RESEARCH LETTER

The *MAT1-2-1* mating-type gene upregulates photo-inducible carotenoid biosynthesis in *Fusarium verticillioides*

Attila L. Ádám[1], Jorge García-Martínez[2], Endre P. Szűcs[1], Javier Avalos[2] & László Hornok[1]

[1]Agricultural Biotechnology Center, Mycology Group of the Hungarian Academy of Sciences, Institute of Plant Protection, Szent István University, Gödöllő, Hungary; and [2]Department of Genetics, Faculty of Biology, University of Seville, Seville, Spain

Correspondence: László Hornok, Agricultural Biotechnology Center, Mycology Group of the Hungarian Academy of Sciences, Institute of Plant Protection, Szent István University, H-2100 Gödöllő, Páter K. u.1., Hungary. Tel.: +36 28 522 000; fax: +36 28 410 804; e-mail: hornok.laszlo@mkk.szie.hu

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Abstract

Filamentous ascomycetes, including mitotic holomorphs, have constitutively transcribed *MAT* (mating type) genes. These genes encode transcription factors considered to be the major regulators of sexual communication. The proven targets of the MAT transcription factors are pheromone precursor and pheromone receptor genes. However, recent studies demonstrated that MAT proteins may also affect other genes not involved directly in the mating process. When grown in the light, *Fusarium verticillioides* produces the acidic xanthophyll neurosporoxanthin and lower amounts of nonpolar precursor carotenes, such as phytoene, torulene, β-carotene, and γ-carotene. Depending on the illumination conditions, a drastic decrease or the absence of light-inducible carotenoid accumulation was detected in three independent Δ*FvMAT1-2-1* knockout mutants of *F. verticillioides* as compared with the parental wild-type strain. Transcript levels of the *carB*, *carRA*, and *carT* genes, encoding key enzymes of the carotenoid biosynthetic pathway, were also significantly reduced in the mutants. The downregulation of these genes in the Δ*FvMAT1-2-1* mutant indicates that *MAT* genes play a role in the control of carotenogenesis in *Fusarium*. The finding that mating-type genes regulate important processes unrelated to sex helps to understand the presence of functional *MAT* genes in asexually reproducing fungus populations.

Introduction

In heterothallic species of filamentous ascomycetes, sexual reproduction requires interaction between two strains belonging to opposite mating types, while homothallic species are self-fertile and can complete the sexual cycle by mating within the same thallus. Mating type in heterothallic species is controlled by two idiomorphic alleles, designated *MAT1-1* and *MAT1-2*, that contain three (*MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*) and one (*MAT1-2-1*) genes, respectively, whereas homothallic species have all these four *MAT* genes, linked at the same locus (Yun *et al.*, 2000). The translation products of *MAT1-1-1* and *MAT1-2-1*, the major motors of sexual communication (Turgeon, 1998), are regulatory proteins that contain DNA-binding motifs with conserved regions of the α-box domain and the HMG-box (high mobility group) domain, respectively. These proteins act as transcriptional factors and regulate pheromone precursor and pheromone receptor genes in heterothallic ascomycetes (Debuchy, 1999; Pöggeler & Kück, 2001; Kim & Borkovich, 2004). Pheromone communication is required between mating partners (Bistis, 1983) in heterothallic species. On the contrary, in homothallic species, such as *Fusarium graminearum*, the expression of the pheromone precursor and pheromone receptor genes is nonessential in sexual development, although these genes are controlled by the *MAT* locus (Kim *et al.*, 2008; Lee *et al.*, 2008).

Fusarium species are well known because of the richness of their secondary metabolism including the production of a range of pigments. Relevant examples are the carotenoids, fat-soluble terpenoid pigments produced by photosynthetic organisms and a variety of heterotrophic bacteria and fungi (Britton *et al.*, 1998). In response to light, different *Fusarium* species produce the carboxylic apocarotenoid neurosporaxanthin (Avalos & Estrada, 2010; Jin *et al.*, 2010). The genes and enzymes needed for the synthesis of this xanthophyll have been investigated in detail in *Fusarium fujikuroi* (Linnemannstöns *et al.*, 2002; Prado-Cabrero et *al.*, 2007a). The enzymatic steps from the diterpenoid precursor geranylgeranyl pyrophosphate, i.e., a condensation, five

