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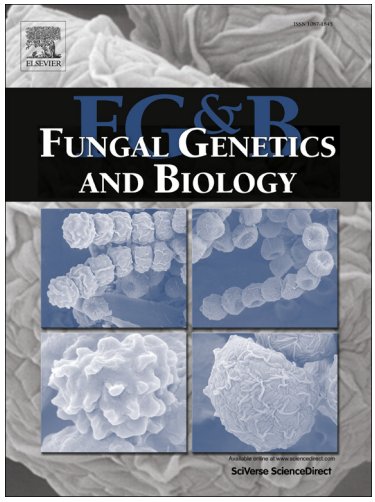

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A RALDH-like enzyme involved in *Fusarium verticillioides* development

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ABSTRACT

Retinaldehyde dehydrogenases (RALDHs) convert retinal to retinoic acid, an important chordate morphogen. Retinal also occurs in some fungi, such as *Fusarium* and *Ustilago spp.*, evidenced by the presence of rhodopsins and β-carotene cleaving, retinal-forming dioxygenases. Based on the assumption that retinoic acid may also be formed in fungi, we searched the *Fusarium* protein databases for RALDHs homologs, focusing on *Fusarium verticillioides*. Using crude lysates of *Escherichia coli* cells expressing the corresponding cDNAs, we checked the capability of best matches to convert retinal into retinoic acid *in vitro*. Thereby, we identified an aldehyde dehydrogenase, termed CarY, as a retinoic acid-forming enzyme, an activity that was also exerted by purified CarY. Targeted mutation of the *carY* gene in *F. verticillioides* resulted in alterations of mycelia development and conidia morphology in agar cultures, and reduced capacity to produce perithecia as a female in sexual crosses. Complementation of the mutant with a wild-type *carY* allele demonstrated that these alterations are caused by the lack of CarY. However, retinoic acid could not be detected by LC-MS analysis either in the wild type or the complemented *carY* strain *in vivo*, making elusive the connection between CarY enzymatic activity and retinoic acid formation in the fungus.

Keywords: *Fusarium verticillioides*; RALDH enzyme; Retinoic acid; Apocarotenoids; Conidia; Hyphal development; Sexual fertility

Abbreviations: ALDH, aldehyde dehydrogenase; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; ORF, open reading frame; PFU, perithecia-forming units; RALDH, retinaldehyde dehydrogenase; RT-PCR, reversed transcription PCR

1. Introduction

Carotenoids are fat-soluble terpenoid pigments widespread in photosynthetic organisms, where they act as essential protective and accessory pigments of the photosynthetic apparatus (Telfer, 2005). Most animals are unable to produce carotenoids but they obtain them through the diet, particularly β-carotene (Lu and Li, 2008; Maiani et al., 2009), which is cleaved symmetrically by β-carotene cleavage oxygenases into retinal, the chromophore of the visual opsins (von Lintig, 2012). In chordates, retinal is the source of retinoic acid, an important morphogen that interacts with specific receptors to control the expression of many genes involved in development and organogenesis (Duester, 2008; Ross et al., 2000). Likewise, retinoic acid is involved in proliferation and differentiation of epithelial tissues and induction of apoptosis of diverse cell types in numerous organs. Moreover, retinoic acid is an inducer of meiosis of female and male germ cells in mammals (Griswold et al., 2012).

Carotenoids are subject of a complex metabolism in different biological systems, from microorganisms to plants and animals (Moise et al., 2005). In addition to photosynthetic organisms, they are also produced by many non-photosynthetic bacteria and fungi (Avalos and Cerdá-Olmedo, 2004; Britton et al., 1998). Because of their ease of culture and genetic manipulation, some of these microorganisms have been used as model systems to investigate carotenoid biosynthesis and regulation (Avalos et al., 2014). Among these models are species of the genus *Fusarium*, which produce neurosporaxanthin (β-apo-4'-carotenoic acid) (Avalos et al., 2012), a carboxylic apocarotenoid first identified in *Neurospora crassa* (Zalokar, 1957). In *Fusarium*, the neurosporaxanthin biosynthetic pathway (Fig. S1) starts with the formation of phytoene from geranylgeranyl diphosphate (GGPP) followed by four desaturations and one cyclization to yield γ-carotene. An additional desaturation produces torulene, that is subsequently cleaved by a specific carotenoid cleavage oxygenase to form the aldehyde β-apo-4'-carotenal, which is finally oxidized by an aldehyde dehydrogenase to the acid neurosporaxanthin (Avalos et al., 2014). These reactions are sequentially catalyzed by the phytoene synthase/carotene cyclase CarRA (Linnemannstöns et al., 2002), the desaturase CarB (Prado-Cabrero et al., 2007a), the torulene-cleaving dioxygenase CarT (Prado-Cabrero et al., 2009) and the ALDH CarD (Díaz-Sánchez et al., 2011). As a lateral branch of the pathway (Fig. S1), the CarRA

enzyme may catalyze a second cyclization of γ-carotene to yield β-carotene, the substrate of the cleavage dioxygenase CarX. This enzyme produces the apocarotenoid retinal (Prado-Cabrero et al., 2007b; Thewes et al., 2005), the expected chromophore for the two rhodopsins encoded in the *Fusarium* genomes, called CarO and OpsA in *F. fujikuroi* (Estrada and Avalos, 2009; Prado et al., 2004). Orthologs for the *Fusarium carRA* and *carB* genes are found in other fungal species, such as the zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides*, the ascomycetes *N. crassa*, or the basidiomycetes *Xanthophyllomyces dendrohorus* and *Ustilago maydis* (Avalos et al., 2014). However, *carT* and *carD* orthologs were only found in *N. crassa*, where they were called *cao-2* and *ylo-1* (Estrada et al., 2008; Saelices et al., 2007).

Besides CarT and CarX in *F. fujikuroi*, and CAO-2 in *N. crassa*, the only carotenoid cleavage oxygenases described in fungi are Cco1, a CarX-like retinal-forming enzyme in *U. maydis* (Estrada et al., 2010), and CarS and AcaA, that cleave asymmetrically β-carotene to yield the precursors of trisporic acids in *P. blakesleeanus* (Medina et al., 2011). The case of *U. maydis* is particularly interesting, since it has three genes coding for putative retinal-binding rhodopsins (Brefort et al., 2011), two of them possible orthologs of *Fusarium* CarO and OpsA. However, no retinal biosynthetic activity has been found so far in *N. crassa*, in spite of the presence of NOP-1 (Bieszke et al., 1999a), a photoactive rhodopsin for which the ability to bind retinal has been demonstrated *in vitro* (Bieszke et al., 1999b). The putative retinal-forming enzyme of *N. crassa*, the oxygenase CAO-1, is unable to cleave β-carotene but it efficiently cleaves resveratrol (Díaz-Sánchez et al., 2013), a biphenolic phytoalexin produced by some plants.

Aldehyde dehydrogenases (ALDHs) comprise a large family of enzymes that catalyze the oxidation of a wide range of aldehydes to the corresponding acids. Relevant members from this family are retinaldehyde dehydrogenases (RALDHs), which convert retinal to retinoic acid in chordates (Duester, 2000; Duester, 2001). The ability of *Fusarium* to produce retinal led us to consider the occurrence of a RALDH enzyme in this fungus and its ability to convert this apocarotenoid into retinoic acid. For this purpose, we selected *F. verticillioides* because of its high sexual fertility and its close relatedness to *F. fujikuroi*. A search for RALDH homologs in the *F. verticillioides* genome allowed us to identify several candidates, including an enzyme that we called CarY. Here, we show that CarY exhibits retinal dehydrogenase activity and that Δ*carY* mutant displays patent phenotypic alterations compared to the wild-type progenitor,

suggesting the participation of the CarY product in the development of this fungus. To our knowledge, this is the first report on a retinoic acid biosynthetic activity by an enzyme from a non-vertebrate organism. However, retinoic acid was not detected in fungal cell extracts, and therefore the *in vivo* function of CarY remains to be confirmed.

2. Material and Methods

2.1. Fungal strains and growth conditions

Fusarium verticillioides wild-type strains FGSC 7600 (mating type MATA-1) and FGSC 7603 (mating type MATA-2) were kindly provided by Dr. Attila L. Ádám. *Fusarium fujikuroi* FKMC1995 (Kansas State University Collection, Manhattan, KS) was kindly provided by Prof. John. F. Leslie. *Neurospora crassa* wild type strain Oak Ridge 74-OR23-1A was obtained from the Fungal Genetics Stock Center (http://www.fgsc.net).

Unless otherwise stated, *F. verticillioides* experiments were performed on DG minimal medium (composition per liter: glucose, 30 g; $NaNO_3$, 3g; KH_2PO_4, 1g; $MgSO_4 * 7H_20$, 0.5 g; KCl, 0.5 g; HBO_4, 10 µg; $CuSO_4$, 100 µg; $FeCl_3$, 200 µg; $MnCl_2$ 20 µg; MoO_4Na, 20 µg; and $ZnSO_4$, 2 mg; Avalos et al., 1985) or DGasn medium, whose composition is that of DG minimal medium with 3 g l^{-1} L-asparagine instead of $NaNO_3$ as nitrogen source. When required, the medium was supplemented with 100 µg ml^{-1} hygromycin or 125 µg ml^{-1} geneticin G418. For carotenoid analysis, the strains were grown on DG minimal agar for 7 days as described (Prado et al., 2004). Incubations were performed at 30ºC, either in the dark or under illumination (7 W m^{-2} white light). When indicated, the agar surface was covered with sterile cellophane discs (Scotch, Cergy Pontoise, France). For expression analyses, 500-ml Erlenmeyer flasks with 250 ml of culture medium were inoculated with 10^8 conidia and incubated at 30ºC in the dark at 150 rpm for 2 days. When indicated, 80 ml samples were transferred from each flask to Ø 140 x 20 mm Petri dishes and illuminated at 7 W m^{-2} for different times before mycelia filtration. For large-scale DNA extraction, 250-ml Erlenmeyer flasks with 50 mL DGasn were inoculated with 10^8 conidia and incubated for 2 days at 30ºC before filtration. In all cases, the mycelial samples were separated from the media with filter paper, frozen in liquid nitrogen and stored at -80ºC before DNA or RNA

extraction. For the same purpose, *N. crassa* was grown in Vogel's minimal media with 1.5% sucrose as carbon source following standard procedures (Davis, 2000).

For biomass and conidiation analyses, colonies were grown on DG agar covered with cellophane under white light (7 W m^{-2}) or in the dark. After 3-day incubation, the mycelia were separated from the cellophane and dried for 2 hours in an oven at 60°C. In parallel colonies, the conidia were obtained by adding water and scraping the mycelial surface with a spatula. Conidia concentrations in the samples were estimated by counting in a haemocytometer.

2.2. Cloning of ALDH cDNA sequences and enzymatic assays

For ALDHs comparison with RALDH enzymes, BLASTp analyses against the *F. verticillioides* genome (Broad Institute) were done through the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using RALDH1 and RALDH2 retinal dehydrogenases from *Mus musculus* (accession numbers P24549 and Q62148). From more than 30 putative aldehyde dehydrogenases, the five with the highest similarity with MmRALDH1, FVEG_00585, FVEG_11973, FVEG_07427, FVEG_10377 and FVEG_00151, were expressed in *Escherichia coli* cells to perform *in vitro* assays. Their coding sequences were amplified by PCR with the primer sets indicated in Table 1. Forward primers begin with ATG start codon of the coding sequence, while reverse primers include the stop codon.

