin was recorded (lower blue line). Afterwards 10 µM HoxA was added. Subsequently repeatedly 1 µM hemin was added and the spectrum recorded each time. A peak at 408 nm appeared (dashed line). **B,** HoxB was expressed in *P. pastoris* for 2.5 days. Purification was performed using a Mono S column. Measurement like in **A**. **C,** HPLC analysis of the products obtained in the heme oxygenase assay. 25 µM HoxA and HoxB were incubated with 10 µM hemin, 1.5 mg/ml BSA, 4.6 µM petF (spinach ferredoxin), 0.025 U/ml petH (spinach ferredoxin reductase), 10 µM catalase, 5 mM Tiron, 1.05 mM glucose-6-phosphate, 0.105 µM NADP+ and 0.15 U/ml glucose-6-phosphate-dehydroxygenase. The resulting peak in the HPLC profile (asterisk) was analyzed spectroscopically and resembled biliverdin **D**. **E,** To the heme oxygenase assay (from **C**) 300 µg PGP was added. Spectra were recorded every 30 sec. for 10 min. The Soret band of hemin decreased, while the Q-band of the PGP Pr form (705 nm) increased (arrows). **F**, Calibration of the SEC column. Standards were alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). (Ve = volume elution; Vo = void volume).



Appendix Fig. S4: Analysis of the calculated dissociation constant (Kd) for each concentration of the interaction of HO and the phytochrome photosensory domain *in vitro* from Fig. 6. Biolayer interferometry analysis with Apo-PGP **(A)** and holo-PGP **(B)**. Each Kd was calculated for the indicated concentration separately to compare consistency over the range.

Appendix Table S1: Strains used in this study.

Strain	Genotype	Reference
A. nidulans		
SKV103	*pyrG89; pyroA4; veA+*	(Vienken & Fischer, 2006)
SKV103	*pyrG89; pyroA4; veA+*	(Vienken *et al*, 2005)
SChS27	*alcA::GFP-hoxA ::pyr4 in SKV103*	This study
SChS28	*alcA::GFP-hoxB ::pyr4 in SKV103*	This study
SChS29	*alcA::NYFP-hoxA ::pyro and alcA::CYFP-hoxA::pyr4 in SKV103*	This study
SChS30	*alcA::NYFP-hoxA ::pyro and alcA::CYFP-fphA::pyr4 in SKV103*	This study
SChS31	*alcA::NYFP-hoxB ::pyro and alcA::CYFP-fphA::pyr4 in SKV103*	This study
SChS32	*alcA::NYFP-hoxB ::pyro and alcA::CYFP-hoxA::pyr4 in SKV103*	This study
SChS33	*alcA::NYFP-hoxB ::pyro and alcA::CYFP-hoxB::pyr4 in SKV103*	This study
SChS37	*alcA::mEOS-hoxA ::pyr4 in SKV103*	This study
SChS38	*alcA::mEOS-hoxB ::pyr4 in SKV103*	This study
SChS40	*αlcA(p)::GFP-HoxAΔCTA::pyroA in SKV103*	This study
SChS41	*αlcA(p)::GFP-CTA::pyroA in SKV103*	This study
SChS43	*αlcA(p)::GFP-HoxAΔCTA::pyroA in SKV103*	This study
SChS44	*αlcA(p)::GFP-CTA::pyroA in SKV103*	This study
A. alternata		
ATCC 66981	*A. alternata* wild type	Christopher Lawrence (Blacksburg,

		VA)
SChS25	Δ*hoxA in SMW24*	This study
SChS26	Δ*hoxB in SMW24*	This study
SOI1	*fphA* mutant Δ*fphA528* 528 nucleotides deleted by CRISPR Cas9	This study
SMW24	Δ*pyrG in* ATCC66981	(Wenderoth *et al*, 2019)
E. coli		
Top10	F- *mcrA* cr *mrr-hsd*RMS-*mcr*BC) Φ 0*lac*Zac hsd *lac*X74 *rec*A1 *ara*D139 sd *araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, Leek, NL
BL21	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen, Darmstadt
P. pastoris		
G115	His-	Invitrogen
SChS39	Aox1::HoxB-Strep::His in GS115	This study

Appendix Table S2: Oligonucleotides used in this study.

