

# The White Collar Complex is Essential for Sexual Reproduction but Dispensable for Conidiation and Invasive Growth in *Fusarium verticillioides*

Á. M. BODOR<sup>†</sup>, V. STUBNYA<sup>†</sup>, A. L. ÁDÁM<sup>1</sup>, M. LÁDAY AND L. HORNOK\*

Agricultural Biotechnology Centre, Mycology Group of the Hungarian Academy of Sciences,  
Institute of Plant Protection, Szent István University, H-2100 Gödöllő, Hungary

<sup>1</sup>Present address: Plant Protection Institute, Centre for Agricultural Research,  
Hungarian Academy of Sciences, 1525 Budapest, P.O.Box 102.

(Received: ??????????; accepted: ??????????)

*Fvwc1* and *Fvwc2*, orthologues of the *wc-1* and *wc-2* genes encoding for proteins of the white collar complex (WCC) in *Neurospora crassa* were cloned from *Fusarium verticillioides* and lack-of-function *wc* mutants were obtained by targeted gene disruption. Photo-conidiation was found to be absent in *F. verticillioides*, on the contrary, the wild type strain produced less conidia under continuous illumination than in the dark. Inactivation of any of the *wc* genes led to total female sterility, without affecting male fertility or asexual conidiation. No loss in colonization capability/invasive growth of the *wc* mutants was observed, when assessed on tomato fruits. Both *Fvwc1* and *Fvwc2* showed constitutive expression in the wild type cultures incubated in the dark and exposure to light caused only negligible increases in their transcription. Both *Fvwc1* and *Fvwc2* were down-regulated in a  $\Delta Fvmat1-2-1$  gene disruption mutant, lacking a functional mating type (*mat1-2-1*) gene, suggesting that the MAT1-2-1 product has a positive regulatory effect on the white collar genes.

Keywords: colonization, conidiation, female sterility, sexual reproduction, white collar gene.

Light influences a variety of physiological processes in fungi. In the model fungus, *Neurospora crassa* all light responses, including entrainment of the circadian rhythm, biosynthesis of carotenoids, photo-induction of conidiation, induction of protoperithecium formation, perithecial development, phototropism of conidiophores, and the direction of ascospore release are sensitive only to blue or near ultraviolet light (Liu et al., 2003). The central light signaling component in *Neurospora* is the heterodimeric white collar complex (WCC), formed by WC-1 and WC-2 (Chen and Loros, 2009). The WC-1 protein contains a zinc-finger domain and three PAS (Per-Arnt-Sim) domains, of which the first is a chromophore-binding domain (LOV, light-oxygen-voltage) (Ballario et al., 1998), whereas the WC-2 protein has a zinc-finger and a PAS domain (Linden and Macino, 1997). By using the third PAS domain, WC-1 forms a complex with the PAS domain of WC-2, yielding WCC (Cheng et al., 2002). The LOV domain of WC-1 binds the flavin, FAD and serves as a blue light photoreceptor (Froehlich et al., 2002).

\* Corresponding author; e-mail: Hornok.Laszlo@mkk.szie.hu

<sup>†</sup> These authors contributed equally to this work.

WCC, acting as a putative transcription factor activates a range of genes in *Neurospora*, including early light responsive genes, like the circadian clock gene *frq*, several conidiation (*con-6*, *con-8*, *con-10*) and carotenoid biosynthesis genes (*al-1*, *al-2*, *al-3*), some of the clock-controlled genes (*ccg-4*, *ccg-6*), the so-called blue light induced genes (*bli-3*, *bli-4*) (reviewed by Linden et al., 1997) and *sub-1*, a transcription factor encoding gene, which, in turn regulates the expression of a set of late light responsive genes, such as *ccg-1* and *ccg-2* (reviewed by Liu et al., 2003; Chen and Loros, 2009).

Colonies of the *wc* mutants of *Neurospora* has a non-pigmented white border around the dark yellow core of the colony: this is the origin of the name 'white collar'. This specific appearance of the mutant colonies is due to the impaired carotenoid biosynthesis in the mycelium (that remains white) and the WCC-independent carotenoid production in the conidia (that become orange-colored).

The *wc-1* and *wc-2* mutants of *N. crassa* have been found to be impaired in most light regulated responses, including photo-conidiation (Lauter and Russo, 1991), induction of protoperithecium formation (Degli-Innocenti et al., 1984), mycelial carotenoid biosynthesis (Harding and Turner, 1981), and light resetting of the circadian clock (Linden et al., 1997).

WCC-based light regulatory systems exist in other filamentous ascomycetes, including phytopathogenic fungi (Corrochano, 2007; Avalos and Estrada, 2010), but the behavior of the *wc* mutants have been found to vary among species. The *wcoA* (ortholog of *wc-1*) gene disruption mutants of *Fusarium fujikuroi* exhibited phenotypes, partially different from that of the *wc-1* mutants of *Neurospora*: the *wcoA* mutants retained the light-induced carotenogenesis, but showed drastic changes in secondary metabolite production. Conidia production of the mutants varied depending on culture conditions: the mutants produced lower amounts of conidia when grown on minimal agar medium, but in shaken cultures, under nitrogen limited conditions their conidia production exceeded that of the wild type (Estrada and Avalos, 2008). Knockout *wc1* mutants of *Fusarium oxysporum* were deficient in surface hydrophobicity and virulence on immune-depressed mice, but retained their virulence on tomato plants (Ruiz-Roldán et al., 2008). Deletion of the *blr-1* (*wc-1*) and *blr-2* (*wc-2*) genes of *Trichoderma atroviride* resulted in increased vegetative growth and blocked photo-conidiation; furthermore, the two *blr* genes proved to be essential for the light-induced expression of the photolyase encoding gene, *phr-1* (Casas-Flores et al., 2004). In *Bipolaris oryzae*, no differences were found in hyphal development and conidiophore formation between the wild type and its *blr1* (*wc-1*) and *blr2* (*wc-2*) gene disruption mutants, but the *blr1* and *blr2* disruptants were unable to produce conidia from the seemingly normal, light-induced conidiophores (Kihara et al., 2006; Moriwaki et al., 2008). Contrary to the situation in *Neurospora*, sexual development in *Aspergillus nidulans* occurs mainly in the dark. Disruption of *lreA* (*wc-1*) and *lreB* (*wc-2*) in *Aspergillus nidulans* caused 70% and 30% reduction, respectively in cleistothecia formation, as compared to that of the wild type, when fungi were grown in the dark. White light illumination caused a drastic, but still not complete reduction of cleistothecia formation in the *lreA* and *lreB* disruptants (Purschwitz et al., 2008).

Reasons of the diversity of phenotypes associated with mutations of the *wc* genes in different fungal taxa are not completely understood. Phylogenetic comparison of fungal

proteins from the WC1 family revealed high conservation of the relevant functional domains (Estrada and Avalos, 2008), suggesting that sources of phenotypic divergences are probably others than structural differences of the *wc* genes. Regulation of the white collar based photoreception, on the other hand is highly complicated. Studies on transcription of *wc-1* in *N. crassa* led to the identification of three distinct promoters on this gene: one of them is induced by light, the other is activated by WCC and the third is presumably involved in the production of a truncated WC1 isoform. Furthermore, the WC-1 protein positively regulates the expression of the *wc-1* gene, contributing thus to the stabilization of the circadian clock (Káldi et al., 2006). Just an opposite self-regulatory role of the functionally active WcoA protein was reported in *F. fujikuroi*, where the expression of *wcoA* was suppressed by the WcoA protein (Estrada and Avalos, 2008) indicating that species-specific regulatory differences may contribute to the functional diversity of *wc* genes found for different fungal taxa.