PCRs were carried out with cDNA obtained from total RNA of the FGSC 7600 wild-type strain and the Superscript III First Strand System for RT-PCR (Invitrogen, Paisley, UK). The PCR products were cloned into pBAD/THIO-TOPO TA vector (Invitrogen), yielding plasmids pThio-PAL1 to pThio-PAL5 (PAL from "Putative ALDH enzyme"), respectively. The inserted cDNA fragments were sequenced to confirm integrity and orientation. For protein expression, the *E. coli* BL21 strain was transformed independently with the five pThio-PAL plasmids. Crude lysates from each transformant, and subsequent *in vitro* assays were achieved as described (Díaz-Sánchez et al., 2011).

The coding sequence of the only ALDH enzyme able to convert retinal to retinoic acid, FVEG_00585, called thereafter CarY, was cloned in vector pGEX-5x-1 (Amersham Biosciences, NJ, USA) to give plasmid pGEX-PAL1, carrying the CarY protein fused to glutathione-S-transferase. pGEX-PAL1 was introduced into *E. coli* GRO7 strain and the transformed bacteria were grown in 2 x YT medium at 28ºC, induced with 0.1 mM

IPTG, and used for CarY purification as described (Scherzinger et al., 2006). Protein concentration was determined using the BioRad protein assay kit (BioRad, Hercules, CA) and enzymatic assays were performed as described (Díaz-Sánchez et al., 2011).

2.3. Generation and complementation of ΔcarY mutant

A plasmid was constructed in which most of the *carY* coding sequence was replaced by a hygromycin-resistance cassette. A 2.9-kb DNA PCR product, containing *carY* and 598 bp and 643 bp of upstream and downstream noncoding sequences, was obtained by PCR from *F. verticillioides* FGSC 7600 genomic DNA with primer set 6 and cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany). The resulting plasmid was digested with *Hind*III and *Bgl*II to delete 980 bp of the 1591-bp *carY* coding sequence, and ligated with a 3.8-kb segment containing the *hph* cassette obtained by digestion of vector pAN7-1 (Punt et al., 1987) with the same restriction enzymes, to yield plasmid pVIO5. The orientation of the insert was confirmed by restriction analysis.

To get the Δ*carY* mutant, 10^8 protoplasts from the same *F. verticillioides* strain were obtained as described (Estrada and Avalos, 2009) and exposed to *Dra*I-linearized pVIO5 plasmid, following a transformation protocol formerly described (Proctor et al., 1997). The resulting hygromycin-resistant colonies were passed through single conidia to ensure homokaryosis, checked for conservation of the hygromycin-resistant phenotype, and analyzed by Southern blot hybridizations (Sambrook and Russell, 2001) with a probe covering a segment of the *carY* ORF and a 3' downstream sequence. The probe consisted in a 872-bp fragment, obtained after removal by *Nco*I digestion of 371 bp from the 5' end of the 1,243-bp PCR amplification product generated with primer set 7 from wild-type genomic DNA.

For complementation of the Δ*carY* mutation, a 3.5-kb PCR fragment obtained from wild-type genomic DNA with primer set 8 was cloned into the pGEM-T Easy vector. A segment containing the *carY* coding sequence and 1.9 kb of its promoter region was removed from this plasmid by digestion with *EcoR*I and cloned into the pNTP2 vector, containing the geneticin-resistance cassette (Rodríguez-Ortiz et al., 2012), to yield plasmid pVIO8. The orientation of the insert was determined by restriction analysis and the integrity of the promoter and the *carY* coding sequence was checked by sequencing. About 10^8 protoplasts from the Δ*carY* strain were obtained as described above, exposed to *Spe*I-linearized plasmid pVIO8 and incubated for one week on

selective medium. The geneticin-resistant colonies were passed through single conidia, checked for conservation of hygromycin and geneticin resistances and analyzed for *carY* expression by real time RT-PCR analysis, as described in the next section. For this purpose total RNA samples were obtained from mycelia incubated only in the dark or following 30 min or 1 h under light, as described in section 2.1.

2.4. Expression analyses

Real-time RT-PCR analyses were performed on total RNA samples extracted with the RNeasy® Plant Mini Kit (Qiagen). Reaction mixtures contained 12 µl of SYBR Green PCR Master Mix 2X (Applied Biosystems, Branchburg, NJ, USA), 0.125 µl of MultiScribe Reverse Transcriptase (50 U ml^{-1}), 0.125 µl of RNase Inhibitor (10 U ml^{-1}), 50 ng of RNA and 5 mM each primer. The reactions, carried out in 25 µl volumes on an ABI 7500 (Applied Biosystems), consisted of 30 min of retrotranscription at 48ºC, 10 min at 95ºC, 40 cycles of 95ºC denaturation for 15 s and 60ºC polymerization for 1 min. Dissociation curves were obtained afterwards. Transcripts of the genes *carY*, *carRA*, *carB*, *carO*, *carX*, *carT*, and *carD* were detected with the primer sets 9-15 (Table 1), designed with the Primer Express v2.0.0 software (Applied Biosystems) and synthesized by StabVida (Oeiras, Portugal). $MgCl_2$ and primer concentrations, and annealing temperatures, were optimized as recommended by the manufacturer. The ß-tubulin gene from *F. verticillioides* (primer set 16, Table 1) was used as a control for constitutive expression. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method with Sequence Detection software v1.2.2 (Applied Biosystems). For each biological replicate, RT-PCR reactions were performed twice to ensure statistical accuracy.

2.5. Carotenoid analyses

Carotenoids were extracted with acetone from *ca.* 20 mg lyophilized mycelia samples in a Fast-Prep-24 homogenizer (MP Biomedicals LLC Europe, France) using sea sand (Panreac Química SAU, Barcelona, Spain) and two pulses of 30 s at 6 m s^{-1}. In each pulse, 1 ml acetone was added and the extracted fractions were collected and vacuum dried. Total amounts of colored carotenoids were estimated from absorption maxima in hexane, using an average maximal ε (1 mg ml^{-1} cm^{-1}) of 0.2.

For *in vitro* analyses, HPLC separations were performed in a Waters System (Waters, Eschborn, Germany) equipped with a photodiode array detector (model 996)

and a C30 reverse-phase column (YMC Europe, Schermbeck, Germany), using solvent A (acetonitrile:tetrahydrofuran:NH_4acetate 1% H_2O 4:1:5, v/v/v) and B (acetonitrile: tetrahydrofuran:H_2O 50:40:6, v/v/v). The column was developed at a flow rate of 1 ml min^{-1}, with a linear gradient from 100% A to 100% B within 20 min, followed by 5 min at a flow rate of 2 ml min^{-1} and another 10 min to 100% A.

2.6. Crossing experiments

The crossing procedure was based on methods formerly described (Leslie and Summerell, 2006). Crosses were done in transparent plastic boxes with hermetic covers and breathing strips (Eco2box / white filter, oval model 80mm H, Duchefa, Haarlem, The Netherlands) with 80 ml carrot agar. 5 x 10^6 conidia of the strain used as a female were spread on the carrot agar and incubated for 4 days at 30ºC under constant illumination. Formerly, 5 x 10^6 conidia of the strain used as a male were incubated on water agar (20 g l^{-1} agar) plates for 7 days at 30ºC under constant illumination. The resulting conidiating mycelia surface was mixed with 6 ml distilled water using a spatula and added to the surface of the 4-day-old female cultures. The 6-ml samples were scrubbed on the female mycelia with the tip of a 10-ml glass pipette and the boxes were incubated for four weeks at 30ºC with a photoperiod of 12 h. In this case, illumination was achieved with 2 white light fluorescents (Philips Master TL-D 18W/840) and a black light fluorescent (Sylvania Blaklite-Blue F18W/BLB/ES), at a distance of 30 cm. Perithecia started to appear after 2-3 weeks.

2.7. Retinoic acid assays

Retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) or methyl retinoate were added to the medium at a final concentration of 0.75 mM. To obtain methyl retinoate, 100 mg of retinoic acid were dissolved in a solution of methanol:benzene (1:4 v/v). To avoid precipitation, methanol was added after retinoic acid was dissolved in the benzene fraction. To introduce methyl groups to retinoic acid, 300 µl of a 2M (trimethylsilyl)diazomethane solution (Sigma-Aldrich) were slowly added as several drops. After methylation, the sample was vacuum-dried in a rotoevaporator and resuspended in diethyl ether. (Trimethylsilyl)diazomethane was removed by washing five times with water, allowing separation between water and solvent and recovering the upper layer containing methyl retinoate. Once washed, the sample was vacuum-

dried and resuspended in ethanol when added directly on mycelia surfaces, or in a mixture of ethanol/tween 80 4:5 (v/v) when added to the medium before inoculation.

For chemical assays of retinoic acid in cell extracts, mycelial samples grown for 6 days over cellophane sheets on DG minimal medium under light were crumbled in liquid nitrogen, lyophilized and ground to very fine powder using a ball mill (Retsch MM200, Germany). 100-mg grounded samples were extracted for 2 h with 4 ml 80 % acetone 1 % acetic acid using an overhead shaker at room temperature. For internal standardization, the samples were spiked with 3 pmol of deuterated retinoic acid (D6-RA, Buchem B.V., The Netherlands) prior to extraction. After centrifugation (3200 g, 10 min) the supernatants were transferred to new 15 ml tubes and partitioned against 2 ml petrol ether:diethyl ether 2:1 (v/v). After centrifugation (3200 g, 10 min) the organic solvent phase was collected, dried under vacuum and dissolved in 50 µl dichloromethane:methanol 1:1 (v/v). From this, 2 µl were injected into the LC-MS system. Separation was carried out using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 1.9 µm Hypersil Gold C18 reversed-phase column (150 x 2.1 mm; Thermo Fisher Scientific). A gradient system was used, consisting of solvent A, 0.05% (v/v) aqueous acetic acid, and solvent B, 0.05% (v/v) acetic acid in acetonitrile. The starting condition of 30% A was maintained for 1 min and then a gradient to 100% B was developed within 4 min. This condition was maintained for 15 min.

The qExactive mass spectrometer (Thermo Fisher Scientific) was equipped with a heated electron spray ionization (HESI) source and operated in the positive ion mode. Spectra were recorded using a spray voltage of 3 kV and a capillary temperature of 350°C. Nitrogen sheath gas flow and auxiliary gas flow were set to 40 and 15 arbitrary units, respectively. MS1 data (m/z 50-600) and data-dependent MS2 spectra were recorded and the normalized collision energy was set to 30 %. Internal standard-based quantification was performed using the MS data and the Tracefinder 3.1 software (Thermo Fisher Scientific). The retinoic acid/D6-retinoic acid calibration curve showed a linear behavior from 0.5 pmol to 15 pmol. Retention times of the authentic references served as confirmation of identity in addition to using the following exact mass precursor and MS2 fragment ions: m/z [M+H]=301.21621 (159.11687, 201.16374).