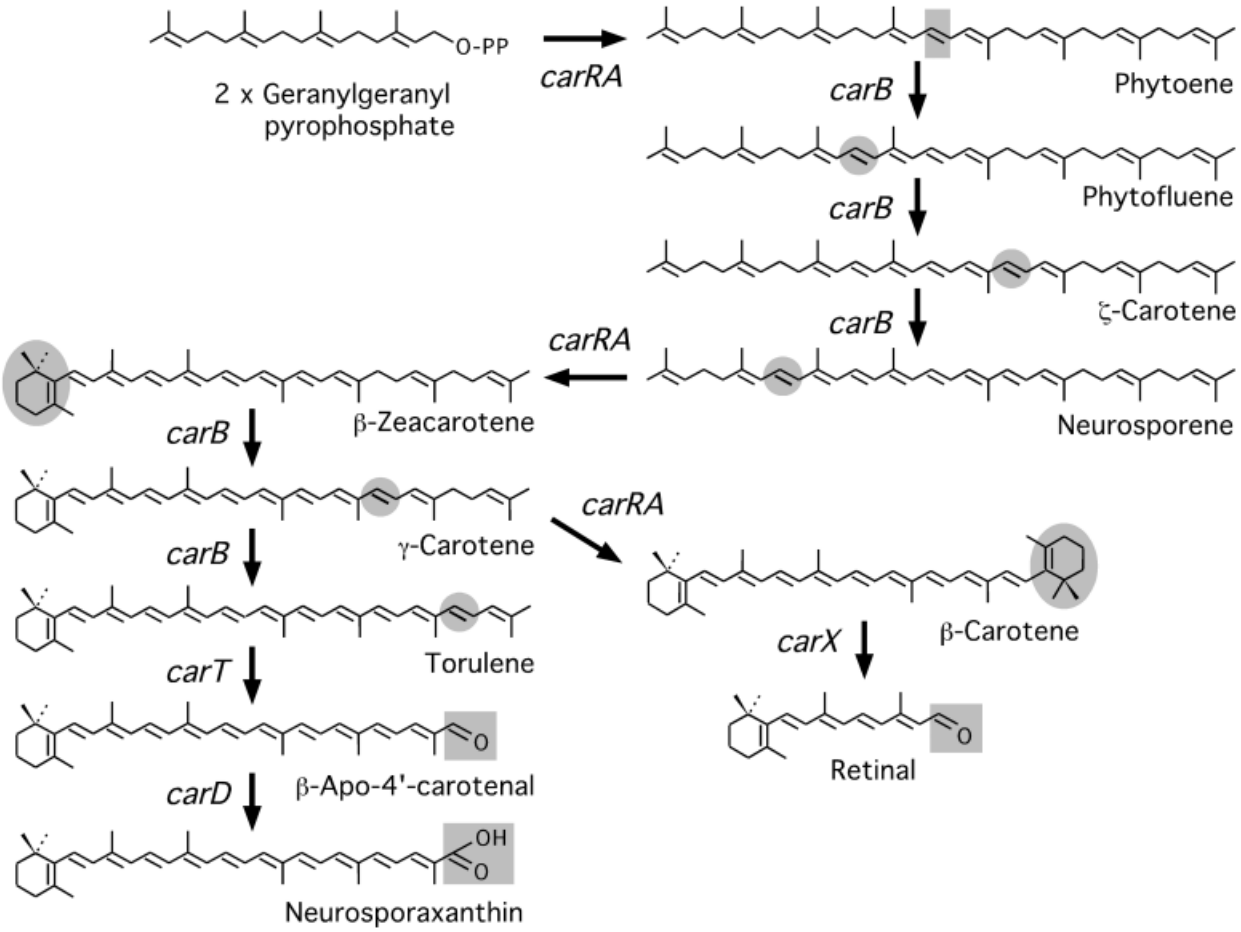

Fig. 1. Enzymatic reactions of neurosporaxanthin and retinal biosynthesis in *Fusarium fujikuroi*. The genes responsible for catalysis of each biosynthetic step are indicated. *carRA*, *carB*, and *carT* encode a bifunctional enzyme with phytoene synthase and carotene cyclase activities, a dehydrogenase, and a carotene-cleaving oxygenase, respectively. *carD* codes for a postulated dehydrogenase currently under investigation. Cyclizations catalyzed by CarRA and other enzymatic steps are indicated with ellipse or quadratic marks, respectively. Desaturations introduced by the CarB enzyme, highlighted in gray circle, provide the chain of conjugated double bonds causing visible light absorption. Except for geranylgeranyl pyrophosphate, phytoene, phytofluene, and retinal, all the depicted molecules are colored carotenoids.

desaturations, a cyclization and an oxidative cleavage reaction, are depicted in Fig. 1. The pathway includes a side branch through a second cyclization reaction to produce β-carotene, the substrate of the retinal-forming enzyme CarX (Prado-Cabrero *et al.*, 2007b). Retinal is the light-absorbing prosthetic group of opsins (Spudich, 2006).

Cultures of *Fusarium verticillioides* (teleomorph: *Gibberella moniliformis*), a cosmopolitan pathogen of maize that produces fumonisins, exhibit an orange pigmentation when grown in the light, not apparent in dark-grown cultures, suggesting the occurrence of a similar regulation of carotenoid biosynthesis as described in *F. fujikuroi*. Interestingly, when the wild type and its Δ*FvMAT1-2-1* mutants were cultured on synthetic minimal medium, marked morphological differences were observed between the wild type and the mutants: the mutant colonies became pale and they seemingly lost their ability to produce carotenoids.