Modification of the WCC at protein level is also an important factor in its regulation: blue light induces a rapid post-translational phosphorylation of WC-1 (Schwerdtfeger and Linden, 2000). Hyper-phosphorylation of WCC was subsequently shown to alter its binding activity to promoters of the target genes, a cascade of events playing important role in photo-responses (He and Liu, 2005). Furthermore, other photoreceptor proteins, like cryptochromes, phytochromes, and rhodopsins can also modify the activity of the white collar complex, as has recently been shown in the case of the WCC-based photo-activation of the conidiation specific *con10* gene in *N. crassa* (Olmedo et al., 2010). This complex regulation of the WCC-driven photoreception certainly contributes to the diversity of phenotypes found for the WCC-deficient mutants of different fungi.

To extend knowledge on phenotypes associated with WCC mutations in phytopathogenic fungi we cloned the *wc-1* and *wc-2* orthologues from *Fusarium verticillioides* (teleomorph: *Gibberella moniliformis*), a cosmopolitan pathogen of maize that synthesizes a range of secondary metabolites, including fumonisins and carotenoids. We produced gene disruption mutants for these two genes with the aim to examine the effects of inactivation of the WCC on conidiation, sexual reproduction, and plant tissue colonization capability. Our results suggest further, that *MAT1-2-1*, the mating type gene up-regulates transcription of the *wc* genes under conditions favoring sexual reproduction.

## Materials and Methods

### *Fungal strains, growth conditions, phenotypic analyses*

*F. verticillioides* strain FGSC 7603 (genotype: MATA-2) wild type, its  $\Delta FvMAT1-2-1/M15$  mutant, lacking a functional *FvMAT1-2-1* gene, produced by Keszthelyi et al. (2007) and three independent *Fvwc1* ( $\Delta Fvwc1$ -KO1,  $\Delta Fvwc1$ -KO3,  $\Delta Fvwc1$ -KO6) and *Fvwc2* ( $\Delta Fvwc2$ -KO1,  $\Delta Fvwc2$ -KO10,  $\Delta Fvwc2$ -KO11) mutants were maintained as conidial suspensions in 15% glycerol at -70 °C. Complete medium (CM), carrot agar (CA) (Leslie and Summerell, 2006), DG agar (Estrada and Avalos, 2008), and potato-dextrose agar (PDA, Reanal, Budapest, Hungary) were used to compare growth, morphology, co-

nidiation, and mating capability of the fungi. Incubations occurred at 25°C either in the dark, or under continuous illumination, or under a diurnal cycle of 12/12 h light and darkness at 22/20 °C. Light-grown cultures were exposed to 100 lux illumination produced by a battery of three cool white and one black light tubes.

Conidia were washed off by intense agitation in standard amounts of physiological saline and were quantified by direct counting on a Bürker-chamber. (Macroconidia that are rare in most isolates of *F. verticillioides* were not observed under the culture conditions used in this research and, therefore the term conidia, as we use refers to microconidia throughout the text.) Sexual crossings were performed according to the protocol described by Leslie and Summerell (2006). Colonization capability of the fungi was assessed by inoculating 10 µl conidium suspension (10<sup>8</sup> conidia ml<sup>-1</sup>) into surface-disinfected tomato fruits according to Di Pietro et al. (2001). Inoculated fruits were incubated at 25 °C under a diurnal cycle of 12/12 h light and darkness. Fungal growth was assessed by measuring colony diameters at 24-h-intervals.

#### *Cloning and disruption of the white collar genes of F. verticillioides*

Degenerate oligonucleotide primers (Table 1) were used to PCR-amplify *wc1* and *wc2* sequences from genomic DNA of *F. verticillioides* FGSC 7603. The entire genes, designated as *Fvwc1* and *Fvwc2*, respectively were amplified by using single oligonucleotide nested (SON) PCR (Antal et al., 2004) and cloned into pGEM-t Easy (Promega, Madison, WI, USA) as described previously (Ádám et al., 2008). As there is no *XbaI* restriction site on the original *Fvwc1* sequence, the entire *wc1* gene amplified with the WC1\_f8/WC1\_SON2 primer pair was blunt-end-ligated into the *EcoRV* site of pBS. This plasmid was digested with *XbaI-SacI* and an *XbaI-HindIII* fragment of the *hygB* cassette and a *HindIII-SacI* fragment of the C-terminal part of the *Fvwc1* gene (amplified by WC1\_2827/WC1r\_3822 primers) were ligated into this restriction site, yielding the plasmid pBSFvWC1/hph (Fig. 1A). To produce  $\Delta Fvwc2$  gene disruption mutants, a 747 bp *XbaI-ClaI* fragment of the *Fvwc2* sequence was replaced with a 3805 bp hygromycin expression cassette containing the *hygB* (hygromycin B phosphotransferase, *hph*) gene from *Escherichia coli* yielding the plasmid, pBSFvWC2/hph (Fig. 1B). PCR fragments, amplified with the primer pair, M13for/M13rev from the plasmids, pBSFvWC1/hph and pBSFvWC2/hph were used to transform fungal protoplasts. The site specific integration of the *hph* cassette in stable transformants obtained after repeated transfers to hygromycin-containing medium was confirmed by Southern hybridization (Fig. 1C) using a fragment of the *hph* gene amplified with the *hph\_check1* and *hph\_check2* primers and PCR (Fig. 1D) using the primer pairs WC1for1549/Wc1rev3009, Test4519for/WC1rev3848, WC2for2266/WC2rev3506, and Test4519for/WC2rev3989 (Table 1).

#### *Measurement of Fvwc1 and Fvwc2 expression by quantitative real time (qrt)-PCR*

Expression levels of *Fvwc1*, *Fvwc2*, and *FvMAT1-2-1* were measured by quantitative real-time (qrt)-PCR as described earlier using the  $\Delta\Delta C_T$ -method with some modifications (Livak and Schmittgen, 2001). RNA polymerase II was used as a reference

**Table 1**  
Primers used for PCR amplification of *F<sub>wc1</sub>*, *F<sub>wc2</sub>* and *hph* sequences

Primer	Nucleotide sequence (5' → 3')	Description
dWc1for	GTID TCH GAY AAY TTC CAR AAC C	to amplify W1, a fragment of <i>F<sub>wc1</sub></i> , forward
dWc1rev	CKG GVG TRT TYC KNG TRT GGCA	to amplify W1, a fragment of <i>F<sub>wc1</sub></i> , reverse
dWc2for	TKA CCG ART TYA CMA AGC G	to amplify W2, a fragment of <i>F<sub>wc2</sub></i> , forward
dWc2rev	AGG CRT TGC ABA GYG TCT T	to amplify W2, a fragment of <i>F<sub>wc2</sub></i> , reverse
RNApII_for	GAT AGT CTG CCA CAA CTG TA	to normalize gene expression in quantitative RT-PCR with the gene RNA
RNApII_rev	TCT TCA TCG ACT GTA ACT TC	polymerase II
Wc1_for	TCA CCT CTG AIT GGC ATA AGC	to quantify the expression of the <i>F<sub>wc1</sub></i> gene, forward
Wc1_rev	AAT GAT GCA CTT TCG ACC CTT	to quantify the expression of the <i>F<sub>wc1</sub></i> gene, reverse
Wc2_for	CGT CTC CTC GGG AAC CTT	to quantify the expression of the <i>F<sub>wc2</sub></i> gene, forward
Wc2_rev	GAT CTC GCT CCA TCG GAA TTG	to quantify the expression of the <i>F<sub>wc2</sub></i> gene, reverse
Wc1for1549	GAC TCG AAG GAA AAG ACG AT	to check <i>F<sub>wc1</sub></i> deletion, forward
Wc1rev3009	AGC AGG CTG TGA AAT ATGG	to check <i>F<sub>wc1</sub></i> deletion, reverse
Test4519for	CAG TTC TTC TCG GCG TTC TGG	to check site-specific integration of the <i>hph</i> cassette
Wc1rev3848	AGC GTG AAG AAA GAA AAG TG	to check site-specific integration of the <i>hph</i> cassette
Wc2for2266	CCA ATC CAA ATA ATC AAT CG	to check <i>F<sub>wc2</sub></i> deletion, forward
Wc2rev3506	GTA TTG GCC CAC AAT GAA TA	to check <i>F<sub>wc2</sub></i> deletion, reverse
Wc2rev3989	GAT GGC GAA TGA ATT TGT AT	to check site-specific integration of the <i>hph</i> cassette
hph_check1	GGC GCA GAC CGG GAA CACA	<i>E. coli hph</i> (Z32698.1) forward
hph_check2	CAC GGC GGG AGA TGC AAT AGG TC	<i>E. coli hph</i> (Z32698.1) reverse
Wc1_8for	CTTCTTGGCATAFACCCGTCCT	to amplify the N-terminal part of the entire <i>wc1</i> gene, forward
Wc1_SON2rev	CTTGTCTTGGTTATCGCGGAC	to amplify the N-terminal part of the entire <i>wc1</i> gene, reverse
Wc1_for2827	TTGCAAGCCCAGACGACTCT	to amplify the C-terminal part of the entire <i>wc1</i> gene, forward
Wc1_rev3822	GTATCACAAAATGCCGTTTAATC	to amplify C-terminal part of the entire <i>wc1</i> gene, reverse
M13 for	GTA AAA CGA CGG CCA GT	to amplify full length disruption fragment for protoplast transformation
M13 rev	CAG GAA ACA GCT ATG AC	to amplify full length disruption fragment for protoplast transformation