3. Results

3.1. Identification of a RALDH enzyme in Fusarium

Two representative mammal retinal dehydrogenase genes, RALDH1 (Accession No. P24549) and RALDH2 (Q62148) from *Mus musculus*, were used for BLAST analyses against the genome of *F. verticillioides*. The searches retrieved more than 30 ALDH genes with high sequence similarities, whose alignments showed identity percentages usually exceeding 45%. Because of the vast knowledge on *N. crassa* carotenogenesis, the genome of this fungus was also included in the analysis. To evaluate the phylogenetic relations among the proteins with higher similarity to RALDHs, a phylogram was generated from a Clustal alignment between the two isozymes from *M. musculus* and the five proteins from *F. verticillioides* and *N. crassa* with the lowest *e* values in the BLAST search (Fig. 1A). As predicted, the *M. musculus* paralogs are within a common clade in the phylogram; however, they were not markedly separated from fungal proteins. *F. verticillioides* FVEG_00585 and *N. crassa* NCU03415 showed a similarity of 53% of 493 aa, and 54% of 494 aa to RALDH1, respectively (Fig. 1B). In general, the four proteins share 79% of conserved residues. Higher identity percentages of the remaining proteins against RALDH1 were 49% of 501 aa for FVEG_11973, 48% of 495 aa for FVEG_07427, 47% of 500 aa for FVEG_10377, and 47% of 501 aa for FVEG_00151. Based of the high similarity of these enzymes with animal RALDHs (Fig. S2), biochemical assays were conducted.

To determine the enzymatic activities of the five putative RALDH enzymes from *F. verticillioides*, the corresponding cDNAs were cloned and expressed as a fusion to thioredoxin in *E. coli*. Crude protein extracts from each *E. coli* culture were incubated with retinal and analyzed by HPLC. The results revealed the conversion of retinal into retinoic acid by a crude extract of *E. coli* expressing gene FVEG_00585 (Fig. 2), but not by those expressing any of the other investigated genes (FVEG_11973 shown as example in Fig. 2). The identity of the enzymatic product was confirmed by comparison with commercial retinoic acid. Since retinal is the product of the *Fusarium* CarX enzyme, we renamed gene FVEG_00585 as *carY*.

To confirm the retinoic-forming activity of the *F. verticillioides* CarY, the enzyme was expressed as a GST-fusion protein in *E. coli*, purified with glutathione-Sepharose and released by the protease factor Xa (Fig. 3A). *In vitro* incubation of CarY with retinal resulted in the formation of retinoic acid, as confirmed by HPLC analysis (Fig.

3A). However, no retinoic acid was detected upon incubation with retinal of *N. crassa* NCU03415 protein obtained with the same protocol (Fig. S3A). Former reports showed that some ALDHs are highly specific for a limited range of substrates while others exhibit a broader substrate specificity (Yoshida et al., 1998). To characterize the substrate specificity of CarY, the purified enzyme was incubated with different apocarotenals: β-apo-11'-carotenal (C_{15}), β-apo-14'-carotenal (C_{22}), β-apo-12'-carotenal (C_{25}), β-apo-10'-carotenal (C_{27}) and β-apo-8'-carotenal (C_{30}) and, as a positive control, retinal (β-apo-15'-carotenal, C_{20},). HPLC analyses showed the conversion of β-apo-14'-carotenal, besides retinal (Fig. 3A), but not of any other substrate (Fig. 3B and Fig. S3B). Thus, these data indicate a narrow specificity regarding the chain length of the substrates.

The gene *carY* is highly conserved in other *Fusarium* species. The orthologs in *F. oxysporum* 4287 (FOXG_00928), *F. fujikuroi* (FFUJ_00634) and *F. graminearum* (FGSG_00979), exhibit 99%, 99% and 96% identity at the protein level with *F. verticillioides* CarY, respectively. Its closest relative is the *F. fujikuroi* protein, which differs in only two non-conserved amino acids (Fig. 1B).

3.2. Carotenogenesis in F. verticillioides

The enzymatic activity of CarY implies the availability of the substrate retinal in *F. verticillioides*. The genes involved in retinal and neurosporaxanthin formation *carRA*, *carB*, *carX*, *carT*, and *carD*, have been described in *Fusarium fujikuroi* and are conserved in other *Fusarium* species [reviewed by (Avalos et al., 2014)], such as *F. oxysporum* or *F. graminearum*. The genome of *F. verticillioides* contains all orthologous genes required for carotenogenesis: *carRA* (FVEG_10718), *carB* (FVEG_10717), *carX* (FVEG_10719), *carT* (FVEG_09251) and *carD* (FVEG_02675). Similarly, it contains orthologs for the presumed retinal-binding rhodopsins, *carO* (FVEG_10716) and *opsA* (FVEG_12735). As in the *F. fujikuroi* genome, *carRA*, *carB*, *carX* and *carO* are clustered in *F. verticillioides*, while *carT*, *carD* and *opsA* are located in other genomic regions.

Light is the major environmental stimulatory signal in *Fusarium* carotenogenesis (Avalos and Estrada, 2010). As formerly described (Ádám et al., 2011), surface cultures of wild type FGSC7600 of *F. verticillioides* grown on DGasn medium were faintly pigmented in the dark, and exhibited a deeper orange color when incubated under light. In the dark, the carotenoid analyses revealed higher levels of carotenoids

in FGSC7600 than in the wild type FKMC1995 of *F. fujikuroi* irrespective of the temperature of incubation (Fig. 4A), indicating regulatory differences between these strains. Under constant illumination, *F. verticillioides* accumulated *ca.* 400-500 µg of carotenoids g^{-1} dry weight, depending on the temperature, exceeding again the values found in *F. fujikuroi* under the same culture conditions.

To confirm the photoregulation of carotenogenesis in *F. verticillioides*, we analyzed by real-time RT-PCR the effect of light on mRNA levels of different *car* genes. Time-course experiments showed a rapid photoinduction of the mRNA of *carRA*, *carB*, *carX* and *carO*, reaching the maximum amounts approximately 60 min after light onset, and decreasing slowly during the following 60 min (Fig. 4B). However, only a minor photoinduction was observed for *carT* and even lower for *carD* and *carY*, with less than 3-fold increase in mRNA levels after one hour of illumination.

3.3. Phenotype of the ΔcarY mutant

To gain insights into the biological function of CarY, we investigated the effect of *carY* mutation in *F. verticillioides*. The wild-type strain FGSC 7600 was transformed with a plasmid in which the central region of gene *carY* was replaced by a hygromycin resistance cassette (Fig. S4A), and six transformants were obtained under selective conditions. Southern blot analysis with a probe covering deleted and non-deleted *carY* sequences (Fig. S4B) showed the band corresponding to the wild-type *carY* gene in five transformants (T1-T4 and T6), and the replacement of the *carY* gene by the deletion construct in transformant T5, hereafter referred to as Δ*carY* mutant. As a control for the phenotyping of this mutant, the wild-type *carY* sequence preceded by a 1.9-kb promoter region (Fig. S4C) was reintroduced into this strain, leading to the isolation of three transformants under selective conditions, that we called C1, C2, and C3. The absence of *carY* in the Δ*carY* mutant and its recovery in the three transformants were confirmed by PCR, either with genomic DNA or with cDNA (Fig. S4C). Moreover, expression analysis (Fig. 5A) showed that the *carY* mRNA levels were significantly enhanced in two complementing strains investigated (C1 and C3) compared to those in the wild type, while very low expression was found in the Δ*carY* mutant. The enhanced *carY* expression in the complementing strains is possibly due to multiple integrations or positional effects of the integrated sequence.

The wild type, the Δ*carY* mutant and the complemented strains were grown under different culture conditions for detailed phenotypic comparison. After growth on

minimal DG medium for four days at 30ºC in the dark or under illumination, visual inspection of the colonies revealed morphological differences: those of the Δ*carY* mutant exhibited a more compact aspect than wild-type colonies under illumination (Fig. 5B). The differences were less apparent in the dark, or in DGasn medium irrespective of illumination, albeit a more compact appearance was noticeable in the Δ*carY* mutant colonies also under these conditions (Fig. S5A). The phenotypic differences were reverted in the complemented strains, whose aspect was more similar to that of the wild type (Fig. 5B). Only minor phenotypic variations were observed between the different complemented strains and the wild type, likely due to differences in *carY* expression.

The mutation of the *carX* gene results in a partial deregulation of carotenoid biosynthesis in *F. fujikuroi* (Thewes et al., 2005). To check a possible impact of CarY activity on carotenoid metabolism, we determined the carotenoid content of the wild type, the Δ*carY* mutant and the complemented strains C1 and C3 in the dark or under constant illumination on DG medium (Fig. S5B). At 30°C, the four strains exhibited similar carotenoid levels, with concentrations of *ca.* 100 and 350 μg g^{-1} dry weight in the dark or under light, respectively. Carotenoid compositions were also comparable in the four strains, as indicated their similar absorption spectra (Fig. S5C). In accordance with their similar carotenoid content, no significant changes were found in the mRNA levels for the *car* genes in the Δ*carY* mutant compared to those of the wild type, either in the dark or after illumination (data not shown). These results are not unexpected, considering that the CarY enzyme is not predictably involved in neurosporaxanthin biosynthesis, and contradict a possible regulatory role for CarY on carotenogenesis. To check a possible relation with oxidative stress, we tested if the Δ*carY* mutation affects the sensitivity to H_2O_2. The wild type, the Δ*carY* mutant and the complemented strain C1 were grown on plates with DG medium supplemented with 0, 2, 4, 8, 16 and 32 mM H_2O_2 at 30ºC under illumination. After three days of incubation, no difference in sensitivity to H_2O_2 was appreciated among the tested strains.

3.4. Effect of the ΔcarY mutation on growth, conidiation and hypha development

When the fungus grows on agar, a part of the hyphae develops aerially and another part grows below the agar surface, hindering the calculation of mycelial biomass. To facilitate this analysis, new experiments were carried out growing the fungus on

cellophane sheets. Under these conditions the appearance of the colonies was similar to those on agar, with more compact progression and slightly slower radial growth of the colonies of the the Δ*carY* mutant compared to those of the wild type and the complemented strain C1 (Fig. 6A). As expected from their more compact aspect, the colonies of the Δ*carY* mutant contained a higher mycelial mass than those of the control strains (wild-type and complemented transformants) (Fig. 6B). Accordingly, the colonies of the Δ*carY* mutant contained more conidia (Fig. 6C), but because of their higher mycelial contents, we conclude that there are not patent differences in conidiation among the tested strains. However, the conidia of the Δ*carY* mutant were smaller in size compared to those of the control strains, while such difference was less evident in the dark (Fig. 6D). The result was similar irrespective of the location of the mycelium in the colony, and the difference was more apparent on cellophane than on the agar surface.