The objective of the present work was to demonstrate that inactivation of the *MAT1-2-1* gene causes a drastic reduction of carotenoid production paralleled with a significant decrease in the photo-induced mRNA levels of the *carB*, *carRA*, and *carT* genes encoding key enzymes of the carotenoid biosynthetic pathway. These findings provide experimental proof of previous hypotheses suggesting that *MAT* genes may regulate important processes not directly related to sexual reproduction. This study helps to understand the role of functional mating-type genes in fungi where sexual reproduction is durably suspended or absent.

Materials and methods

Fungal strains and growth conditions

Fusarium verticillioides wild-type strains FGSC 7600 (genotype: MATA-1) and FGSC 7603 (MATA-2) and three

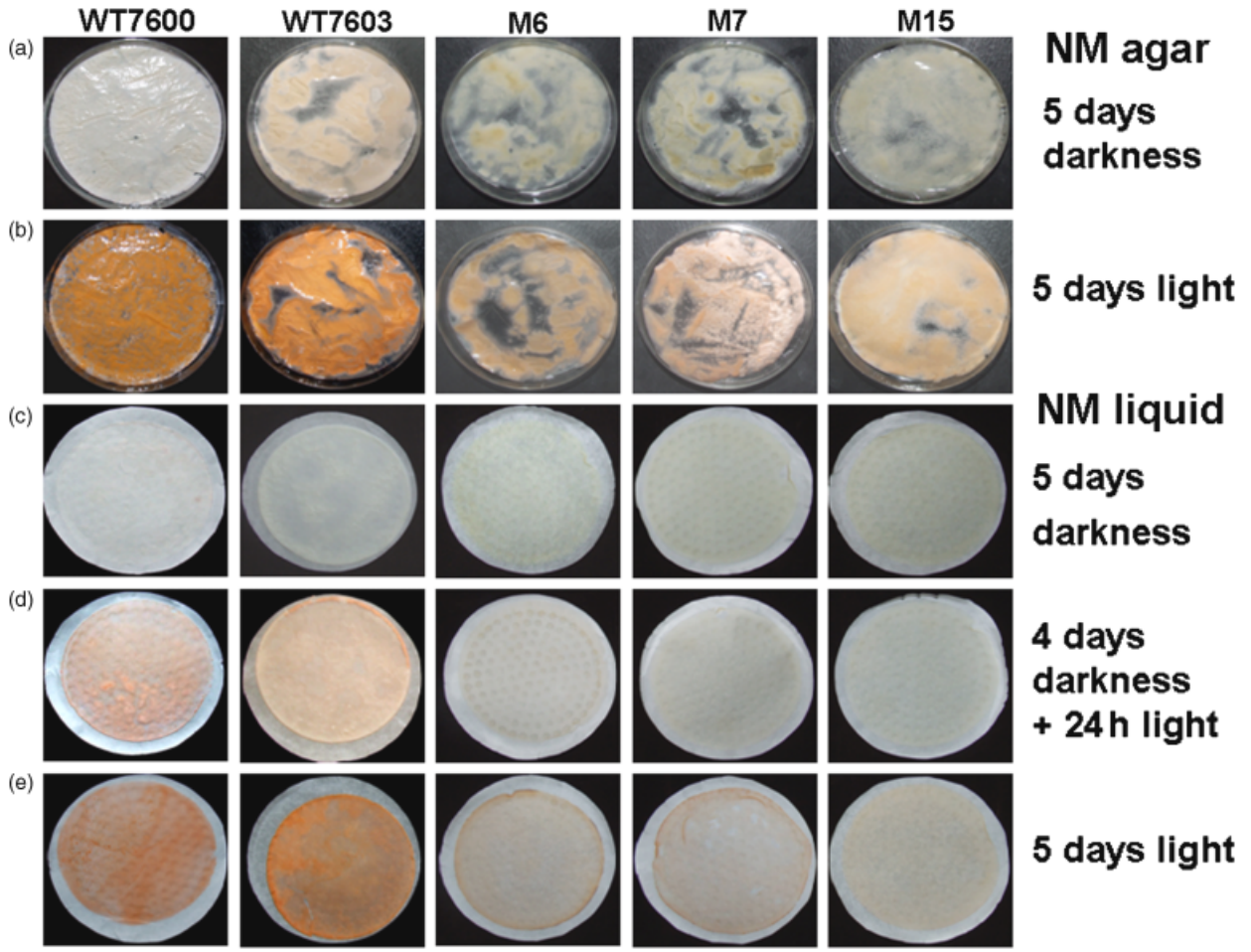

Fig. 2. Pigmentation of two wild-type strains of *Fusarium verticillioides* (FGSC 7600 and FGSC 7603, referred to here as WT7600, WT7603) and three independent Δ*FvMAT1-2-1* mutants of WT7603 (M6, M7, and M15), grown under different conditions. Lack of pigmentation in the dark indicates that the orange color is mainly due to the photo-induced accumulation of carotenoids. Color intensities of cellophane-supported colonies removed from the NM agar plates after 5 days of growth were compared to rule out the disturbing effects of agar discoloration. Cultures in (a) and (b) and (c), (d), and (e) were grown on NM agar plates supported with cellophane sheets and in liquid NM, respectively. Cultures in (a) and (c) were incubated for 5 days in the dark; cultures in (b) and (e) were incubated in continuous light (100 lx) for 5 days and cultures in (d) were exposed to 24-h continuous light after 4-day incubation in the dark. All pictures were taken after 5-day incubation. Experiments were repeated at least three times. Photographs of one representative experiment are presented.