gene (Radonić et al., 2004). Validation of reference gene and target gene primers was performed according to Livak and Schmittgen (2001). Qrt-PCR was carried out using the Biorad MiniOpticon system with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The primer pairs WC1\_for/ WC1\_rev and WC2\_for/WC2\_rev (Table 1) amplified a 338 and a 302 bp DNA fragment, respectively. Identity of the fragments was checked by sequencing.

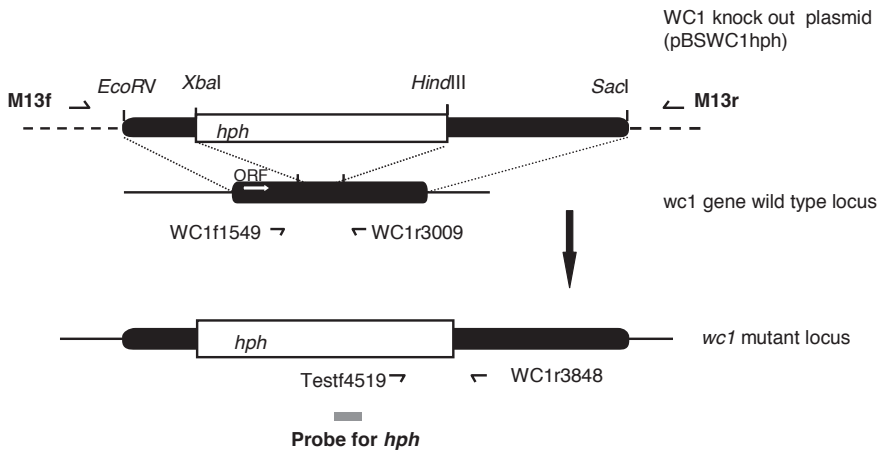


Fig. 1A. Targeted disruption of *Fvwc1* and *Fvwc2* genes. Schematic illustration of the *Fvwc1* gene replacement strategy. Primer pairs used for the PCR analysis of site-specific integration of hph cassette are depicted.

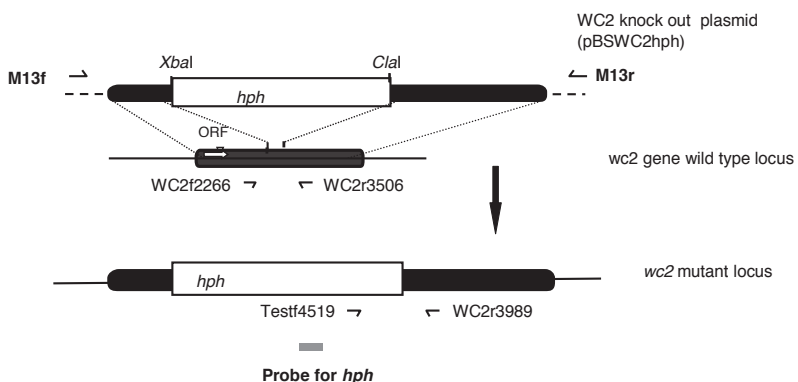


Fig. 1B. Targeted disruption of *Fvwc1* and *Fvwc2* genes. Schematic illustration of the *Fvwc2* gene replacement strategy. Primer pairs used for the PCR analysis of site-specific integration of hph cassette are depicted.



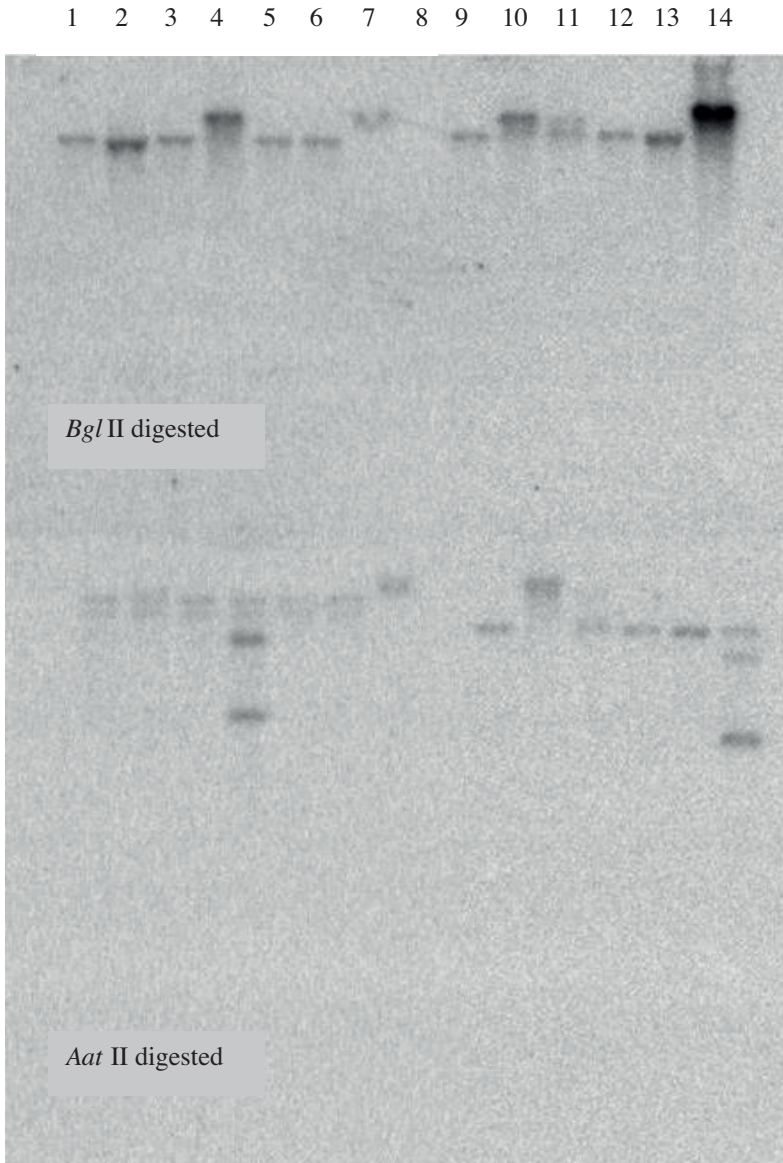


Fig. 1C. Targeted disruption of *Fvwc1* and *Fvwc2* genes. Southern analysis indicates single copy integration of the *hph* cassette into the genome of the transformed wild type strain, FGSC 7603. *Bgl*III and *Aat*II digested DNA samples of the wt strain (lane 7), the  $\Delta FvMAT1-2-1$  mutant (lane 8) and 12 putative  $\Delta Fvwc1$  and  $\Delta Fvwc2$  mutants of *F. verticillioides* (lanes 1-6 and 9-14, respectively) were probed with a 661 bp fragment of the *hph* gene generated with the *hph\_check1* and *hph\_check2* primers. *Aat*II cuts once in the *hph* sequence, whereas *Bgl*III has no recognition site on this sequence. Mutants  $\Delta Fvwc1$ -KO1,  $\Delta Fvwc1$ -KO3,  $\Delta Fvwc1$ -KO6 and  $\Delta Fvwc2$ -KO1,  $\Delta Fvwc2$ -KO10,  $\Delta Fvwc2$ -KO11 (lanes –1, 3, 6 and 9, 12, 13), that gave one and two (or two co-migrating) bands after *Bgl*III and *Aat*II digestion, respectively were used in further experiments.