Mycelial organization in the colonies of the investigated strains was examined in more detail under a stereoscopic microscope (Fig. 7). Noticeable differences were appreciated in the border of the colonies, thicker in the Δ*carY* mutant compared to the control strains (two representative cuts shown for each strain and condition in Fig. 7A, column 1). Moreover, a combination of top and back illumination revealed a larger border of thin expanding mycelia in the control strains, which was less marked in the dark (Fig. 6A, column 2). The comparison between agar and cellophane cultures showed similar results, except that the wild type and the complemented strain exhibited more irregular borders on cellophane than on agar, while those of the Δ*carY* mutant were basically unaffected. A detailed inspection of the hyphae in the colony borders showed different aspects, with the hypha growing in a straighter manner in the control strains compared to those of the Δ*carY* mutant, that exhibited a more erratic pattern (Fig. 7A, column on the right). A higher magnification of the hyphal tips allowed to observe more clearly the difference, and revealed the occurrence of some entwined hyphae forming thicker strands in the Δ*carY* mutant, which were not noticed in the control strains (Fig. 6C). The straighter growth of the hyphal tips in the borders of the control colonies may explain its faster expansion on the cellophane sheets (Fig. 6A), also observed on agar.

3.5. Effect of the ΔcarY mutation on sexual fertility

The phenotypic characteristics of the Δ*carY* mutant suggest that the enzymatic CarY product plays a regulatory role in development. Therefore, we analyzed the possible function of CarY in sexual development of *F. verticillioides*. For this purpose, the Δ*carY* mutant and the complemented strain C1 were crossed on carrot agar medium, either as males or females, with a wild-type strain of the opposite sex. After four-week incubation, perithecia were formed and counted. Irrespective of the cross, detailed observation under a stereoscopic microscope revealed that some perithecia developed individually, but others were clustered, each one containing several perithecia whose number was difficult to ascertain. For quantification purposes, both individual perithecia and clustered perithecia were considered as perithecia-forming units (Fig. 7A and 7C), abbreviated hereafter PFUs.

When acting as a male partner, the Δ*carY* mutant produced similar PFU amounts as the wild type, but much lower when it acted as a female partner (Fig. 7B). Moreover, acting as a female the mutant produced abundant aerial hyphae, resulting in a cotton-like surface on the carrot agar, while such hyphae were mostly absent in the control strain. Total PFU numbers varied between different experiments, but the same tendency was always observed. However, the CarY enzyme was not needed for perithecia maturation, as suggested by the presence of cirri protruding from the ostioles in the few observed perithecia, which is characteristic of their ripe state (Fig. 7A). Interestingly, the female-acting complemented strains recovered their ability to form PFUs at wild-type levels and reduced their formation of aerial hyphae, indicating that these phenotypic alterations are produced by lack of a functional CarY enzyme. We conclude that CarY activity stimulates the formation of perithecia but it is not needed for their development.

To check if the phenotype of the Δ*carY* mutant is due to a lack of retinoic acid, the strains were crossed on carrot agar supplemented with commercial retinoic acid. The polar nature of the acidic group of retinoic acid may hinder its passage through the membrane into the cell. Therefore, parallel experiments were also achieved with methyl retinoate. However, the addition of any of these compounds into the media did not affect the reduced capacity to develop PFUs by the Δ*carY* mutant (data not shown). Moreover, addition of these chemicals to DG cultures did not lead to the recovery of a wild-type phenotype by the Δ*carY* mutant. However, these experiments were not conclusive; the lack of effect might be the result of incapability of retinoic

acid to enter the cells or, in case of methyl retinoate, of a negative impact of the esterification on its possible biological activity.

3.6. Lack of retinoic acid detection in F. verticillioides

Our data are consistent with a role of CarY in the production of retinoic acid in *F. verticillioides*. To test this hypothesis, we made attempts to detect retinoic acid in the fungal cells. Cell extracts were obtained from the wild type, the Δ*carY* mutant and the complemented strain C1 under the conditions shown in Fig. 6A, i.e. those resulting in noticeable phenotypic differences between the Δ*carY* mutant and the control strains. For better sensitivity, a LC-MS method was used based on the identification and quantification of target analyte peaks using full MS1 data recorded from m/z 50 – 600. This analytical method showed the presence of approximately 0.1 ng retinal mg^{-1} dry mass in the three strains. Verification of retinoic acid was achieved by screening for the presence of two characteristic MS2 fragment ions (m/z 159.11687, 201.16374) after fragmentation of the MS1 parent ion *m/z* [M+H]=301.21621. Using commercial standard for retinoic acid (Sigma-Aldrich, Germany), the presence of these fragment ions was clearly detectable up to a limit of 1 pmol on column (Fig. S6, top row). The extracts from the *Fusarium* samples showed a small signal in MS1 (Fig. S6, left column) but failed to produce the confirming fragment ions in MS2 (Fig. S6, right row). According to the threshold for unequivocal MS2 confirmation (1 pmol on column), we conclude that the samples analyzed contain less than 0.075 ng retinoic acid mg^{-1} dry mass.

4. Discussion

We have shown that the gene FVEG_00585 of *F. verticillioides* (EWG36660), that we called *carY*, encodes an aldehyde dehydrogenase capable of converting retinal into retinoic acid. We focused our attention on this species because of its amenability to sexual crossing. The capacity of CarY to produce retinoic acid was solidly established *in vitro*. Its substrate specificity was found to be unexpectedly high, contrasting with the relaxed substrate recognition by CarD from *F. fujikuroi* (Díaz-Sánchez et al., 2011) and YLO-1 from *N. crassa* (Estrada et al., 2008), the ALDH enzymes that convert the structurally related β-apo-4'-carotenal into neurosporaxanthin. Interestingly, despite their wider substrate specificity, neither CarD nor YLO-1 were active in converting

retinal into retinoic acid. On the other hand, the lack of activity of *N. crassa* NCU03415 on retinal in our assays was unexpected, considering its high sequence similarity with CarY. However, it could be due also to technical drawbacks, such as incorrect folding or insolubility. Interestingly, no retinal-forming enzyme has been found so far in *N. crassa* (Díaz-Sánchez et al., 2013), despite the ability of its rhodopsin NOP-1 to bind retinal *in vitro* (Bieszke et al., 1999b).

The biochemical capacity of CarY to produce retinoic acid *in vitro*, its high substrate specificity, its sequence similarity to animal RALDHs, and the former demonstration of retinal-forming activity by the CarX enzyme in the close relative *F. fujikuroi* (Prado-Cabrero et al., 2007b) suggests the formation of retinoic acid in *Fusarium*. Retinal should be presumably produced in *F. verticilliodes* to provide the chromophore for the CarO and OpsA rhodopsins, formerly investigated in *F. fujikuroi*. The expression of the *F. verticillioides* genes *carT* and *carD*, involved in late steps of neurosporaxanthin biosynthesis, is barely affected by light, while those presumably involved in the production of β-carotene and retinal, *carRA*, *carB* and *carX*, as well as the rhodopsin gene *carO*, are strongly photoinduced (Fig. 4B). Therefore, retinal should be produced in higher amounts under light. Interestingly, the marked phenotypic alterations of the Δ*carY* mutant are more apparent under illumination. Moreover, light stimulates sexual crossing in *Fusarium* (Leslie and Summerell, 2006), a regulatory effect consistent with the participation of a retinal-dependent activity.

Despite the predicted occurrence of retinoic acid-forming activity, we could not detect retinoic acid in cell extracts of light-grown *F. verticillioides*. Different hypotheses may explain this result. First, the CarY substrate, retinal, is found in very low amounts in the same cell extracts, even in those of the Δ*carY* mutant, allowing to predict a very low retinoic acid content. In fact, a substantial proportion of the identified retinal might be originally bound to rhodopsins. Accumulation of aldehyde compounds has toxic effects due to their chemical reactivity (Lindahl, 1992), and so concentrations of free retinal in the cell should be kept low. Moreover, even if retinoic acid were formed in very low amounts, its possible fate in the cell would be also unknown. Several reasons could explain the lack of retinoic acid detection in the cell extracts, including its rapid degradation, its conversion to a derivative compound, its secretion out of the cell (possibly bound to a carrier molecule to facilitate solubility) or its covalent linkage to a target molecule. In addition, we should not disregard ideas that retinal is not the substrate for CarY *in vivo*, and that this enzyme

participates in the metabolism of another physiologically active compound in the fungus. The lack of effect of exogenously added retinoic acid or the lack of activity of the putative *N. crassa* CarY ortholog favors this hypothesis.

The developmental alterations exhibited by the Δ*carY* mutant, affecting hyphal development on agar and sexual fertility, are consistent with the participation of CarY in the production of a morphogenetic signal, either retinoic acid or a different compound. This signal may act in the fungus via signaling cascades, which could imply G protein, cAMP/PKA, and mitogen activated protein (MAP) kinases. Some studies have shown that these signaling pathways work together to regulate sexual fertility, development and virulence in fungi (Bölker, 1998; Lengeler et al., 2000). In contrast to the *Fusarium* pheromones, which are small diffusible peptides (Martin et al., 2011), the CarY-dependent signal could play an intracellular regulatory role affecting hypha progression and differentiation. Such regulatory role for the CarY enzymatic product would be consistent with a biological effect at very low concentrations. This is the case of retinoic acid in animals: in humans, physiological levels in serum are ca. 3-4 ng ml^{-1} (De Leenheer et al., 1982). Moreover, production of retinoic acid in excessive amounts or in an inappropriate cell stage results in malformations or toxicity (Maden, 1994). Therefore, if we consider the hypothesis of retinoic acid as a morphogenetic signal in *F. verticillioides*, its concentration could be finely regulated at very low levels. In mammals, retinoic acid is a ligand for two families of retinoid receptors, which regulate gene expression (Petkovich, 1992). BLAST searches through the *Fusarium* genome database against several animal retinoic acid receptors (CAB60726 from *Homo sapiens*, isoforms NP_033050, NP_035373 and 1510302C from *Mus musculus*, and NP_001083723 from *X. laevis*) yield no hit using the default 10^{-3} e value as a statistical significance threshold. Therefore, if *F. verticilloides* has a retinoic acid-detecting mechanism, it would be seemingly unrelated to animal retinoic acid receptors.

The involvement of CarY in sexual fertility of *Fusarium* adds an unexpected component to the complex regulation of this developmental process. Different reports have implicated other genes in perithecium formation or maturation. In the homothallic species *F. graminearum,* the loss of the pheromone precursor and receptor genes *ppg1* and *pre2* results in significant reductions in the formation of mature perithecia (Lee et al., 2008). Moreover, no perithecia are produced in mutants of MAP kinase gene *map1* (Urban et al., 2003), APSES-type transcription factor gene

stu1 (Lysøe et al., 2010), ceramide synthase gene *bar1* (Rittenour et al., 2011), or *fsr1* gene, encoding a multimodular protein that includes caveolin- and calmodulin-binding domains (Shim et al., 2006). Other genes, as that encoding the b-ZIP transcription factor Zif1, are needed for later stages of perithecia maturation (Wang et al., 2011). In the heterothallic species *F. fujikuroi*, mutants of the Gα subunit gene *ffg1* do not develop perithecia in the *MAT*1-1 mating background (Studt et al., 2013) and mutants of the Sfp-type phosphopantetheinyl transferase gene *ppt1* do not develop perithecia in a *MAT*1-2 mating background (Wiemann et al., 2012). The Δ*carY* phenotype resembles the mutation in the precursor and pheromone receptor genes in that it reduces but it does not totally abolish the formation of perithecia. In these examples, the mutants usually exhibit pleiotropic phenotypes, with alterations in other traits, such as secondary metabolism or pathogenesis. Our data with CarY joins to the increasing information indicating that regulatory networks participating in different basic processes of the fungus are also involved in the control of perithecia development.