independent *MAT1-2-1* gene disruption mutants (Δ*FvMAT1-2-1*/M6, M7, M15) of the latter wild-type strain, produced earlier (Keszthelyi *et al.*, 2007), were maintained as conidial suspensions in 15% glycerol at −70 °C. Complete medium (CM) and carrot agar (CA) (Leslie & Summerell, 2006), liquid nitrate minimal (NM) medium and NM agar with 3.0 g L^{-1} $NaNO_3$ as the N-source (Avalos & Cerdá-Olmedo, 1987) were used to compare the growth and morphology of these strains. Agar plates, covered by cellophane sheets and inoculated with 10^5 conidia, were incubated for 5 days in different illumination regimes (Fig. 2), at 25 °C. Light-grown cultures were exposed to 100 lx illumination in all experiments produced by a battery of three cool white fluorescent light tubes. Chemicals were from Sigma Chemical Co. (St. Louis, MO).

Determination of carotenoids

For the determination of carotenoids and measurement of *car* gene expression, fungi were cultured under various light regimes (Figs 3–5) in 20 mL liquid NM inoculated with 4×10^6 conidia. Samples were harvested, filtered, and frozen in liquid nitrogen after different time intervals (as indicated in Figs 3–5). Carotenoids were extracted from freeze-dried samples (0.05 g). The total amounts of colored carotenoids and the amounts of polar and nonpolar carotenoids were determined according to Arrach *et al.* (2002). The term polar carotenoids refers to the fraction containing neurosporaxanthin and minor amounts of its direct precursor β-apo-4′-carotenal; the UV-absorbing retinal is not included. The term nonpolar carotenoids includes all the colored

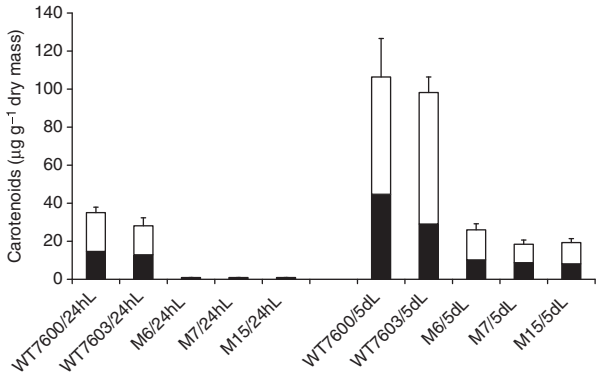

Fig. 3. Nonpolar (black columns), polar (white columns), and total (black+white columns) carotenoid contents in the wild-type strains of *Fusarium verticillioides*, FGSC 7600 (WT7600) and FGSC 7603 (WT7603) and three independent Δ*FvMAT1-2-1* mutants (M6, M7, and M15) of FGSC 7603, grown in liquid NM under different illumination conditions. Samples were run in triplicate and experiments were repeated at least two times. 24hL–24-h light exposition after 4-day incubation in the dark, 5dL–5-day incubation in continuous light. Dark-grown samples contained no detectable amounts of carotenoids in any strain (data not shown). Vertical bars indicate the SEs.

carotene precursors from γ-carotene to torulene, and the side product β-carotene (Fig. 1). HPLC was carried out using a Hewlett Packard Series 1100 Chromatographer (Agilent Technologies, Palo Alto, CA) equipped with a G1322A degasser, a G1311 quaternary pump, and a G1315A diode array detector. Samples were resolved in 20 μL hexane and 10 μL aliquots were run through an analytic ProntoSIL Spheribond ODS (octadecyl-silyl) column (5 μm particle diameter; 250 × 4.6 mm; Bischoff Chromatography, Leonberg, Germany). For nonpolar carotenoids, isocratic separations were carried out eluting with methanol/acetonitrile/chloroform (47 : 47 : 6) (1 mL min^{-1}). For polar carotenoids, the method used for *ylo-1* analysis by Estrada *et al.* (2008) was followed.