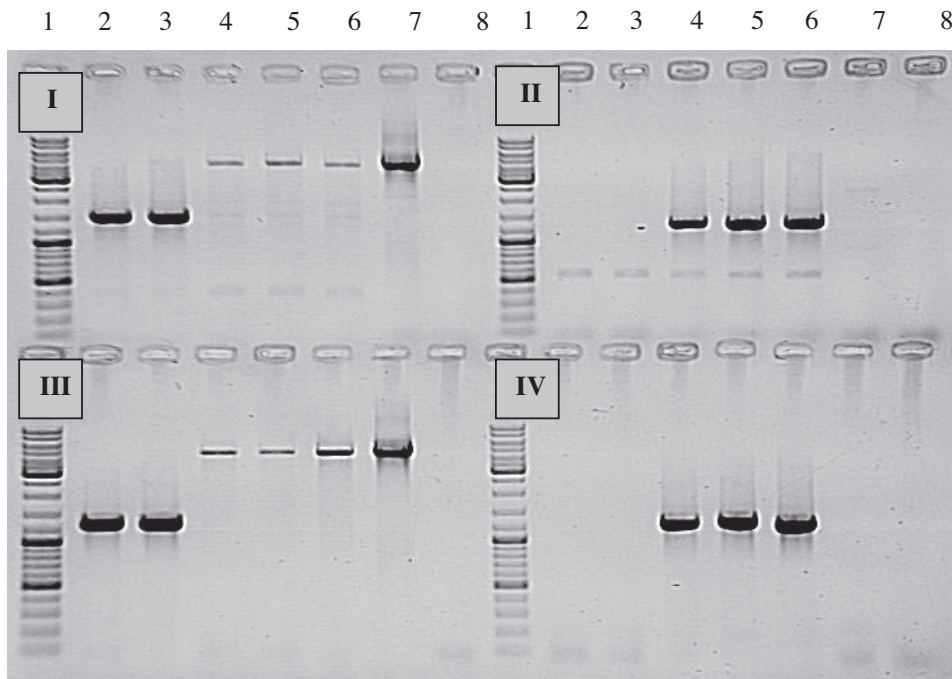


Fig. 1D. Targeted disruption of *Fvwc1* and *Fvwc2* genes. Site-specific integration of the *hph* cassette as demonstrated by PCR analysis. (I) Amplification by the primer pair, WC1for1549/Wc1rev3009 amplified the expected 1479 and 4276 bp fragments in the wild type and the  $\Delta Fvwc1$  mutants, respectively. (II) Amplification with the primer pair, Test4519for/WC1rev3848 resulted in a 1287 bp fragment in the  $\Delta Fvwc1$  mutants, but no fragment appeared in the wt. (III) Primers, WC2for2266/WC2rev3506 amplified the expected 1260 and 4312 bp fragments in the wild type and the  $\Delta Fvwc2$  mutants, respectively. (IV) Primers, Test4519for/WC2rev3989 gave no fragment in the wt, but amplified a 1233 bp fragment in the  $\Delta Fvwc2$  mutants. Lanes are: 1- Fermentas gene ruler ladder mix, 2, 3 – wild type strain, 8 – blank; I-II/4 -  $\Delta Fvwc1$ -KO1, I-II/5 -  $\Delta Fvwc1$ -KO3, I-II/6 -  $\Delta Fvwc1$ -KO6, I-II/7 – plasmid pBSFvWC1/hph; III-IV/4 -  $\Delta Fvwc2$ -KO1, III-IV/5 -  $\Delta Fvwc2$ -KO10, III-IV/6 -  $\Delta Fvwc2$ -KO11, III-IV/7 – plasmid pBSFvWC2/hph.

## Results

### *Cloning and sequence of Fvwc1 and Fvwc2 genes of F. verticillioides*

By using degenerate primer pairs, dWC1for/dWC1rev and dWC2for/dWC2rev designed from white collar sequences of *Gibberella zeae* (*wc-1*: XM\_388117, *wc-2*: XM\_380886), *Magnaporthe grisea* (*wc-1*: XM\_360995, *wc-2*: XM\_362076), *Hypocrea jecorina* (*wc-1*: AY823264, *wc-2*: AY823265), *Trichoderma atroviridae* (*wc-1*: AY628431, *wc-2*: AY628432) and *Neurospora crassa* (*wc-1*: XM\_954684, *wc-2*: XM\_958726) two ~1,000 bp sequences, designated W1 and W2, respectively were PCR amplified from *F. verticillioides* FGSC 7603. The W1 and W2 sequences shared significant similarity with sequences of white collar genes of the above mentioned fungus species. The flanking re-



gions of these potential *wc* gene tags were amplified by using SON PCR and the products were cloned and sequenced. The GenBank accession numbers for the nucleotide sequence of *Fvwc1* and *Fvwc2*, putative white collar genes of *F. verticillioides* are HM045019 and HM045020, respectively. Both low stringency Southern hybridization and *in silico* genome mining ([http://www.broadinstitute.org/annotation/genome/fusarium\\_verticillioides/MultiHome.html](http://www.broadinstitute.org/annotation/genome/fusarium_verticillioides/MultiHome.html)), indicated that *Fvwc1* and *Fvwc2* are single copy genes and there are no other closely related sequences in the genome of *F. verticillioides*.