Name	Sequence (from 5' to 3')	Description
AlccgA-RT-F	GTCAACTCTGTCAAGAACGC	RT-qPCR primers for *A. alternata*
AlccgA-RT-R	TTGATCTTGTCACCAGCAGC	
Alh2b-RT-F	ACAAGAAGAAGCGCACCAAG	
Alh2b-RT-R	CGTTGACGAAAGAGTTGAGAA	
Alt_HemeO1_proto1_for	GTC CGT GAG GAC GAA ACG AGT AAG CTC GTC AGG GCA GAG ACG CTG TGC CGG TTT TAG AGC TAG AAA TAG CAA GTT AAA	Deletion of *hoxA* and *hoxB*
Alt_HemeO1_proto1_HH_rev	GAC GAG CTT ACT CGT TTC GTC CTC ACG GAC TCA TCA GAG GGC ACG GTG ATG TCT GCT CAA GCG	
Alt_HemeO1_proto2_for	GTC CGT GAG GAC GAA ACG AGT AAG CTC GTC GAC ATT TCT CAG GAG GCC AGG TTT TAG AGC TAG AAA TAG CAA GTT AAA	
Alt_HemeO1_proto2_HH_rev	GAC GAG CTT ACT CGT TTC GTC CTC ACG GAC TCA TCA GGA CAT TCG GTG ATG TCT GCT CAA GCG	
Alt_HemeO2_proto1_for	GTC CGT GAG GAC GAA ACG AGT AAG CTC GTC GGC ATG CCA ACC CTG ACA TAG TTT TAG AGC TAG AAA TAG CAA GTT AAA	
Alt_HemeO2_proto1_HH_rev	GAC GAG CTT ACT CGT TTC GTC CTC ACG GAC TCA TCA GGG CAT GCG GTG ATG TCT GCT CAA GCG	
Alt_HemeO2_proto2_for	GTC CGT GAG GAC GAA ACG AGT AAG CTC GTC AAC GAT AAC CGG AAT CCA TTG TTT TAG AGC TAG AAA TAG CAA GTT AAA	

Alt_HemeO2_proto2_HH_rev	GAC GAG CTT ACT CGT TTC GTC CTC ACG GAC TCA TCA GAA CGA TCG GTG ATG TCT GCT CAA GCG	
HoxA_check_for	ACG AGG AGG AGA CCA AGT ACC	Sequencing of the deletion
HoxA_check_rev	GCT TGC ACA TGA TAG TCA CAT CAC	
HoxB_check_for	GGC GAT GAT GTG GGG ATC G	
HoxB_check_rev	CCG ATG CAT CAC TCG CTT G	
pASK_AltHOXAsyn fwd	GTG AAA TGA ATA GTT CGA CAA AAA TCT AGA TGC TTG AGA AGC AAC AAC A	Expression of HoxA
pASK_AltHOXAsyn rev	GGT GGC TCC AAG CGC TGA GAC CAT GTT GAA TCT TGA AAA GGG TTA	
HoxA_Anchordel_for	CAT GGT CTC AGC GCT TGG AG	Construction of HoxAΔCTA for Expression
HoxA_Anchordel_rev	CGG GCG GCG TAA AAA CGT C	
pASK_AltHOXBsyn fwd	GTG AAA TGA ATA GTT CGA CAA AAA TCT AGA TGG GTC GTC ACG CTA ACC C	Expression of codon optimized HoxB in *E. coli*
pASK_AltHOXBsyn rev	GGT GGC TCC AAG CGC TGA GAC CAT GTG AGC CCT GCC CAA CGG ATT	
HoxB_unopt_SalI_for	GAG GTC GAC ATG GGC AGG CAT GCC AAC	Expression of native HoxB in *E. coli*
HoxB_unopt_PstI_rev	CTC CTG CAG CGA GCC CTG GCC CAC ACT C	
pPIC_HoxB_unopt_for	AAA AAC AAC TAA TTA TTC GAA GGA TCC GCC ACC ATG GGC AGG CAT GCC A	Expression of HoxB in *P. pastoris*
pPIC_HoxB_unopt_rev	CTA AGG CGA ATT AAT TCG CGG CCG CCT TAT TAT TTT TCG AAC TGC GGG TGG	
pACYC_AltHoxA	TTA AGT ATA AGA AGG AGA TAT ACA	Coexpression