The reduced perithecia formation activity of the Δ*carY* mycelia may be causally connected to their developmental phenotype. When the Δ*carY* mutant acts as a female partner, the surface of the crossing cultures exhibits a "fluffy" appearance, i.e., it develops more aerial hyphae than those of the wild-type and complemented strains acting as female (Fig. 8B). This developmental alteration is probably related to the distinct hyphal growth exhibited by surface colonies (Figs. 5B and 7). The Δ*carY* conidia exhibit wild-type fertility acting as male partner. Therefore, the Δ*carY* hyphae may be affected in its capacity to interact with conidia, which may in turn result in a defective capacity to start the sexual process that leads to the formation of perithecia. The recognition of a spore (ascospore, microconidium or macroconidium) of the strain acting as a male partner by the hypha of the strain acting as a female is a major point in the process of sexual crossing in *Fusarium* sp. (Leslie and Summerell, 2006). However, there is no information on the molecular mechanism underlying such hypha/spore interaction. The interaction between spores and early sexually differentiated hyphae, including the formation of a cytoplasmic bridge, was visualized in *Neurospora* long ago (Backus, 1939), but even in this model fungus, in which an extensive amount of genetic and molecular knowledge has been accumulated (Davis, 2000), the molecular basis for the interaction mechanism between gametic conidia and receptive hyphae in the sexual cycle is unknown.

Our results represent the first report on retinoic acid biosynthetic activity in the fungal kingdom and, in combination with retinal formation, open the way for the identification of similar biosynthetic activities in other fungi or lower eukaryotes. The biological role of the other branch of the carotenoid pathway, leading to neurosporaxanthin biosynthesis and competing with retinal production, is unknown. However, the identification of CarY provides a new perspective to the possible biological roles of retinal in *Fusarium* in addition to its predicted participation in light-dependent rhodopsin activity. The high similarity of CarY to RALDHs and its biochemical activity in our enzymatic assays suggest a possible function in retinal metabolism, presumably associated to the production of a morphogenetic signal whose chemical nature and mechanism of action remain to be elucidated.

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Figure legends

Fig. 1. Sequence analysis of aldehyde dehydrogenases from *Fusarium* and *Neurospora*. **(**A) Phylogram of the five ALDHs from *F. verticillioides* and *N. crassa* and two retinal dehydrogenases from *M. musculus*. The bootstrap NJ tree was achieved with the ClustalX 1.83 program (Thompson et al., 1997), excluding gaps and applying the correction for multiple substitutions. The tree was represented with the NJPlot program (http://doua.prabi.fr/software/njplot). Numbers in nodes indicate bootstrap values. (B) Clustal alignment of CarY from *F. verticillioides* (Fv: FVEG_00585) with the RALDHs from *M. musculus* (Mm1: NP_03849.2; Mm2: NP_033048.2) and a putative RALDH from *N. crassa* (Nc: NCU03415). Residues present at the same position in at least two proteins are shaded in grey, and those conserved in the four ALDHs are shaded in black. The two non-coincident residues in the *F. fujikuroi* CarY homologue FFUJ_00634 are encircled over the sequences.

Fig. 2. *In vitro* activity of CarY and other ALDHs on retinal. HPLC analyses of the incubation of crude lysates of *E. coli* expressing FVEG_00585 (CarY) or FVEG_11973 (upper chromatograms) with retinal (peak a). As a control, crude extract of *E. coli* expressing the empty vector was assayed with retinal (control incubation). Absorption spectrum and elution time of the generated product (peak b) coincide with those of commercial retinoic acid, included as a standard. Structures and the enzymatic reaction are shown.

Fig. 3. *In vitro* activity of purified CarY. (A) HPLC analyses of the incubation of purified CarY protein with retinal (above) and β-apo-14'-carotenal (below). The chromatograms show the formation of retinoic acid (peak b), or β-apo-14'-carotenoic acid (peak d), which were not detected in the negative controls containing GST. The inner picture shows the SDS-PAGE monitoring CarY purification. Lanes: 1, total lysate of cells expressing GST (void plasmid, control); 2, total lysate of cells expressing GST-CarY; 3, sample 2 after removal of inclusion bodies; 4, non-bound supernatant of sample 3; 5, eluted CarY protein. The size of the CarY protein in lane 5 is indicated. (B) Apocarotenals that were negative in the CarY activity assays.

Fig. 4. Effect of light on carotenogenesis in *F. verticillioides*. (A) Carotenoids produced by the wild-type strains of *F. verticillioides* (FGSC 7600) and *F. fujikuroi* (FKMC1995). Carotenoid amounts were determined in samples incubated in the dark or under constant illumination after 1 week-incubation either at 22ºC or at 30ºC. (B) Real-time RT-PCR analyses of mRNA of the clustered genes *carRA*, *carB*, *carX* and *carO*, and the unlinked genes *carT*, *carD* and *carY* in total RNA samples of the wild-type strain FGSC 7600 of *F. verticillioides*, grown in the dark or exposed to light for 15 min, 30 min, 1h or 2h. All data show averages and standard deviation of four measurements from two independent biological replicates.

Fig. 5. Phenotypic characterization of the Δ*carY* mutant and two complemented strains. (A) Real-time RT-PCR analyses of *carY* mRNA in total RNA samples of the wild-type strain FGSC 7600, the Δ*carY* mutant, and the complemented strains C1 and C3 grown in the dark or exposed to light for 30 min or 1h. The data show average and standard deviation of four measurements from two independent biological replicates. Data are referred to the values of the wild type in the dark, taken as 1. (B) Above: colonies of the same strains grown for 4 days at 30°C on DG medium under constant illumination (left plate, exposed to 7 W m^{-2}) or in the dark (right plate). Below: pictures of the illuminated cultures taken from the back of the Petri dish (left plate) or from the top with backlight (right plate). The strains have the same locations in all the plates.

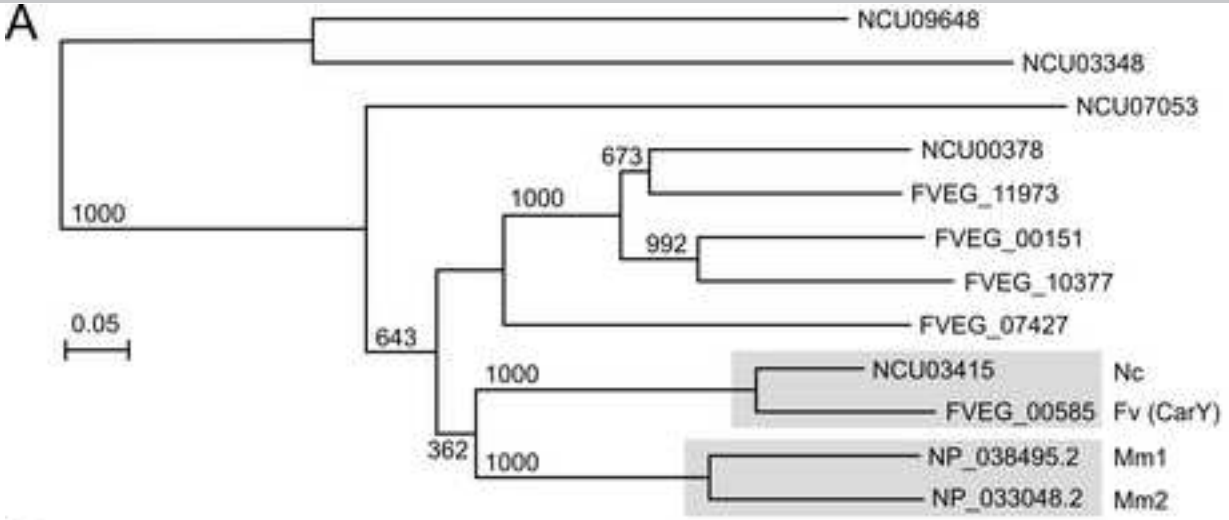
Fig. 6 Effect of the Δ*carY* mutation on growth and conidiation. (A) Time-course of radial growth of colonies of the wild-type-strain FGSC 7600 (WT), the Δ*carY* mutant, and the complemented strain C1 grown at 30°C on DG medium covered with cellophane under constant illumination (7 W m^{-2}). (B) Mycelial dry mass of 3-day-old colonies of the same strains under the same culture conditions under light or in the dark. (C) Conidia produced by colonies grown under the same conditions. Data in A, B and C are average and standard deviation from four independent colonies. (D) Samples under microscope of conidia obtained from outer or inner parts of the colonies of the same strains grown for 3 days in the indicated conditions on DG medium. The black bar represents 20 μm. Approximate locations of inner and outer samples are indicated by black dots in the colonies shown aside. Dotted circles indicate approximate limits of thin colony edges (see Fig. 7). The white bar represents 1 cm.

Fig. 7 Effect of the Δ*carY* mutation on mycelial development on agar cultures. (A) Left column: cross sections of representative colonies of the wild-type strain FGSC 7600, the Δ*carY* mutant, and the complemented strain C1 grown for 4 days at 30°C on DG medium in the dark under constant illumination (7 W m^{-2}). Central columns: borders of the colonies grow with and without cellophane as seen upon simultaneous top and back illumination. Only thin mycelia are illuminated by the back light. Right column: amplification of the border of the colonies on cellophane as seen with back illumination. Bars below picture columns represent 3 mm. (B) Representative examples of expanding hyphae from the light-grown cultures on cellophane shown above. All the pictures were taken with a stereoscopic microscope.

Fig. 8. Effect of the Δ*carY* mutation on perithecial formation. (A) View under stereoscopic microscope of some perithecia produced by the wild-type strain FGSC 7600 and the Δ*carY* mutant after crossing as female parents with the wild type of opposite sex FGSC 7603. The cirri protruding from the perithecium ostiole typical of the ripening process are distinguished by their paler pigmentation. (B) Photographs of the *F. verticillioides* wild-type strain FGSC 7600, the Δ*carY* mutant and the complemented strain C1 crossed with the wild type of opposite sex FGSC 7603. Photographs represent crossing results of each strain, acting either as males (left pictures) or females (right pictures), with the wild type of opposite sex. In A and B, strains were grown for 4 weeks on carrot agar media as described in material and methods. (C) Production of perithecial forming units (PFU) from each cross in the mentioned experiments. Data show average and standard deviation for four measurements from two biological replicates.