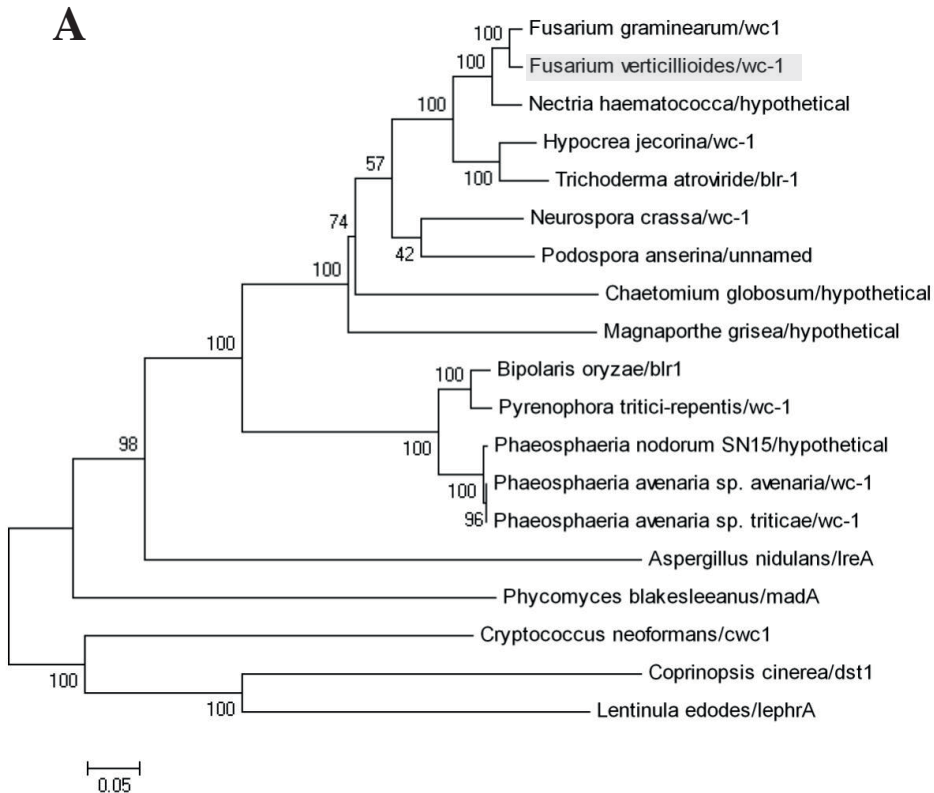


Fig. 2A. Phylograms of 19 fungal proteins from the WC1 family. Species and accession numbers: *Aspergillus nidulans* (LREA, AAP47230), *Bipolaris oryzae* (BLR1, BAF35570), *Chaetomium globosum* (XP\_001219613), *Coprinopsis cinerea* (Dst1, BAD99145), *Cryptococcus neoformans* (Cwc-1, AAT73612), *Fusarium graminearum* (WC1 FGSG\_07941), *Fusarium verticillioides* (FVWC1, HM045019), *Hypocrea jecorina* (wc-1, AAV80185), *Lentinula edodes* (LephRA, BAF56991), *Magnaporthe grisea* (XP\_360995), *Nectria haematococca* (EEU35002), *Neurospora crassa* (WC-1, Q01371), *Phaeosphaeria avenaria* f.sp. *avenaria* (WC-1, ACS74812), *Phaeosphaeria avenaria* f.sp. *triticae* (WC-1, ACS74819), *Phaeosphaeria nodorum* (EAT80456), *Phycomyces blakesleeanus* (MadA, ABB77846), *Podospora anserina* (CAD60767), *Pyrenophora tritici-repentis* (WC-1, XP\_001933567), *Trichoderma atroviride* (BLR-1, AAU14171). Bootstrap values greater than 50% are indicated at branch nodes. Grey shaded quadrates indicate the position of *Fusarium verticillioides* WC1 and WC2 proteins, respectively.

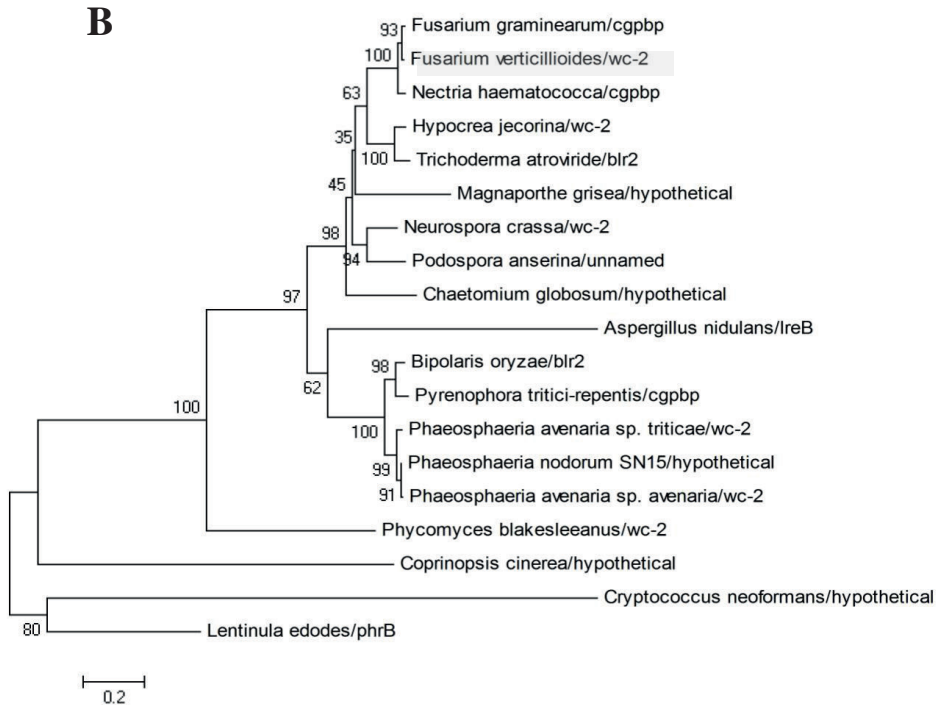


Fig. 2B. Phylograms of 19 fungal proteins from the WC2 family. Species and accession numbers: *Aspergillus nidulans* (LREB, AAP47576), *Bipolaris oryzae* (BLR2, BAF47401), *Chaetomium globosum* (XP\_001221143), *Coprinopsis cinerea* (XP\_001833033), *Cryptococcus neoformans* (AAW72938), *Fusarium graminearum* (CGPBP, cutinase gene palindrome-binding protein, FGSG\_00710), *Fusarium verticillioides* (FVWC2 HM045020), *Hypocrea jecorina* (wc-2, AAV80186), *Lentinula edodes* (phrB, BAH57971), *Magnaporthe grisea* (XP\_362076), *Nectria haematococca* (CGPBP, Q00858), *Neurospora crassa* (WC-2, XP\_963819), *Phaeosphaeria avenaria* f.sp. *avenaria* (WC-2 ACT46731), *Phaeosphaeria avenaria* f.sp. *triticae* (WC-2, ACT46736), *Phaeosphaeria nodorum* (XP\_001804394), *Phycomyces blakesleeanus* (WC-2, CAQ77079), *Podospora anserina* (XP\_001912380), *Pyrenophora tritici-repentis* (CGPBP, XP\_001934846), *Trichoderma atroviride* (BLR-2, AAU14172). Bootstrap values greater than 50% are indicated at branch nodes. Grey shaded quadrates indicate the position of *Fusarium verticillioides* WC1 and WC2 proteins, respectively.

The predicted 1023 aa FvWC1 protein contained all conserved functional domains typical of WC1 proteins from fungi, including the PAS1/LOV domain (aa 348-466), the two other PAS dimerization domains (aa 547-645 and 669-766, respectively), a GATA-type Zn-finger DNA binding domain (aa 879-918) and an NLS (nuclear localization signal) sequence (aa 860-872). The predicted 450 aa FvWC2 protein contained the PAS domain (aa 171-254), the Zn finger domain (aa 463-523), and the NLS sequence (aa 450-461).

The predicted WC proteins of *F. verticillioides* showed various degrees of identity with white collar proteins from other fungi as presented in Fig. 2A, B. Phylogenetic comparison of deduced WC1 and WC2 proteins from 19 fungus species, where sequence

data for both *wc* genes are available demonstrated that (i) the *wc1* and *wc2* orthologues are equally highly conserved and (ii) the phylogenetic relationships of the individual WC proteins match the taxonomic affiliation of the fungus species they had been cloned from.

*Effect of the Fvwc1 and Fvwc2 mutations on conidiation, sexual sporulation and invasive growth in plant tissues*

To examine the function of the WCC in *F. verticillioides*,  $\Delta Fvwc1$  and  $\Delta Fvwc2$  mutants were generated by using the *hph* gene disruption construct. Independent stable transformants that showed PCR and Southern hybridization patterns consistent with disruption of *Fvwc1* and *Fvwc2* by double recombination events were selected as described previously (Keszthelyi et al., 2007). *F. verticillioides* FGSC 7603 and its three independent *wc1* and *wc2* mutants ( $\Delta Fvwc1$ -KO1,  $\Delta Fvwc1$ -KO3,  $\Delta Fvwc1$ -KO6 and  $\Delta Fvwc2$ -KO1,  $\Delta Fvwc2$ -KO10,  $\Delta Fvwc2$ -KO11, respectively) were subjected to phenotype analyses.