Measurement of *carRA*, *carB*, and *carT* expression by quantitative real-time (qrt)-PCR

Expression levels of *carRA*, *carB*, and *carT* genes (FVEG_10718, FVEG_10717, and FVEG_09251, respectively) were measured by qrt-PCR as described earlier

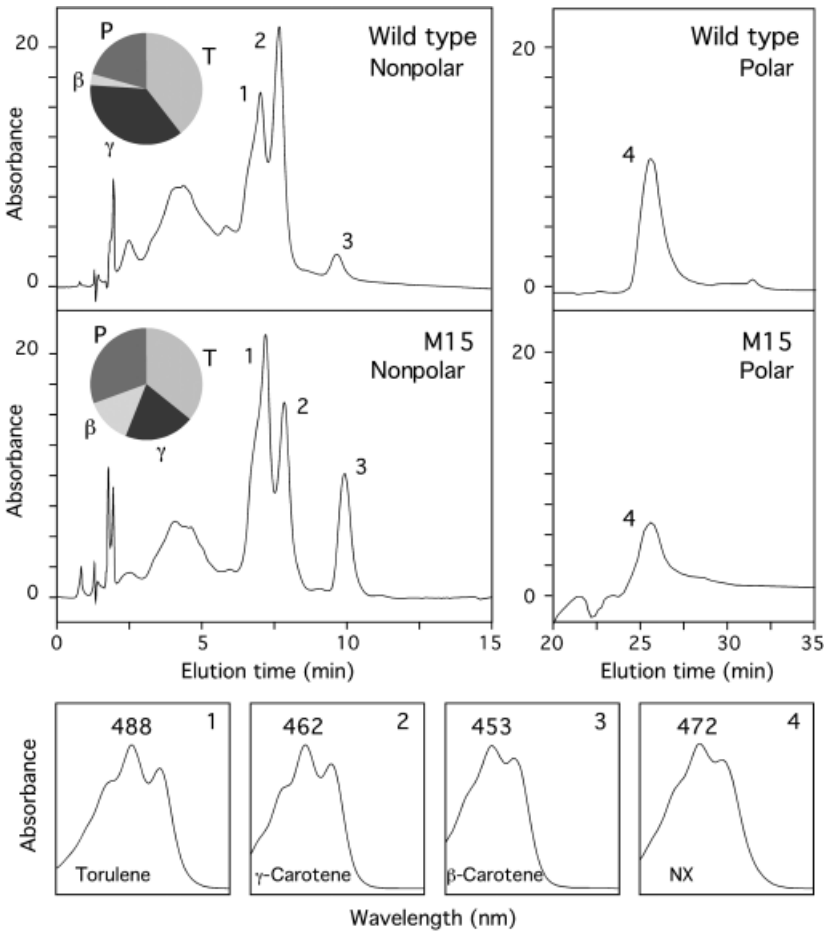

Fig. 4. HPLC analyses of carotenoid samples from 5-day-old cultures (illuminated for 24 h after 4-day incubation in the dark) of FGSC 7603, the wild-type strain of *Fusarium verticillioides* and its Δ*FvMAT1-2-1* M15 mutant grown in liquid NM. Left graph: separation of nonpolar carotenoids. Circle diagrams show the percentage distribution of individual neutral carotenoids. Chromatograms for phytoene (P, $A_{282\,nm}$) are not shown. Right graph: separation of polar carotenoids. The major component is neurosporaxanthin. Below: UVVis spectra (350–550 nm) and maximal absorbance wavelengths (nm) of carotenoids corresponding to the numbered peaks: torulene (T), β-carotene, γ-carotene, and neurosporoxanthin (NX).