Figure 1

A



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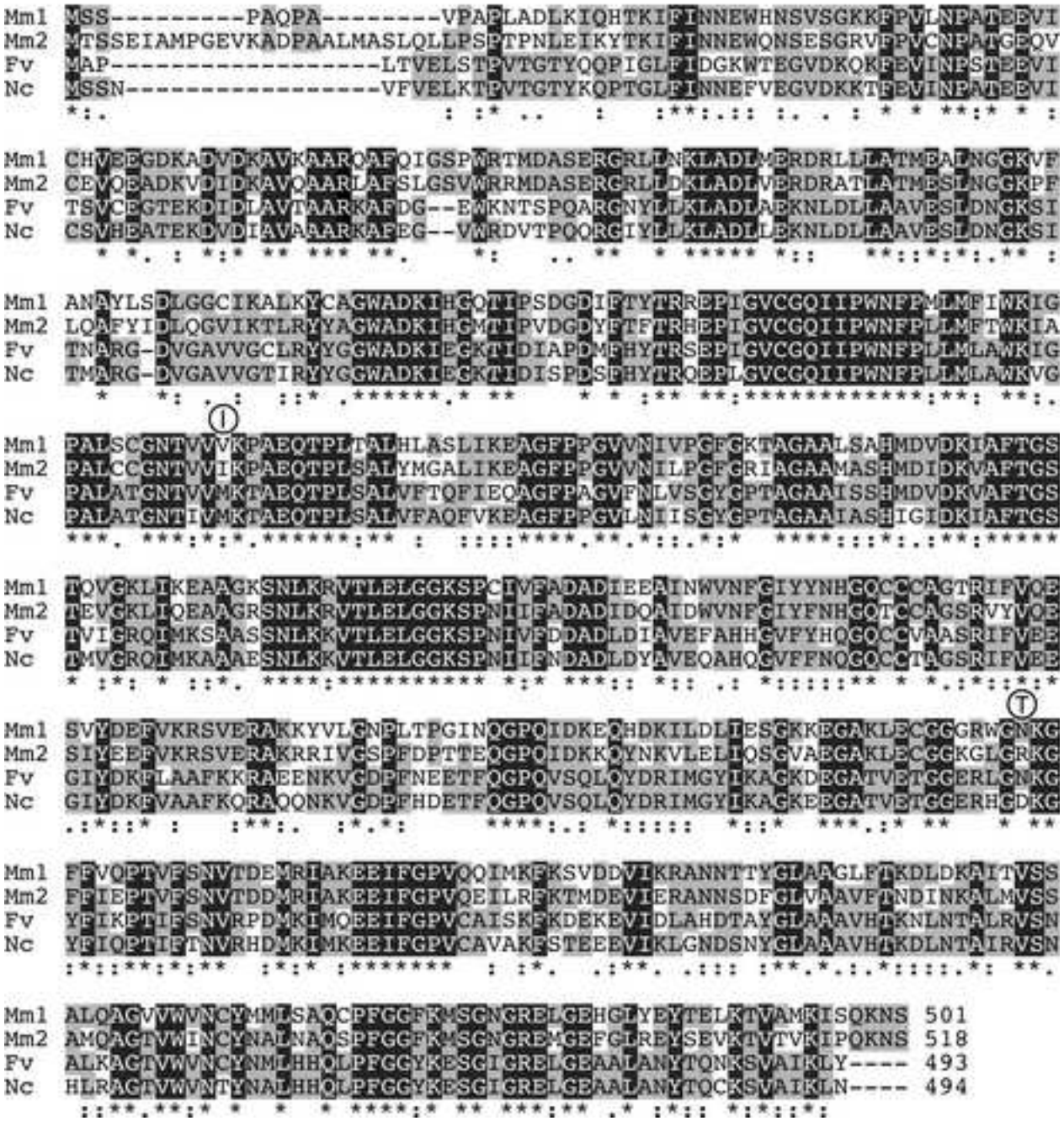


Figure 2

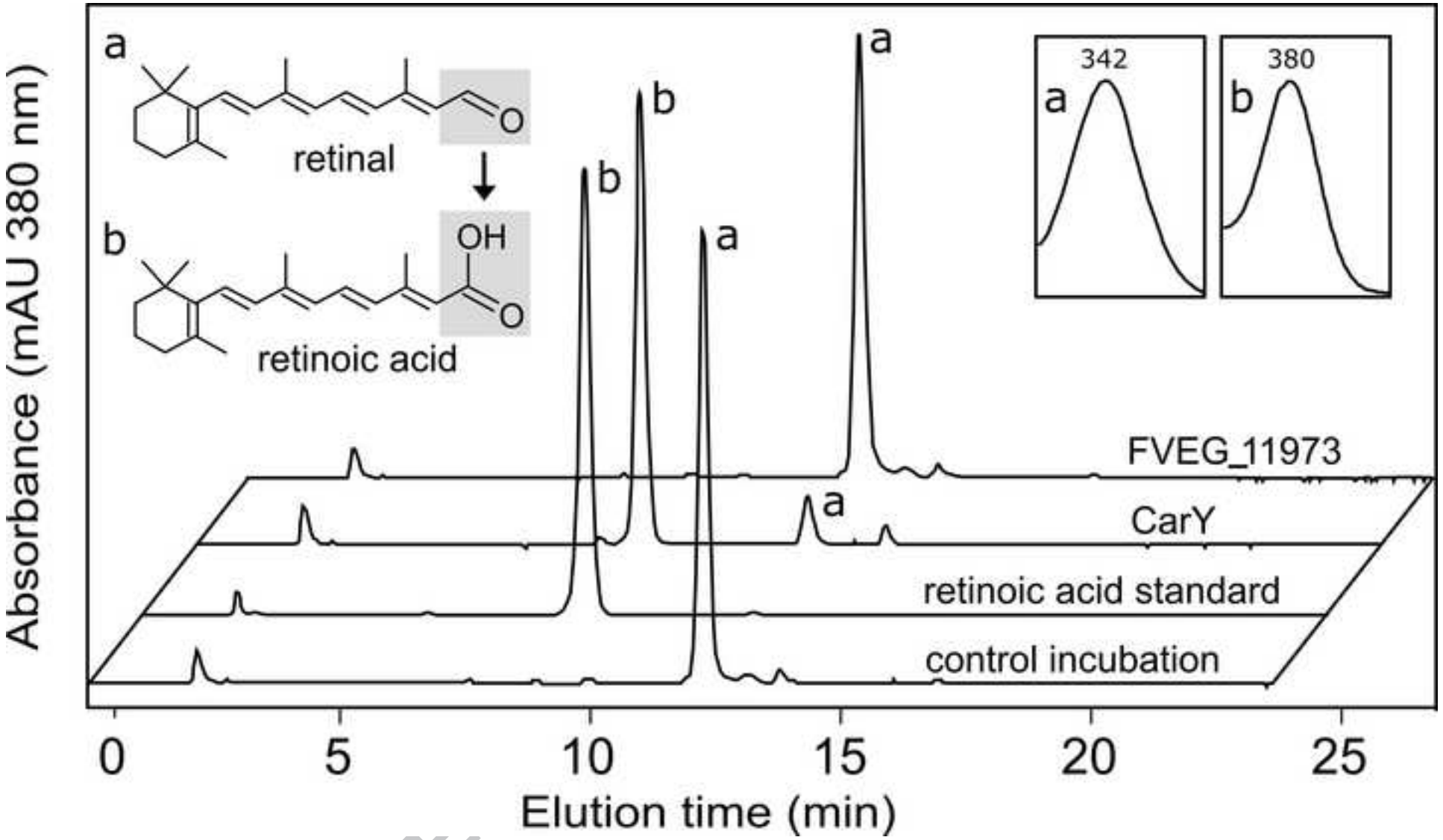
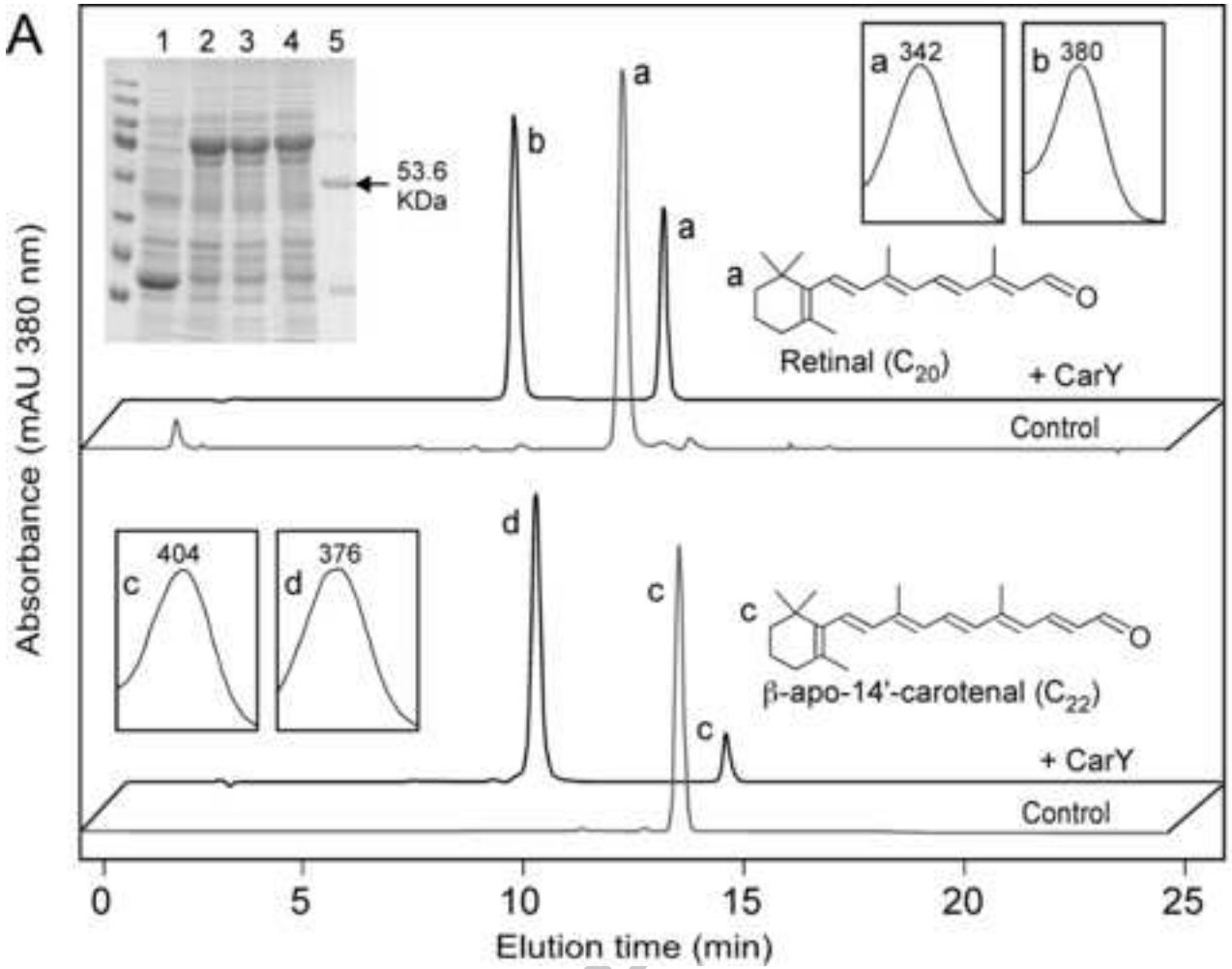