No significant differences were observed in colony morphology, growth rate, and conidium production of the wild type and its  $\Delta Fvwc1$  and  $\Delta Fvwc2$  mutants grown on CA, CM, and PDA under diurnal illumination at 25 °C for seven days. Previous studies on *F. fujikuroi* showed that FKMC1995, a reference strain of this fungus produced higher amounts of conidia in the dark, than under continuous illumination (Estrada and Avalos, 2008). This was an unexpected result as a number of former papers reported that near-uv or cool white light enhances conidiation in most *Fusarium* species studied thus far, including another strain (IMI 58289) of *F. fujikuroi* (Avalos et al., 1985). To see the effect of illumination on conidium production in *F. verticillioides*, the wild type strain, FGSC 7603 and its two WCC-mutants,  $\Delta Fvwc1$ -KO1 and  $\Delta Fvwc2$ -KO11 were grown under the same conditions used by Estrada and Avalos (2008): incubation occurred on DG agar, under N-limited conditions at 25°C for 120 h, either in continuous light or in the dark. In this experiment the wt cultures produced significantly less conidia when grown in continuous light, as compared to cultures incubated in total darkness indicating that, similarly to the situation found for *F. fujikuroi*, light has no stimulatory effect on conidiation in *F. verticillioides*. On the other hand, the *wc* mutants produced nearly equal amounts of conidia when grown either under continuous illumination or in the dark (Fig. 3). To determine whether *wc* mutations altered surface hydrophobicity of the fungal envelop, like found for *F. fujikuroi* by Estrada and Avalos (2008), water droplets were placed on the surface of 5-day-old colonies grown on DG. The water droplets remained in a seemingly intact form on the hydrophobic surface of cultures of both the wild type and its six  $\Delta Fvwc$  gene disruption mutants, but afterwards the droplets had gradually soaked into the surface of the mutant cultures. After 20 h incubation, the water drops were present only on the surface of the wt culture suggesting that functional *wc* genes are required for maintaining hydrophobicity of aerial hyphae (data not shown).

Invasive growth of the wild type and its *Fvwc* gene disruption mutants ( $\Delta Fvwc1$ -KO1,  $\Delta Fvwc1$ -KO6 and  $\Delta Fvwc2$ -KO1,  $\Delta Fvwc2$ -KO10, respectively) was compared on intact tomato fruits, inoculated with conidial suspensions. Diameters of colonies developed were measured at 24-h-intervals for eight days (Fig. 4). All strains produced visible symptoms on the third day after conidial inoculation and colonized the

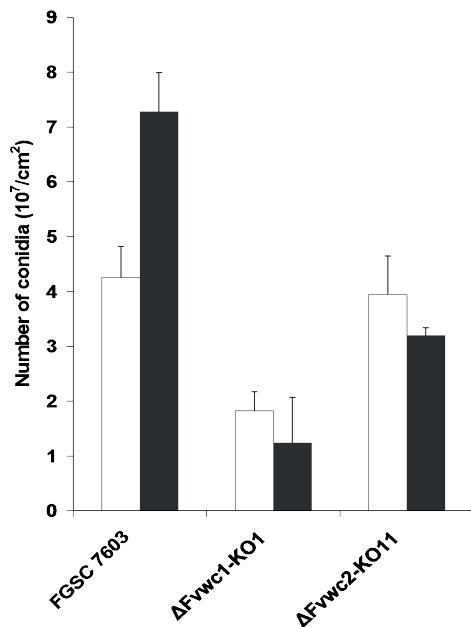


Fig. 3. Effect of deletion of *Fvwc1* and *Fvwc2* on conidium production. Fungi were grown on DG agar, at 24 °C for 120 h in continuous light (white columns) and in the dark (black columns). Average and standard deviations from three independent experiments are shown.

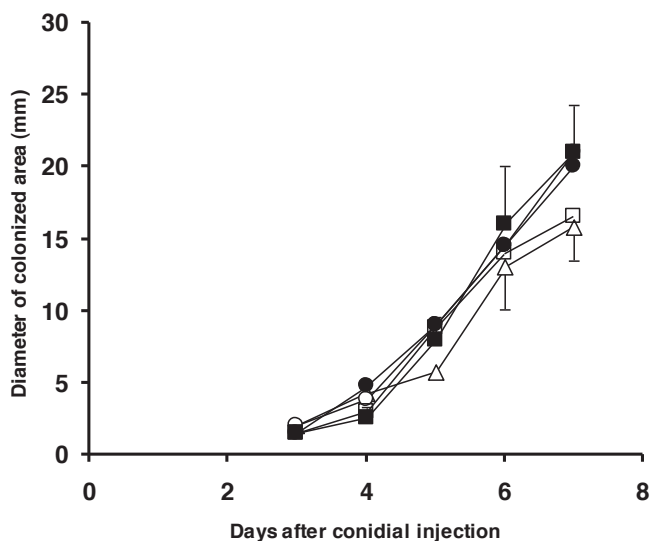


Fig. 4. Diameters of colonies developed after injecting conidial suspensions ( $10^8$  conidia  $\text{ml}^{-1}$ ) of the wild type strain, *F. verticillioides* FGSC 7603 ( $\Delta$ ) and its  $\Delta Fvwc1/1$  ( $\blacksquare$ ),  $\Delta Fvwc1/6$  ( $\bullet$ ),  $\Delta Fvwc2/1$  ( $\square$ ), and  $\Delta Fvwc2/10$  ( $\circ$ ) gene disruption deletion mutants into healthy tomato fruits.

tissues around the site of inoculation, forming a dense aerial mycelial mat on the fruit surface indicating that disruption of the white collar genes caused no loss in colonization capacity of the mutants. Microscopic observation showed that this mycelial mat was produced by colonizing hyphae that emerged from the site of inoculation.

All six  $\Delta Fvwc1$  and  $\Delta Fvwc2$  mutants and the wild type, FGSC 7603 were equally fertile when used as males in crosses with FGSC 7600, the mating type tester strain of *F. verticillioides*. In these crosses abundant mature perithecia (60–110 per cm<sup>2</sup>) developed after three weeks' incubation on carrot agar. When the wild type, FGSC 7603 was used as a female partner in crosses with FGSC 7600 as male, again normal perithecium development was observed. However, when the WCC disrupted mutants were used as females in crosses with FGSC 7600 as male, no perithecia were formed, indicating that inactivation of any component of the WCC led to total female sterility without affecting male fertility.

#### *Expression of the wc genes is up-regulated by the mating type gene, mat1-2-1*

To determine the effect of illumination on the transcription of *Fvwc1* and *Fvwc2*, the wild type strain was grown on DG medium for 120 h in the dark and then illuminated for 1 h. RNA samples were collected either at the end of the dark period or after 1 h illumination. Both *wc* genes showed constitutive expression in the wild type cultures incubated in the dark and exposure to light caused no significant increases in their transcription (Fig. 5).