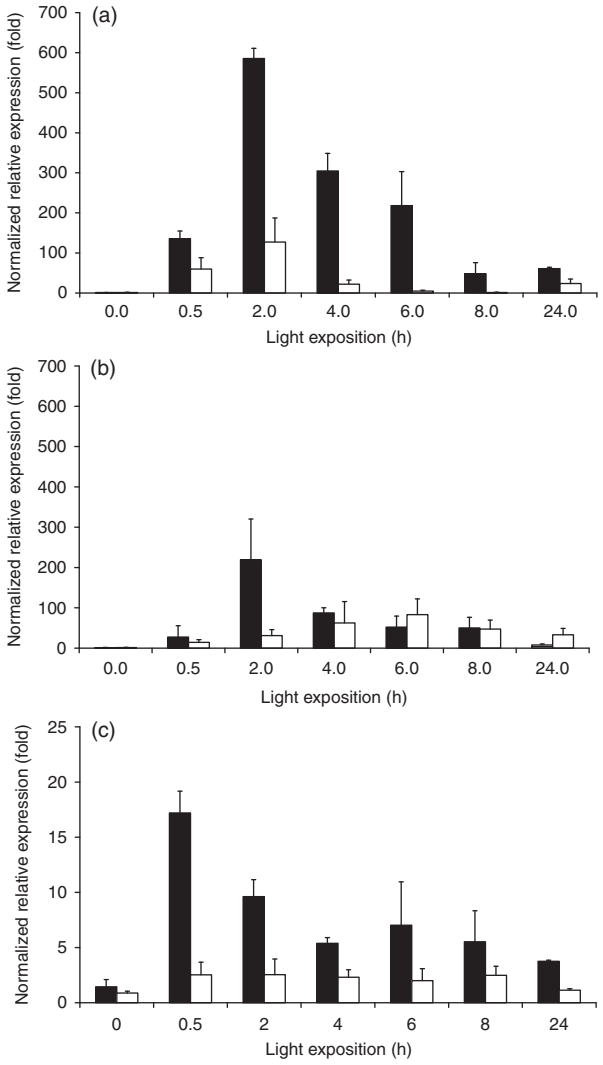

Fig. 5. Expression of the *carRA* (a), *carB* (b), and *carT* (c) genes of the carotenoid biosynthetic pathway in the wild-type strain of *Fusarium verticillioides* FGSC 7603 (black columns) and its Δ*FvMAT1-2-1* M15 mutant (white columns). mRNA levels were monitored by qrt-PCR. Data, calculated using the $\Delta\Delta C_T$ method, were expressed in relative units (zero time expressions at the start of illumination are equal to 1). The results are means of two independent biological repetitions, run in duplicate. Vertical bars indicate the SEs.

(Ádám *et al.*, 2008). qrt-PCR was carried out using the ABI PRISM SDS 7000 system (Applied Biosystem, Foster City, CA) with SYBR Green (Bio-Rad, Hercules, CA) detection. The primer pairs CarBfor/CarBrev (5′-GTCCCTTCGCG CATTGACC-3′/5′-GCTGAGTACCAGGCCGGAAAG-3′), CarRAfor/CarRArev (5′-GGCACCAAATACGGCATTGTC-3′/ 5′-CGGCAGAACGCATACAAACGA-3′), and CarTfor/ CarTrev (5′-GCCTCACTACGCGGTCTAAC-3′/5′-CCGTC ATCTTCTTCCTCTGC-3′) were used to amplify a 358-, a 347-, and a 151-bp DNA fragment, respectively. The identity of the fragments was checked by sequencing.

Results

The Δ*FvMAT1-2-1* mutants have an albino-like phenotype under different culture conditions

Light induction of the orange pigmentation (putative carotenoid accumulation) was assessed in two sexually compatible wild-type strains of *F. verticillioides* FGSC 7600 and FGSC 7603, as well as three independent Δ*FvMAT1-2-1* mutants (M6, M7, and M15) of FGSC 7603, grown on NM agar under different illumination conditions (Fig. 2). On this medium, the two wild-type strains acquired a faint pigmentation in the dark, but this was not apparent in the mutant strains under the same culture conditions (Fig. 2a). When incubation occurred under continuous illumination, the wild-type strains developed an intense orange color, while the three Δ*FvMAT1-2-1* mutants showed a paler pigmentation (Fig. 2b). These findings indicate that (1) the orange pigmentation is light inducible and (2) the synthesis is reduced in the absence of an operational *MAT1-2-1* gene in the *MAT1-2* background. The color development of these five strains on CA and CM agar was similar to that observed on NM (data not shown), suggesting that the deficiency of orange pigmentation in the Δ*FvMAT1-2-1* mutants was not limited to minimal nutrient conditions.

To further analyze the effect of light on pigment accumulation, fungi were grown in liquid NM under different illumination conditions for 5 days. As presented in Fig. 2c, all strains showed an albino phenotype when they were cultured in the dark. Five-day culture under continuous illumination resulted in intense orange coloration in the wild-type strains, but much less pigment accumulation occurred in the three Δ*FvMAT1-2-1* mutants (Fig. 2e). When 4-day-old cultures grown in the dark were exposed to 24-h illumination, a moderate pigment accumulation was observed in the wild-type strains, while the Δ*FvMAT1-2-1* mutants exhibited albino-like phenotypes (Fig. 2d). Similar pigmentation patterns were observed with shorter light exposures (i.e. 8-h illumination, followed by further incubation for 16 h in the dark) after 4-day culturing in the dark (data not shown). To reveal the biochemical bases of the orange pigmentation, the carotenoid contents of the cultures were measured.

Light-inducible carotenoid synthesis is reduced in the Δ*FvMAT1-2-1* mutants

Carotenoids were extracted and analyzed by column chromatography to determine the amounts of both polar and nonpolar carotenoids in the wild-type strains of *F. verticillioides* and the Δ*FvMAT1-2-1* mutants of strain FGSC 7603 grown in liquid NM under different illumination conditions (Fig. 3). As expected, only trace amounts ($< 0.2\ \mu g\ g^{-1}$ dry mass) of carotenoids were found in the albino cultures of

any strain grown in the dark. Conversely, when grown for 5 days under continuous illumination, all strains produced significant amounts of carotenoids, but the total contents of the two wild-type cultures (FGSC 7600 and FGSC 7603) were significantly higher (106 ± 20 and $98 \pm 8\ \mu g\ g^{-1}$ dry mass, respectively) than those measured in the three Δ*FvMAT1-2-1* mutant cultures (from 18 ± 2 to $26 \pm 3\ \mu g\ g^{-1}$ dry mass). Of the cultures grown for 4 days in the dark and then illuminated for 24 h (see Fig. 2e), the wild-type strains contained significant amounts of carotenoids (35 ± 2 and $28 \pm 4\ \mu g\ g^{-1}$ dry mass, respectively), while only trace amounts were found in the three mutants.

