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

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

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"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 ∆*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).
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 *IreA* (*wc-1*) and *IreB* (*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 *IreA* and *IreB* 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 (Schwertfeger 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 synthesize 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 ΔFvMAT1-2-1/M15 mutant, lacking a functional FvMAT1-2-1 gene, produced by Keszthelyi et al. (2007) and three independent Fvwc1 (ΔFvwc1-KO1, ΔFvwc1-KO3, ΔFvwc1-KO6) and Fvwc2 (ΔFvwc2-KO1, ΔFvwc2-KO10, ΔFvwc2-KO11) mutants were maintained as co-nidial 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 \textit{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 (10^8 conidia ml^{-1}) 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.

\textit{Cloning and disruption of the white collar genes of F. verticillioides}

Degenerate oligonucleotide primers (Table 1) were used to PCR-amplify \textit{wc1} and \textit{wc2} sequences from genomic DNA of \textit{F. verticillioides} FGSC 7603. The entire genes, designated as \textit{Fvwc1} and \textit{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 \textit{XbaI} restriction site on the original \textit{Fvwc1} sequence, the entire \textit{wc1} gene amplified with the \textit{WC1}_{f8}/\textit{WC1}_{SON2} primer pair was blunt-end-ligated into the \textit{EcoRV} site of \textit{pBS}. This plasmid was digested with \textit{XbaI}-\textit{SacI} and an \textit{XbaI-HindIII} fragment of the \textit{hygB} cassette and a \textit{HindIII-Sacl} fragment of the C-terminal part of the \textit{Fvwc1} gene (amplified by \textit{WC1}_{2827}/\textit{WC1r}_{3822} primers) were ligated into this restriction site, yielding the plasmid \textit{pBSFvWC1/hph} (Fig. 1A). To produce \textit{ΔFvwc2} gene disruption mutants, a 747 bp \textit{XbaI-ClaI} fragment of the \textit{Fvwc2} sequence was replaced with a 3805 bp hygromycin expression cassette containing the \textit{hygB} (hygromycin B phosphotransferase, \textit{hph}) gene from \textit{Escherichia coli} yielding the plasmid, \textit{pBSFvWC2/hph} (Fig. 1B). PCR fragments, amplified with the primer pair, \textit{M13for}/\textit{M13rev} from the plasmids, \textit{pBSFvWC1/hph} and \textit{pBSFvWC2/hph} were used to transform fungal protoplasts. The site specific integration of the \textit{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 \textit{hph} gene amplified with the \textit{hph}_{check1} and \textit{hph}_{check2} primers and PCR (Fig. 1D) using the primer pairs \textit{WC1}_{for1549}/\textit{WC1}_{rev3009}, Test\textit{4519}_{for}/\textit{WC1}_{rev3848}, \textit{WC2}_{for2266}/\textit{WC2}_{rev3506}, and Test\textit{4519}_{for}/\textit{WC2}_{rev3989} (Table 1).

\textit{Measurement of Fvwc1 and Fvwc2 expression by quantitative real time (qrt)-PCR}