Figure 3



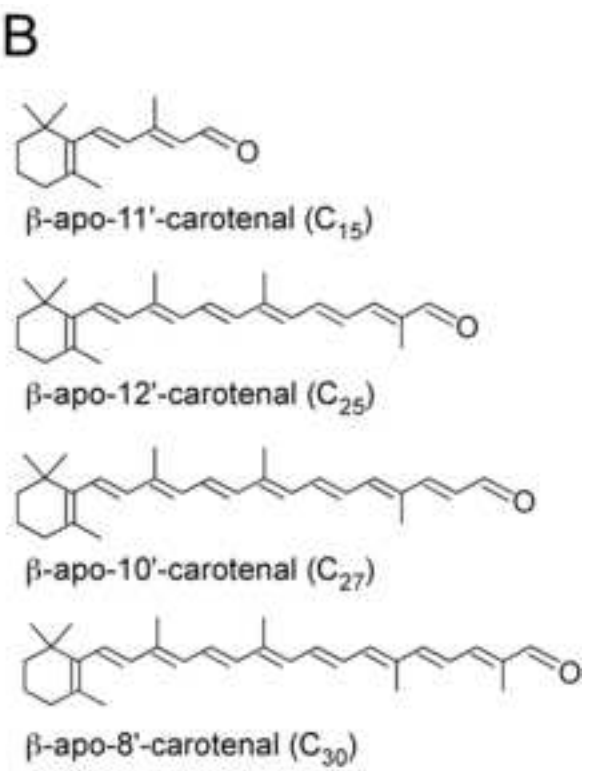
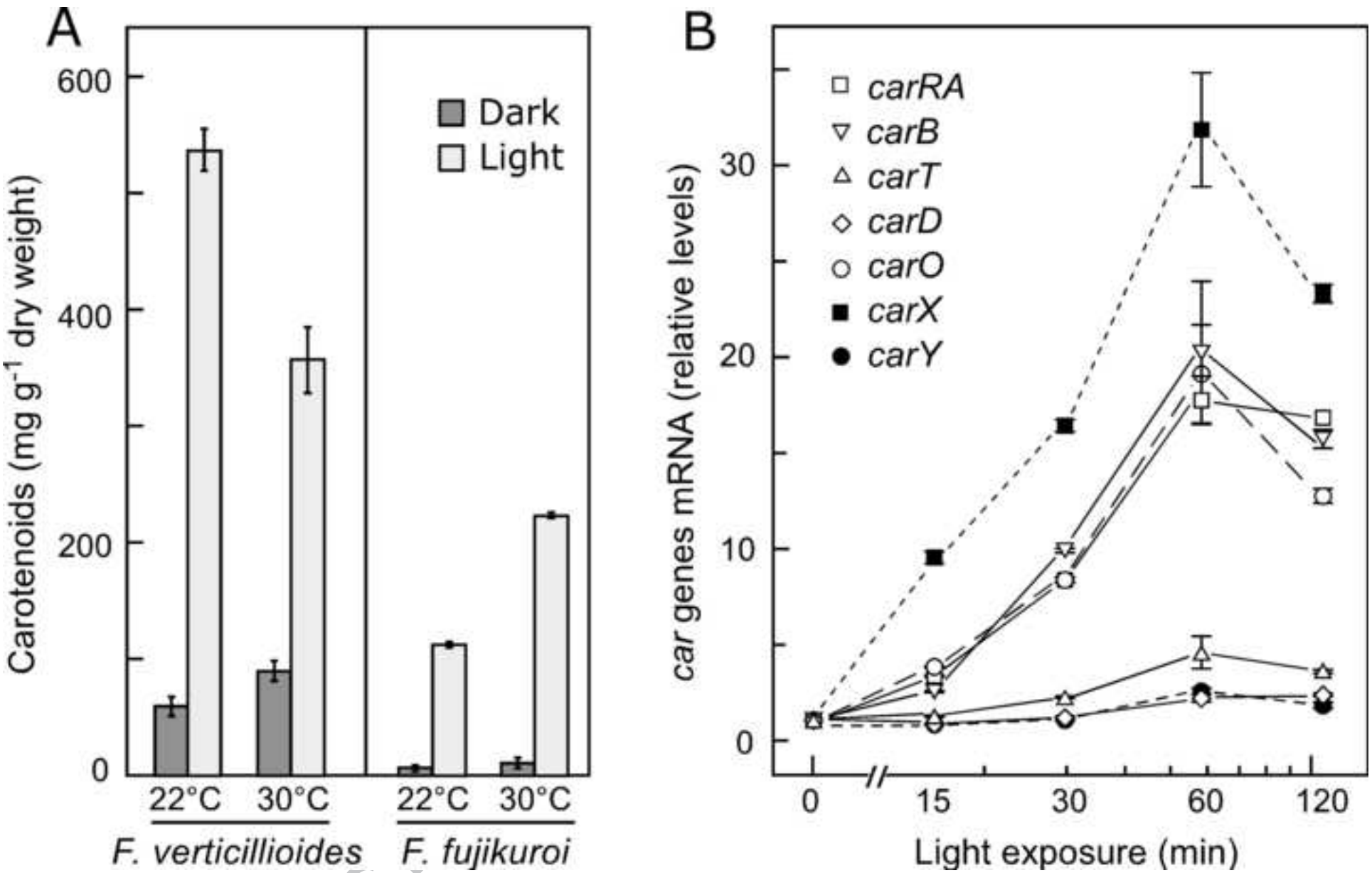


Figure 4



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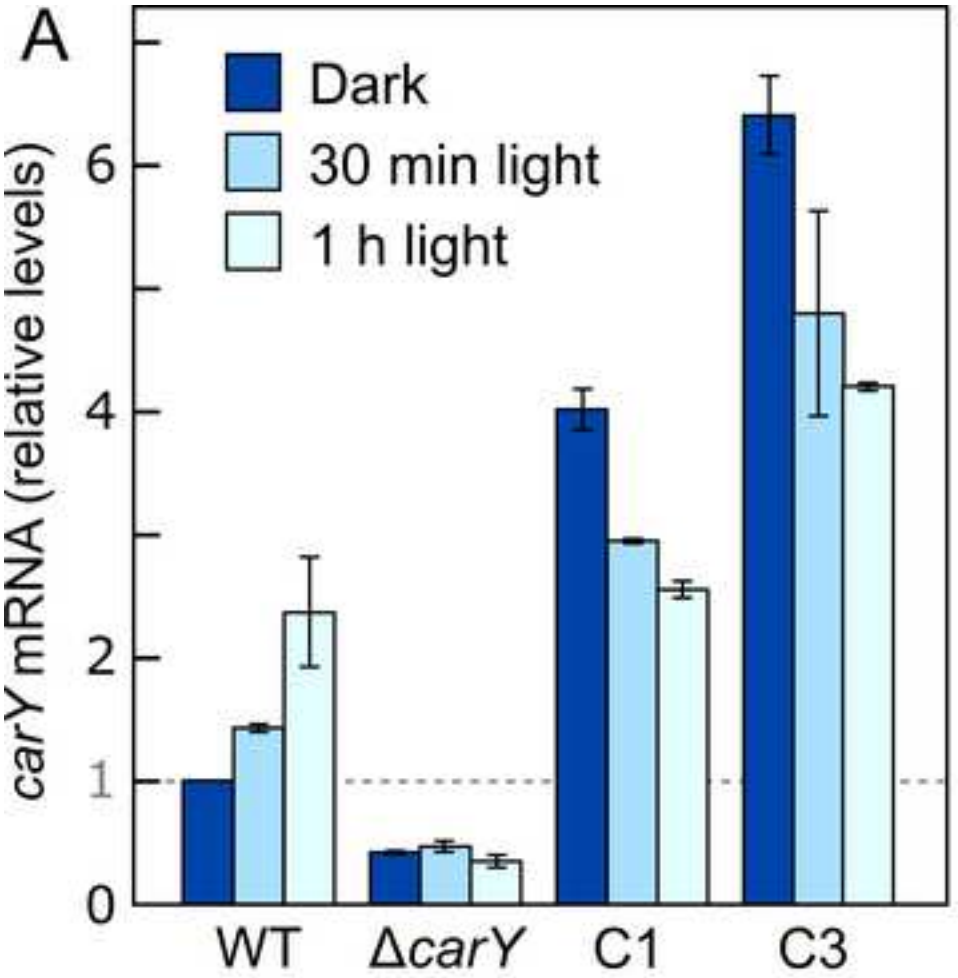