syn fwd	TAT GCT TGA GAA GCA ACA ACA	of HoxA, HoxB or HoxA and HoxB with PGP
pACYC_AltHoxA syn rev	GGT GGC AGC AGC CTA GGT TAA TTA ACG ATG ATG GTG ATG GTG GTG GTG GTG ATG GTG GTG GTG TT	
pACYC_AltHoxB syn fwd	GGT GGC AGC AGC CTA GGT TAA TTA ACG ATG ATG GTG ATG GTG GTG GTG ATG ATG GTG ATG ATG TG	
pACYC_AltHoxB syn rev	TTA AGT ATA AGA AGG AGA TAT ACA TAT GGG TCG TCA CGC TAA CCC	
HoxB_dualexpression_for	GAT CCG AAT TCG AGC TCG GCG CGC CGG ATG GGT CGT CAC GCT AAC C	
HoxB_dualexpression_rev	GTT CGA CTT AAG CAT TAT GCG GCC GCT TAT GAG CCC TGC CCA ACG GAT TCC	
HoxB_unopt_pACYC_for	TTA AGT ATA AGA AGG AGA TAT ACA TAT GGG CAG GCA TGC CAA CCC	
HoxB_unopt_pACYC_rev	GGT GGC AGC AGC CTA GGT TAA TTA ATT AAT GAT GGT GAT GAT GGT GCG AGC CCT GGC CCA CAC TC	
pET_AltHoxAsyn fwd	CTG GTG CCG CGC GGC AGC CAT ATG CTT GAG AAG C	
pET_AltHoxAsyn rev	CAG TGG TGG TGG TGG TGG TGC TCG AGT TGA ATC TTG AAA AG	
pET_AltHoxBsyn fwd	CTG GTG CCG CGC GGC AGC CAT ATG GGT CGT CAC	
pET_AltHoxBsyn rev	CAG TGG TGG TGG TGG TGG TGC TCG AGT GAG CCC TGC C	
HoxA_AscI_for	GTA GGC GCG CCG ATG CTG GAG AAG CAG CAG C	Construction of YFP and GFP plasmids
HoxA_PacI_rev	CAA TTA ATT AAT CAC TGG ATC TTG AAC AGC GTG	
HoxB_AscI_for	GTA GGC GCG CCG ATG GGC AGG CAT	

	GCC AAC
HoxB_PacI_rev	CAA TTA ATT AAC TAC GAG CCC TGG CCC AC
HoxA_GFP_anchor del_rev	TGG CCT CCT GAG AAA TGT C
HoxA_GFP_anchor del_for	TGA TTA ATT AAG GAT CCT CTA GAG TCG
HoxA_onlyCTA_T4 _rev	GCA TTA ATT AAT CAC TGG ATC TTG AAC AGC GTG AC
HoxA_onlyCTA_T4 _for	GTA GGC GCG CCG ATG AGT GAC CCG TTC AAG ACA TTT C

Appendix Table S3: Plasmids used in this study.