In a previous study of this laboratory the possible involvement of the mating type (*mat*) genes in fungal processes unrelated to sexual reproduction was suggested by ana-

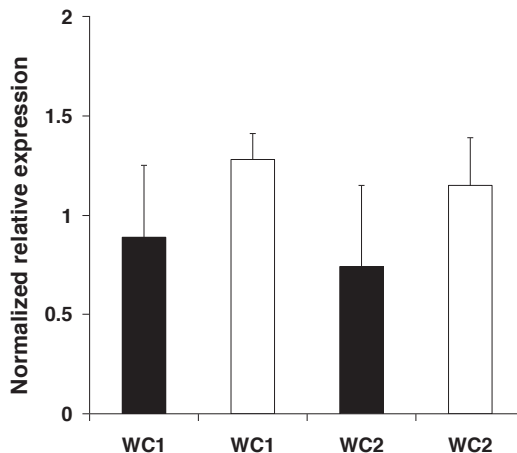


Fig. 5. Expression of *Fvwc1* and *Fvwc2* in *F. verticillioides* FGSC 7603, on DG medium for 120 h in the dark (black columns) and after 1 h illumination (white columns). mRNA levels were monitored by qrt-PCR. Data, calculated by  $\Delta\Delta C_T$  method were expressed in relative units (zero time expression in the wild type strain at the start of illumination is equal to 1). The results are means of two independent biological repetitions, run in duplicates. Vertical bars indicate standard errors.

lyzing transcript profiles of a wild type strain of *F. verticillioides* and its  $\Delta Fvmat1-2-1$  mutant by using a differential cDNA hybridization technique. The majority of the annotated ~200 ESTs found to be either down- or up-regulated in the mutant had no known role in sexual development (Keszthelyi et al. 2007). One of the sequence tags (clone 241) down-regulated in the mutant was a *wc* homologue. To determine the extent of this putative positive stimulatory effect of the MAT1-2-1 product on any of the white collar genes, we compared transcription of *Fvwc1* and *Fvwc2* in the wild type strain, FGSC 7603 and its  $\Delta Fvmat1-2-1$  mutant. Fungi were cultured on CA for six days at 25 °C under a diurnal cycle of 12/12 h light and darkness, conditions favouring mating in *Fusarium* species (Leslie and Summerell, 2006). Samples collected at 126 h (in the middle of the final light period) were subjected to RNA isolation and qrt-PCR was used to measure transcript levels of *Fvwc1* and *Fvwc2*. Both *Fvwc1* and *Fvwc2* were found to be slightly but significantly down-regulated in the  $\Delta FvMAT1-2-1$  mutant as compared to the wild type (Fig. 6A, B) indicating that the MAT1-2-1 gene product has indeed a positive regulatory effect on the white collar genes under conditions that stimulate sexual reproduction and transcription of the *FvMAT1-2-1* gene (Keszthelyi et al., 2007).

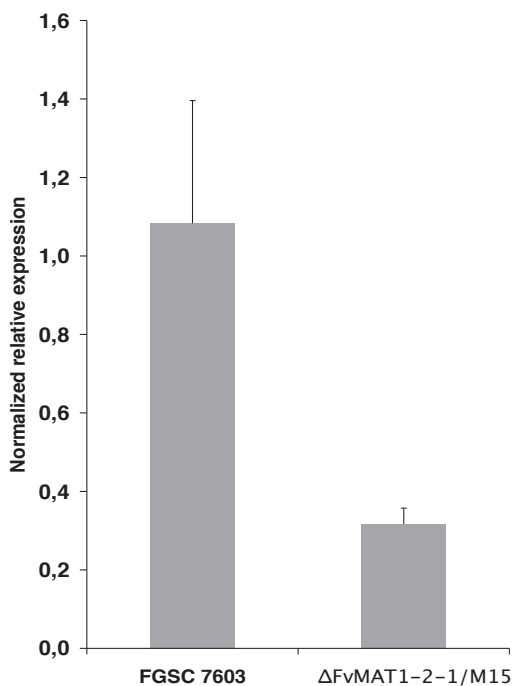


Fig. 6A. Expression of *Fvwc1* in the wild type strain, FGSC 7603 and its  $\Delta FvMAT1-2-1/M15$  gene disruption mutant grown on CA for six days at 25 °C under a diurnal cycle of 12/12 h light/darkness and sampled in the middle of the last light period. mRNA levels were monitored by qrt-PCR. Data, calculated by  $\Delta\Delta C_T$  method were expressed in relative units. The results are means of two independent biological repetitions, run in duplicates. Vertical bars indicate standard errors.



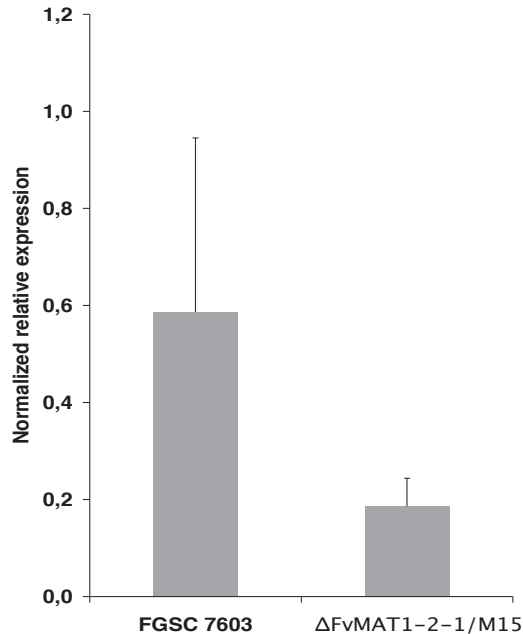


Fig. 6B. Expression of *Fvwc2* in the wild type strain, FGSC 7603 and its  $\Delta FvMAT1-2-1/M15$  gene disruption mutant grown and sampled as given in Fig. 6A. mRNA levels were monitored by qrt-PCR. Data, calculated by  $\Delta\Delta C_T$  method were expressed in relative units. The results are means of two independent biological repetitions, run in duplicates. Vertical bars indicate standard errors.

## Discussion

Contrary to the situation found in *N. crassa* and *A. nidulans*, where mutation of the *wc* genes caused drastic, but not complete loss of sexual fertility (Degli-Innocenti and Russo, 1984; Purschwitz et al., 2008), disruption of the white collar complex in *F. verticillioides* led to complete female sterility without any adverse effect on male fertility. This phenotype was typical of all six independent  $\Delta Fvwc1$  and  $\Delta Fvwc2$  gene disruption mutants studied in this experiment indicating that miss-function of WCC affects sexual reproduction in a species-specific or group-specific manner in filamentous ascomycetes.

Contrary to other fungi, like *B. oryzae*, *N. crassa* or *T. atroviride*, where conidiation is induced by blue light and stimulated by illumination (Casas-Flores et al., 2004; Kihara et al., 2007; Lauter and Russo, 1991; Moriwaki et al., 2008), light had no positive effect on conidium production in *F. fujikuroi* furthermore, both the wild type strain and two *wcA* (= *wc1*) disruption mutants of this fungus produced higher amounts of conidia, when incubated in the dark as compared to incubation in continuous light (Estrada and Avalos, 2008). In our experiments FGSC 7603, the wild type strain of *F. verticillioides* also produced more conidia in the dark than under continuous illumination, confirming that asexual sporulation is not stimulated by cool white and near-uv light in *Fusarium* or at least not in species belonging to the section *Liseola* of this genus. In the absence of pho-

to-conidiation, it was logical to find, that disruption of the blue light photoreceptor system had no adverse effect on conidia production in *F. verticillioides*. Although the present experiments showed that expression of the *wc* genes are not stimulated by light in *F. verticillioides*, this finding does not exclude the involvement of these genes in light-regulated processes, like secondary metabolite production (Estrada and Avalos, 2008) or sexual reproduction (this study).

Due to the prevalence of female sterility in field populations of *F. verticillioides* only a low percentage of the population participates in sexual reproduction and this generally happens at the end of the growing season (Chulze et al., 2000). Asexually produced conidia are, therefore the major source of spread of this pathogen under epidemic conditions, but the conidiation process is stimulated by factors others than light and this have to be considered in epidemiological studies.

To the best of our knowledge, there is a single report on the role of *wc* genes in fungal pathogenicity: Ruiz-Roldan et al. (2008) showed that a  $\Delta wc1$  gene disruption mutant of *F. oxysporum* retained its full virulence in tomato root infection assay. (On the other hand, this mutant showed reduced virulence on immune-suppressed mice.) Our results confirm that the white collar complex is probably dispensable for pathogenicity in plant pathogenic fungi, at least in species that use direct penetration or wounds when initiate infection.