Expression levels of \textit{Fvwc1}, \textit{Fvwc2}, and \textit{FvMAT1-2-1} were measured by quantitative real-time (qrt)-PCR as described earlier using the \textit{ΔΔCt}-method with some modifications (Livak and Schmittgen, 2001). RNA polymerase II was used as a reference.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5' → 3')</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>dWC1for</td>
<td>GTD TCH GAY AAY TTC CAR AAC C</td>
<td>to amplify W1, a fragment of <em>Fvwc1</em>, forward</td>
</tr>
<tr>
<td>dWC1rev</td>
<td>CKG GVG TRT TYC KNG TRT GGC A</td>
<td>to amplify W1, a fragment of <em>Fvwc1</em>, reverse</td>
</tr>
<tr>
<td>dWC2for</td>
<td>TKA CCG ART TYA CMA AGC G</td>
<td>to amplify W2, a fragment of <em>Fvwc2</em>, forward</td>
</tr>
<tr>
<td>dWC2rev</td>
<td>AGG CRT TGC ABA GYG TCT T</td>
<td>to amplify W2, a fragment of <em>Fvwc2</em>, reverse</td>
</tr>
<tr>
<td>RNAplII_for</td>
<td>GAT AGT CTG CCA CAA CTG TA</td>
<td>to normalize gene expression in quantitative RT-PCR with the gene RNA</td>
</tr>
<tr>
<td>RNAplII_rev</td>
<td>TCT TCA TCG ACT GTA ACT TC</td>
<td>polymerase II</td>
</tr>
<tr>
<td>WC1_for</td>
<td>TCA CCT CTG ATT GCC ATA AGC</td>
<td>to quantify the expression of the <em>Fvwc1</em> gene, forward</td>
</tr>
<tr>
<td>WC1_rev</td>
<td>AAT GAT GCA CTT TCG ACC CTT</td>
<td>to quantify the expression of the <em>Fvwc1</em> gene, reverse</td>
</tr>
<tr>
<td>WC2_for</td>
<td>CGT CTC CTC GGG AAC CTT</td>
<td>to quantify the expression of the <em>Fvwc2</em> gene, forward</td>
</tr>
<tr>
<td>WC2_rev</td>
<td>GAT CTC CCT CCA TCG GAA TTG</td>
<td>to quantify the expression of the <em>Fvwc2</em> gene, reverse</td>
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<tr>
<td>WC1for1549</td>
<td>GAC TCG AAG GAA AAG ACG AT</td>
<td>to check <em>Fvwc1</em> deletion, forward</td>
</tr>
<tr>
<td>WC1rev3009</td>
<td>AGC AGG CTG TGA AAT ATGG</td>
<td>to check <em>Fvwc1</em> deletion, reverse</td>
</tr>
<tr>
<td>Test4519for</td>
<td>CAG TTC TTC TCG GCG TTC TGG</td>
<td>to check site-specific integration of the <em>hph</em> cassette</td>
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<tr>
<td>WC1rev3848</td>
<td>AGC GTG AAG AAA GAA AAG TG</td>
<td>to check site-specific integration of the <em>hph</em> cassette</td>
</tr>
<tr>
<td>WC2for2266</td>
<td>CCA ATC CAA ATC AAT AGC</td>
<td>to check <em>Fvwc2</em> deletion, forward</td>
</tr>
<tr>
<td>WC2rev3506</td>
<td>GTA TTG GCC CAC AAT GAA TA</td>
<td>to check <em>Fvwc2</em> deletion, reverse</td>
</tr>
<tr>
<td>WC2rev3989</td>
<td>GAT GGC GAA TGA ATT TGT AT</td>
<td>to check site-specific integration of the <em>hph</em> cassette</td>
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<tr>
<td>hph_check1</td>
<td>GGC GCA GAC CGG GAA CACA</td>
<td><em>E. coli hph</em> (Z32698.1) forward</td>
</tr>
<tr>
<td>hph_check2</td>
<td>CAC GGC GGG AGA TGC AAT AGG TC</td>
<td><em>E. coli hph</em> (Z32698.1) reverse</td>
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<tr>
<td>WC1_8for</td>
<td>CTTCCTTTGCCATACCGTCT</td>
<td>to amplify the N-terminal part of the entire <em>wc1</em> gene, forward</td>
</tr>
<tr>
<td>WC1_SON2rev</td>
<td>CTTGCTTTGTTTATCGCGCAC</td>
<td>to amplify the N-terminal part of the entire <em>wc1</em> gene, reverse</td>
</tr>
<tr>
<td>WC1_for2827</td>
<td>TTGCAAGCCAGACGACTCT</td>
<td>to amplify the C-terminal part of the entire <em>wc1</em> gene, forward</td>
</tr>
<tr>
<td>WC1_rev3822</td>
<td>GTATCAAAAAATCGGTTTATATC</td>
<td>to amplify C-terminal part of the entire <em>wc1</em> gene, reverse</td>
</tr>
<tr>
<td>M13 for</td>
<td>GTA AAA CGA CGG CCA GT</td>
<td>to amplify full length disruption fragment for protoplast transformation</td>
</tr>
<tr>
<td>M13 rev</td>
<td>CAG GAA ACA GCT ATG AC</td>
<td>to amplify full length disruption fragment for protoplast transformation</td>
</tr>
</tbody>
</table>
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*II and *Aat*II digested DNA samples of the wt strain (lane 7), the Δ*FvMAT1-2-I* mutant (lane 8) and 12 putative Δ*Fvwc1* and Δ*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*II has no recognition site on this sequence. Mutants Δ*Fvwc1-KO1*, Δ*Fvwc1-KO3*, Δ*Fvwc1-KO6* and Δ*Fvwc2-KO1*, Δ*Fvwc2-KO10*, Δ*Fvwc2-KO11* (lanes –1, 3, 6 and 9, 12, 13), that gave one and two (or two co-migrating) bands after *Bgl*II and *Aat*II digestion, respectively were used in further experiments.
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), Hypocreja ecorina (wc-1:AY823264, wc-2:AY823265), Trichoderma atroviride (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-
Regions 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), 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.
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 wcI 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*, ΔFvwc1 and ΔFvwc2 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 wcI and wc2 mutants (ΔFvwc1-KO1, ΔFvwc1-KO3, ΔFvwc1-KO6 and ΔFvwc2-KO1, ΔFvwc2-KO10, ΔFvwc2-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 ΔFvwc1 and ΔFvwc2 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, ΔFvwc1-KO1 and ΔFvwc2-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 mutations 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 ΔFvwc 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 (ΔFvwc1-KO1, ΔFvwc1-KO6 and ΔFvwc2-KO1, ΔFvwc2-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 (10⁸ conidia ml⁻¹) of the wild type strain, *F. verticillioides* FGSC 7603 (Δ) and its Δ*Fvwc1*/1 (■), Δ*Fvwc1*/6 (●), Δ*Fvwc2*/1 (□), and Δ*Fvwc2*/10 (○) 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 ΔFvwc1 and ΔFvwc2 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$^2$) 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 ΔΔC$_t$ 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.](image-url)
lyzing transcript profiles of a wild type strain of *F. verticillioides* and its ΔFvmat1-2-1 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 ΔFvmat1-2-1 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 ΔFvMAT1-2-1 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 ΔFvMAT1-2-1/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 ΔΔCt method were expressed in relative units. The results are means of two independent biological repetitions, run in duplicates. Vertical bars indicate standard errors.](image-url)
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 ΔFvwc1 and ΔFvwc2 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 (= wcI) 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 Δ*wcl* 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


Acta Phytopathologica et Entomologica Hungarica 48, 2013