When the carotenoid amounts were sufficient for reliable determinations, nonpolar carotenoids were detected in similar proportions in all the strains, ranging from 30% to 45% of the total carotenoid mixtures (Fig. 3). For more detailed qualitative assays, mycelial extracts of the wild-type strain FGSC 7603 and one representative Δ*FvMAT1-2-1* mutant were subjected to HPLC analysis (Fig. 4). The same major individual carotenoids (mostly neurosporaxanthin, torulene, γ-carotene, β-carotene, and phytoene) were found in *F. verticillioides* as were found previously in other *Fusarium* species (Bindl *et al.*, 1970; Avalos & Cerdá-Olmedo, 1987). However, the mutant contained a higher proportion of phytoene and β-carotene (30.7% and 13.4%, respectively, compared with 20.4% and 3.4% in the wild type) and less of γ-carotene (19.9% against 36.7% in the wild type). This change suggests different patterns of down-regulation of the carotenoid biosynthesis genes in the Δ*FvMAT1-2*-1 M15 mutant in relation to its wild-type parental strain (see the next section and Fig. 5).

Light-induced expression of *carB*, *carRA*, and *carT* is downregulated in the Δ*FvMAT1-2-1* M15 mutant

Parallel to carotenoid biosynthesis, mRNA levels of *carRA*, *carB*, *carT*, and *carX* genes of the carotenoid pathway (Fig. 1) are transiently induced by illumination in *F. fujikuroi* (Prado *et al.*, 2004; Thewes *et al.*, 2005; Prado-Cabrero *et al.*, 2007b). In the *F. verticillioides* genome (http://www.broadinstitute.org/annotation/genome/fusarium_verticillioides/MultiHome.html), highly conserved orthologues of these genes are found (*carRA*: FVEG_10718; *carB*: FVEG_10717; *carT*: FVEG_09251; and *carX*: FVEG_10719.3, with 88%, 99%, 94%, and 85% identity at the protein level with *F. fujikuroi* counterparts), indicating the presence of the same carotenoid pathway in these two closely related fungi.

We compared the transcript levels of *carB*, *carRA*, and *carT* in the wild-type strain, FGSC 7603 of *F. verticillioides* and its Δ*FvMAT1-2-1* M15 mutant using qrt-PCR. Total RNA was isolated from mycelium samples of cultures grown for 4 days in the dark and then illuminated for 0.5, 2, 4, 6, 8, and 24 h, respectively. Very low mRNA levels of either *carB*, *carRA*, or *carT* were found in cultures of both strains when they were grown in the dark and sampled at the start of illumination (0 h), but the levels started to increase as early as 0.5 h following light onset. Expression levels of *carT* peaked at 0.5-h illumination, while expression peaks of *carB* and *carRA* occurred at 2-h illumination; then a continuous decrease was observed with time, and low, but still significant mRNA levels were detected for all the three genes after 24-h illumination. Much smaller increases were measured in the mRNA levels of the three genes in the Δ*FvMAT1-2-1* M15 mutant, suggesting a positive regulatory role of the *MAT1-2-1* gene in the light-induced expression of these carotenoid biosynthesis genes. Interestingly, the light-induced expression of *carB* was delayed compared with that of *carRA* in the M15 mutant, with an induction peak at 6 h instead of 2 h after the start of illumination (Fig. 5). This regulatory difference could explain the different proportions of nonpolar carotenoids found in the mutant (Fig. 4).

Discussion

Sexual reproduction in filamentous ascomycetes is influenced by environmental factors, including nutrients, C/N ratio, pH, temperature, atmospheric conditions, and light (Debuchy *et al.*, 2010). Current standard crossing procedures in the genus *Fusarium* use 12 h light–dark cycles and incubation on a special medium, usually CA (Leslie & Summerell, 2006), rich in carotenoids. Although CA stimulates the development of sexual structures in pairing experiments, the role of carotenoids in sexual reproduction in these fungi is still unclear. Sexual carotenogenesis, described for *Mucorales* fungi (Govind & Cerdá-Olmedo, 1986) has not been observed in ascomycetes. However, indirect evidence suggests that these fungi may also need carotenoids during the development of sexual structures: in many ascomycetes, fruiting bodies show intense yellow or orange coloration (e.g. Samuels, 1988), and bright yellow cirrus development with oozing asci in mature perithecia can be observed in a number of fungi, including species of *Fusarium* (Leslie & Summerell, 2006). Molecular experiments provided additional indirect evidence on a possible role of carotenoids in sexual development in *Fusarium*: a gene encoding a putative opsin-like protein, orthologous to CarO of *F. fujikuroi* (Prado *et al.*, 2004), was downregulated both in the Δ*MAT1-2-1* mutant of *F. verticillioides* (Keszthelyi *et al.*, 2007) and in the *MAT1-2* deleted strain of *F. graminearum* (Lee *et al.*, 2006). Opsins use retinal, a side product of carotenoid biosynthesis (Fig. 1), as a prosthetic group and the gene *carO* is clustered and coregulated with other genes of the carotenoid pathway in *F. fujikuroi* (Prado *et al.*, 2004). A similar gene organization and regulation also seem to be operative in *F. verticillioides*. Furthermore, the