Plasmid	Insert	Source
pFC330	tef1(p)::cas9::tef1(t); AfpyrG; ampR; AMA1	(Nodvig *et al*, 2015)
pFC332	tef1(p)::cas9::tef1(t); hph; ampR; AMA1	(Nodvig *et al.*, 2015)
pFC334	tef1(p)::cas9::tef1(t); gpdA(p)::sgRNA-AnyA::trpC(t); Afpyr4; ampR; AMA1	(Nodvig *et al.*, 2015)
pASK-IBA3_FphAsyn	FphA in pASK-IBA3+	This study
BphO_pACYCDuet	Bpho in pACYC-DUET1	(Brandt *et al*, 2008)
pChS11	Protospacer1 for *ΔhoxA* in 332 with *Pac*I	This study
pChS12	Protospacer2 for *ΔhoxA* in 330 with *Pac*I	This study
pChS13	Protospacer1 for *ΔhoxB* in 330 with *Pac*I	This study
pChS14	Protospacer2 for *ΔhoxB* in 332 with *Pac*I	This study
pChS15	HoxAsyn in pASK-IBA3+ with *Xba*I and *Nco*I	This study
pChS16	HoxAΔCTAsyn in pASK-IBA3+ with *Xba*I and *Nco*I	This study
pChS18	HoxAsyn in pACYC-DUET1 with *Nde*I and *Pac*I	This study

pChS19	HoxBsyn in pACYC-DUET1+ with *Nde*I and *Pac*I	This study
pChS20	HoxAsyn in pET-28a with *Nde*I and *Xho*I	This study
pChS21	HoxBsyn in pET-28a with *Nde*I and *Xho*I	This study
pChS22	alcA::GFP-HoxA, *pyr4* with *Asc*I and *Pac*I	This study
pChS23	alcA::GFP-HoxB, *pyr4* with *Asc*I and *Pac*I	This study
pChS23	alcA::NYFP-HoxA, *pyroA* with *Asc*I and *Pac*I	This study
pChS22	alcA::CYFP-HoxA, *pyr4* with *Asc*I and *Pac*I	This study
pChS23	alcA::NYFP-HoxB, *pyroA* with *Asc*I and *Pac*I	This study
pChS22	alcA::CYFP-HoxB, *pyr4* with *Asc*I and *Pac*I	This study
pChS35	alcA::mEOS-HoxA, *pyr4* with *Asc*I and *Pac*I	This study
pChS36	alcA::mEOS-HoxB, *pyr4* with *Asc*I and *Pac*I	This study

pChS37	HoxB-Strep in PPIC3.5K with *Bam*HI and *Not*I	This study
pKL6	HoxB in pACYC-DUET1 with *Nde*I and *Pac*I	This study
pKL7	HoxAsyn and HoxB in pACYC-DUET1 with *Hin*dIII and *Nco*I	This study
PChS43	*alcA(p)::GFP-HoxAΔCTA::pyr4; ampR*	This study
PChS44	*alcA(p)::GFP-CTA::pyr4; ampR*	This study

References

Brandt S, von Stetten D, Günther M, Hildebrandt P, Frankenberg-Dinkel N (2008) The fungal phytochrome FphA from *Aspergillus nidulans*. *J Biol Chem* 283: 34605-34614

Nodvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One* 10: e0133085

Vienken K, Fischer R (2006) The $Zn(II)_2Cys_6$ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*. *Mol Microbiol* 61: 544-554

Vienken K, Scherer M, Fischer R (2005) The $Zn(II)_2Cys_6$ transcription factor RosA (repressor of sexual development) triggers early developmental decisions in the filamentous fungus *Aspergillus nidulans*. *Genetics* 169: 619-630

Wenderoth M, Garganese F, Schmidt-Heydt M, Soukup ST, Ippolito A, Sanzani SM, Fischer R (2019) Alternariol as virulence and colonization factor of *Alternaria alternata* during plant infection. *Mol Microbiol* 112: 131-146