WC1 positively regulates its own expression in *N. crassa* creating a feedback loop that helps to stabilize the circadian clockwork (Káldi et al., 2006). The novelty of the present study was to demonstrate the stimulatory influence of the mating type gene, *mat1-2-1* on the white collar genes. [The photo-induced carotenoid biosynthesis is also positively regulated by *mat1-2-1* as found recently by Ádám et al. (2011).] The *mat* genes may thus contribute to the complexity of photo-regulation of sexual and asexual sporulation. Such traits greatly vary among individuals of a natural fungus population. Repeated sub-culturing of fungi maintained under laboratory conditions also results in changes either in female fertility or conidiation among the clonal progenies obtained by sub-culturing. Sources of this variation are largely un-known. The complex interactions among the WC proteins, other photoreceptors and additional factors, like the MAT gene product(s) certainly contribute to maintaining the finely tuned photoreception system of fungi. Disturbances in any component of this highly complex regulatory system result in alterations of the photo-induced or photo-stimulated biological traits. The fully harmonized operation of all components of this regulatory system, on the other hand exerts a positive selective impact on natural populations. These considerations help to understand why are functional, constitutively transcribed regulatory genes, like the *wc* and the *mat* genes retained under conditions, where they are seemingly not needed, *i.e.* in the dark or in absence of sexual reproduction.

## Acknowledgements

This research was supported by the grants OTKA, K 76067 and TÁMOP-4.2.1.B-11/2/KMR-2011-0003. We are indebted for support from the Office for Subsidized Research Units of the Hungarian Academy of Sciences.

## Literature

- Antal, Z., Rasclé, C., Fèvre, M. and Bruel, C. (2004): Single oligonucleotide nested PCR: a rapid method for the isolation of genes and their flanking regions from expressed sequence tags. *Curr. Genet.* 46, 240–246.
- Avalos, J. and Estrada, A. F. (2010): Regulation by light in *Fusarium*. *Fungal. Genet. Biol.* 47, 930–938.
- Avalos, J., Casadesús, J. and Cerdá-Olmedo, E. (1985): *Gibberella fujikuroi* mutants obtained with UV radiation and N-methyl-N'-nitro-N-nitrosoguanidine. *Appl. Environ. Microbiol.* 49, 187–191.
- Ádám, A. L., García-Martínez, J., Szűcs, E. P., Avalos, J. and Hornok, L. (2011): The *MAT1-2-1* mating type gene up-regulates photo-inducible carotenoid biosynthesis in *Fusarium verticillioides*. *FEMS Microbiol. Lett.* 318, 76–83.
- Ádám, A. L., Kohut, G., Hornok, L. (2008): Fphog1, a HOG-type MAP kinase gene, is involved in multistress response in *Fusarium proliferatum*. *J. Basic Microbiol.* 48, 151–159.
- Ballario, P., Talora, C., Galli, D., Linden, H. and Macino, G. (1998): Roles in dimerization and blue light photoreponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. *Mol. Microbiol.* 29, 719–729.
- Casas-Flores, S., Rios-Mornberg, M., Bibbins, M., Ponce-Noyola, P., Herrera-Estrella, A. (2004): BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology* 150, 3561–3569.
- Chen, C.-H. and Loros, J. J. (2009): *Neurospora* sees the light. *Commun. Integr. Biol.* 2, 448–451.
- Cheng, P., Yang, Y., Gardner, K. H. and Liu, Y. (2002): PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of *Neurospora*. *Mol. Cell. Biol.* 22, 517–524.
- Chulze, S. N., Torres, A., Ramirez, M. L. and Leslie, J. F. (2000): Genetic variation in *Fusarium* section *Liseola* from no-till maize in Argentina. *Appl. Environ. Microbiol.* 66, 5312–5315.
- Corrochano, L. M. (2007): Fungal photoreceptors: sensory molecules for fungal development and behaviour. *Photochem. Photobiol. Sci.* 6, 725–736.
- Degli-Innocenti, F., Chambers, J. A. and Russo, V. E. (1984): Conidia induce the formation of protoperithecia in *Neurospora crassa*: further characterization of white collar mutants. *J. Bacteriol.* 159, 808–810.
- DiPietro, A., Garcia-Maceira, F. I., Maglecz, E. and Roncero, M. Í. G. (2001): A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. *Mol. Microbiol.* 39, 1140–1150.
- Estrada, A. F. and Avalos, J. (2008): The white collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. *Fungal Genet. Biol.* 45, 705–718.
- Froehlich, A. C., Liu, Y., Loros, J. J. and Dunlap, J. C. (2002): White collar-1, a circadian blue light photoreceptor, binding to the *frequency* promoter. *Science* 297, 815–819.
- Harding, R. W. and Turner, R. V. (1981): Photoregulation of the carotenoid biosynthetic pathway in albino and white collar mutants of *Neurospora crassa*. *Plant Physiol.* 68, 745–749.
- He, Q. and Liu, Y. (2005): Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. *Genes Dev.* 19, 2888–2899.
- Káldi, K., Herreros González, B. and Brunner, M. (2006): Transcriptional regulation of the *Neurospora* circadian clock gene *wc-1* affects the phase of circadian output. *EMBO Rep.* 7, 199–204.
- Keszthelyi, A., Jeney, A., Kerényi, Z., Mendes, O., Waalwijk, C. and Hornok, L. (2007): Tagging target genes of the *MAT1-2-1* transcription factor in *Fusarium verticillioides* (*Gibberella fujikuroi* MP-A). *Anton. Leeuw. Int. J. G.* 91, 373–391.
- Kihara, J., Moriwaki, A., Tanaka, N., Ueno, M. and Arase, S. (2007): Characterization of the *BLR1* gene encoding a putative blue-light regulator in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiol. Lett.* 266, 110–118.
- Lauter, F. and Russo, V. E. A. (1991): Blue light induction of conidiation-specific genes in *Neurospora crassa*. *Nucl. Acids Res.* 19, 6883–6886.

- Leslie, J. F. and Summerell, B. A. (2006): The *Fusarium* Laboratory Manual, Blackwell Publishing, Ames, Iowa.
- Linden, H. and Macino, G. (1997): White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. *EMBO J.* 16, 98–109.
- Linden, H., Rodriguez-Franco, M. and Macino, G. (1997): Mutants of *Neurospora crassa* defective in regulation of blue light perception. *Mol. Gen. Genet.* 254, 111–118.
- Liu, Y., He, Q. and Cheng, P. (2003): Photoreception in *Neurospora*: a tale of two white collar proteins. *Cell Mol. Life Sci.* 60, 2131–2138.
- Livak, K. J. and Schmittgen, T. D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2–Delta C(T) method. *Methods* 25, 402–408.
- Moriwaki, A., Katsube, H., Ueno, M., Arase, S. and Kihara, J. (2008): Cloning and characterization of the *BLR2*, the homologue of the blue-light regulator of *Neurospora crassa* WC-2, in the phytopathogenic fungus *Bipolaris oryzae*. *Curr. Microbiol.* 56, 115–121.
- Olmedo, M., Ruger-Herreros, C., Luque, E. M. and Corrochano, L. M. (2010): A complex photoreceptor system mediates the regulation by light of the conidiation genes *con-10* and *con-6* in *Neurospora crassa*. *Fungal Genet. Biol.* 47, 352–363.
- Purschwitz, J., Müller, S., Kastner, C., Schöser, M., Haas, H., Espeso, E. A., Atoui, A., Calvo, A. M. and Fischer, R. (2008): Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. *Curr. Biol.* 18, 255–259.
- Radonić, A., Thulke, S., Mackay, M. I., Landt, O., Siegert, W. and Nitsche, A. (2004): Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Comm.* 313, 856–862.
- Ruiz-Roldán, M. C., Garre, V., Guarro, J., Mariné, M. and Roncero, M. Í. G. (2008): Role of the white collar 1 photoreceptor in carotenogenesis, UV resistance, hydrophobicity, and virulence of *Fusarium oxysporum*. *Eukaryot. Cell* 7, 1227–1230.
- Schwerdtfeger, C. and Linden, H. (2000): Localization and light-dependent phosphorylation of white collar 1 and 2, the two central components of blue light signalling in *Neurospora crassa*. *Eur. J. Biochem.* 267, 414–422.