data presented in this work confirm that carotenogenesis in *F. verticillioides* is regulated by light as in other *Fusarium* species (Avalos & Estrada, 2010) and, most outstandingly, they demonstrate for the first time a role of a *MAT* gene in regulating the accumulation of these pigments in fungi.

The possible involvement of the *MAT* genes in fungal processes unrelated to the sexual cycle was highlighted by the comparison of the transcript profiles of a wild-type strain of *F. verticillioides* and its Δ*FvMAT1-2-1* mutant. The majority of the > 200 ESTs identified as either down- or upregulated in the mutant have no known roles in sexual development (Keszthelyi *et al.*, 2007). Furthermore, fungi with no known sexual stage still have functional *MAT* genes (Sharon *et al.*, 1996; Kerényi *et al.*, 2004), indicating that the lack of sexual reproduction in mitotic holomorphic species is caused by adverse mutations at loci other than the *MAT* locus. The reasons for the occurrence of functional *MAT* genes in fungi with no known sexual stage are not well understood. One plausible hypothesis is that the MAT transcriptional factors have some functions during the asexual part of the life cycle and may regulate additional genes not involved directly in sexual events (Hornok *et al.*, 2007). The *MAT* genes may thus have a selective impact (e.g. through the stimulation of carotenoid production) on asexually reproducing populations. Another explanation is that these fungi have a cryptic sexual stage, but their teleomorphs have not been identified due to the extreme rarity of mating (Leslie & Klein, 1996; Turgeon, 1998).

The regulatory mechanism(s) for light-inducible carotenogenesis in *Fusarium* species are not fully understood. The white collar (WC) proteins are regarded as a universal photoreceptor system regulating carotenogenesis and other photoregulated processes in fungi (Corrochano & Avalos, 2010). Recent results on WC1-defective mutants in *Fusarium oxysporum* and *F. fujikuroi* indicate, however, that carotenogenesis is regulated differentially in members of the genus *Fusarium* (Avalos & Estrada, 2010). Light-inducible carotenogenesis was retained in WC1 mutants of these *Fusarium* species, suggesting the existence of WC-independent photoreceptor mechanisms and/or the involvement of unknown factors in light-dependent carotenogenesis. Our present results confirm that *F. verticillioides*, like *F. fujikuroi*, has transcriptional control of carotenogenesis in response to light. The induction of *car* gene expression and carotenoid biosynthesis are drastically reduced in the absence of a functional *MAT1-2-1* gene. Thus, the regulation of light-induced carotenogenesis in *F. verticillioides* depends at least in part on *MAT1-2-1*. This gene is absent in the wild strain of the opposite sex, FGSC 7600, which, however, exhibits a normal light induction of carotenogenesis. Presumably, the regulatory role played by *MAT1-2-1* in FGSC 7603 is played in FGSC 7600 by an equivalent *MAT1-1* gene from its *MAT* locus (Yun *et al.*, 2000).

The available information on photoinduction of carotenogenesis in *Fusarium* suggests that it is a transcriptionally controlled mechanism mediated by a still unknown regulatory system. The attenuation of this photoresponse in the Δ*FvMAT1-2-1* mutants of *F. verticillioides* reveals a novel key regulatory element in the carotenoid pathway whose connection with the light-inducing mechanism remains to be identified. Carotenoid production is probably an important selective trait in fungi, as indicated by their widespread occurrence in these organisms (Sandmann & Misawa, 2002). As a likely explanation, different observations support a protective role of these pigments against oxidative stress in taxonomically unrelated fungi, such as *Phaffia rhodozyma* (Schroeder & Johnson, 1993), *Blakeslea trispora* (Jeong *et al.*, 1999), or *Neurospora crassa* (Iigusa *et al.*, 2005). The finding that *MAT* genes stimulate carotenoid production in *F. verticillioides* during its asexual propagation helps to understand the function of mating-type genes in the absence of sexual reproduction. *MAT* genes have a positive selective impact on fungal populations by stimulating important processes unrelated to sexual reproduction and, therefore, they are retained in an operable form during the asexual part of the life cycle that can be extremely long in fungi where sexual reproduction is durably suspended.

Acknowledgements

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