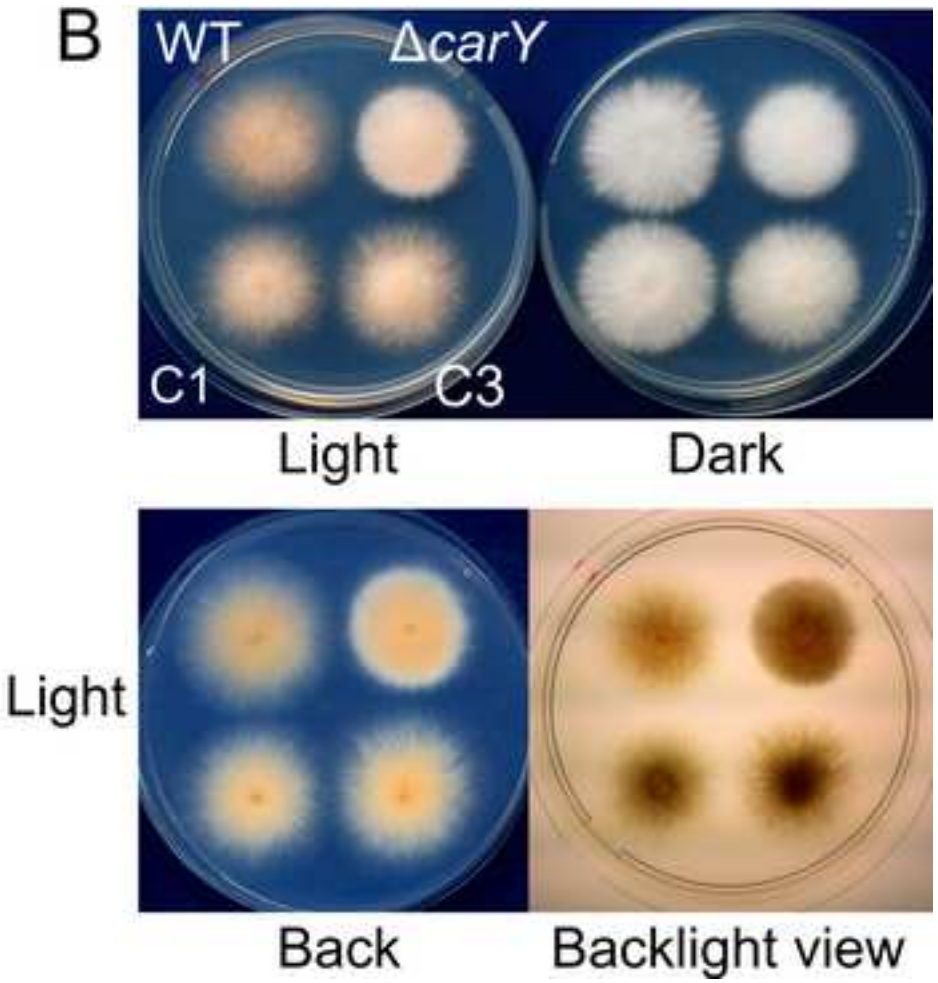

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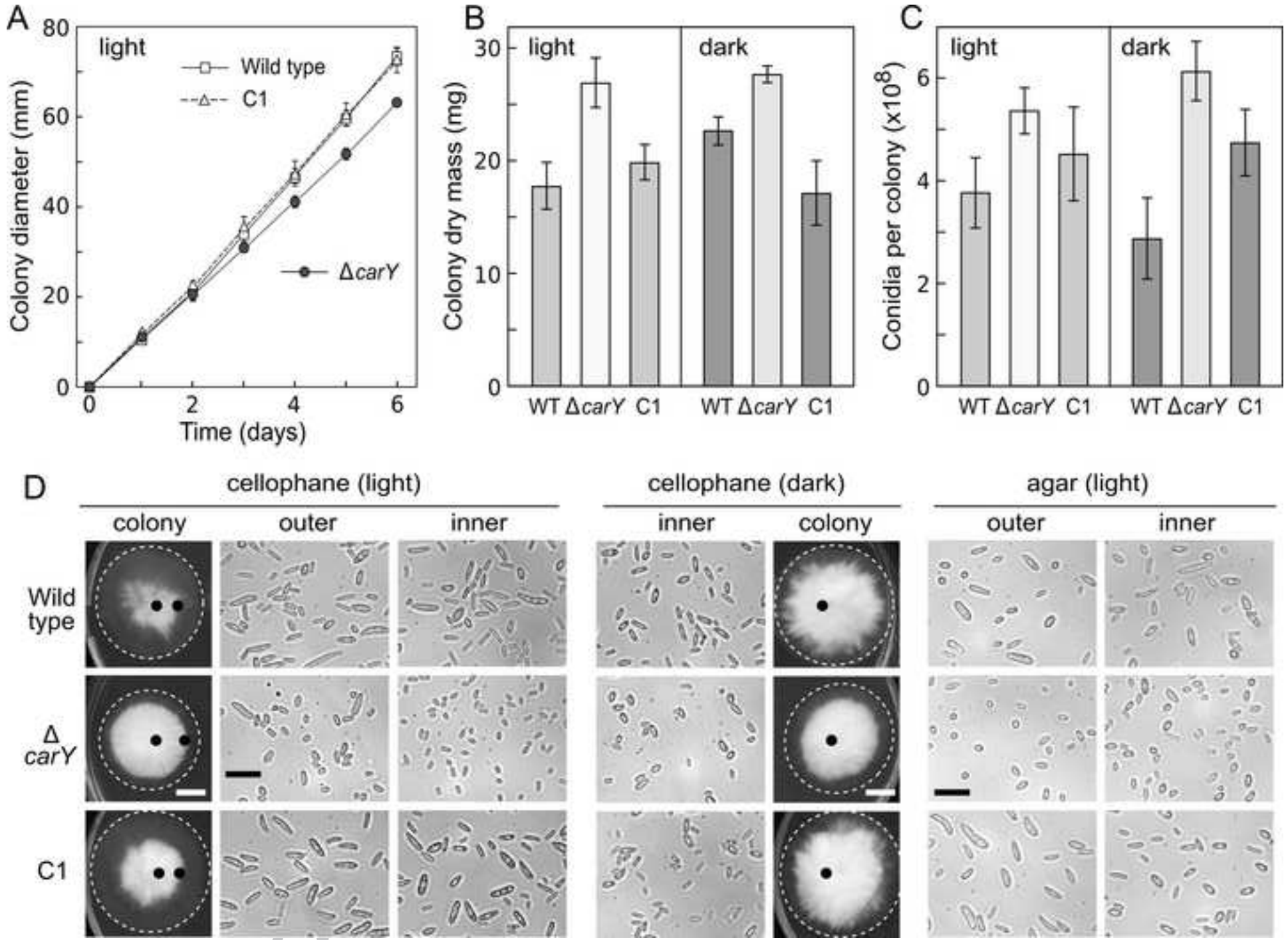

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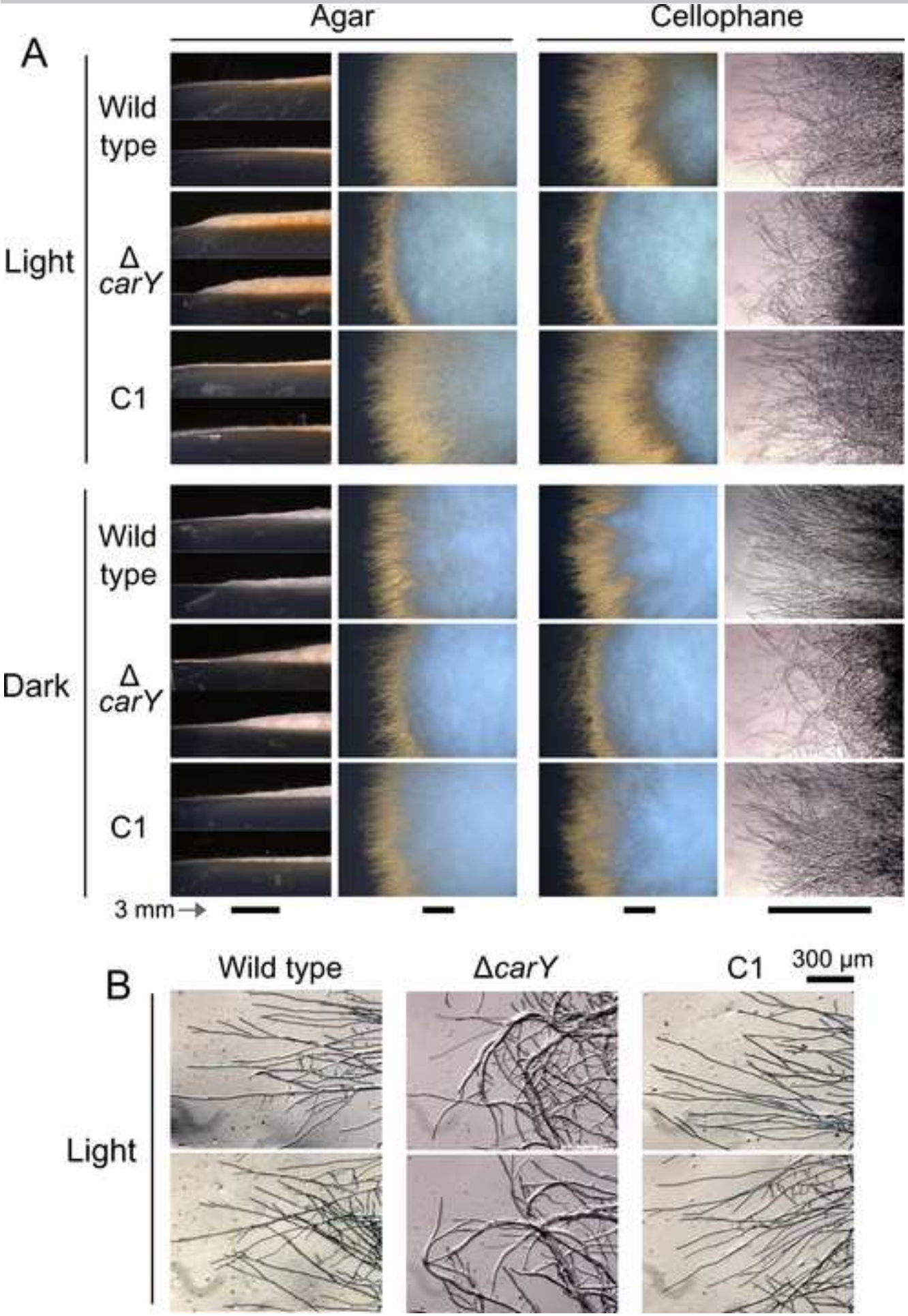

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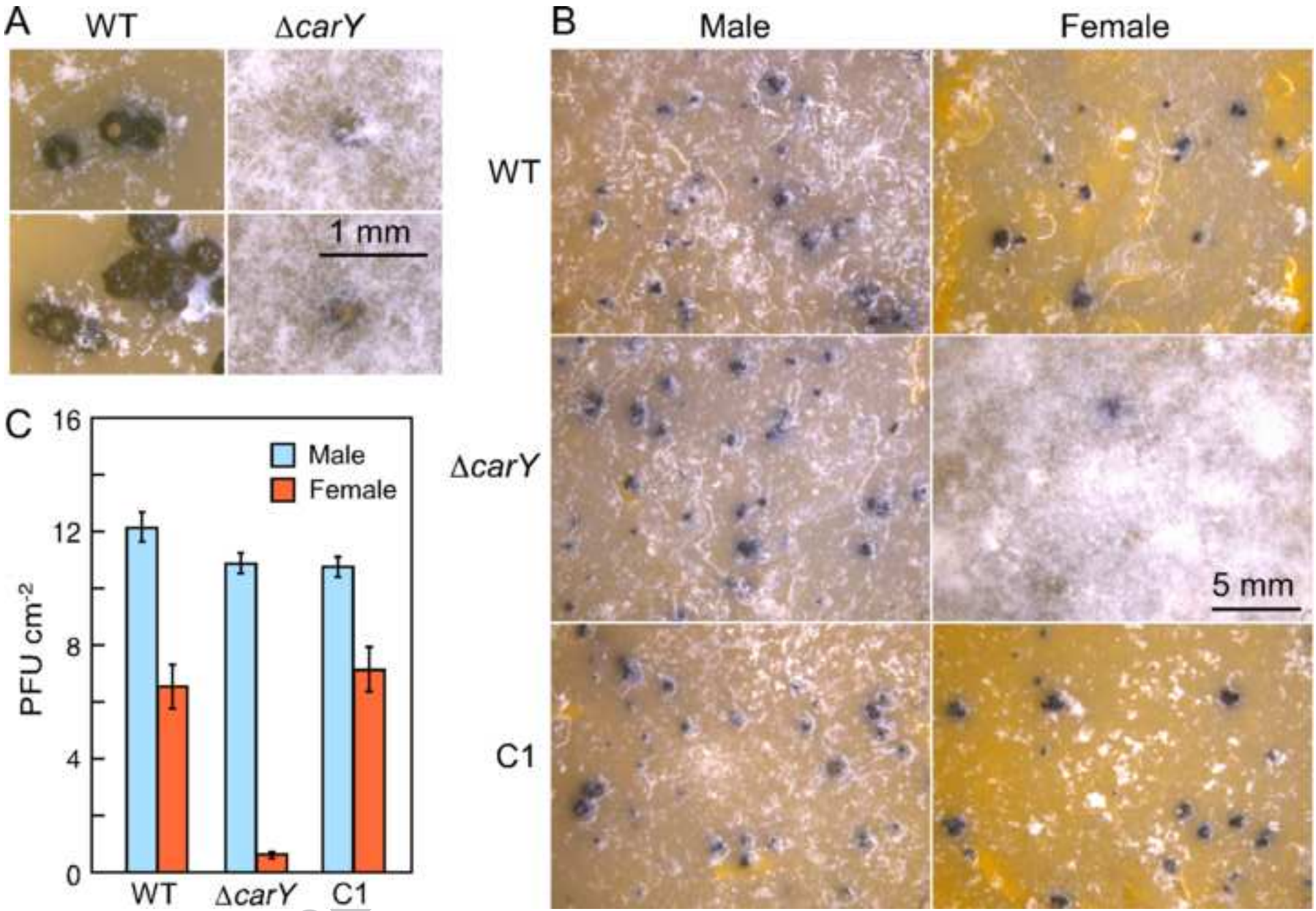
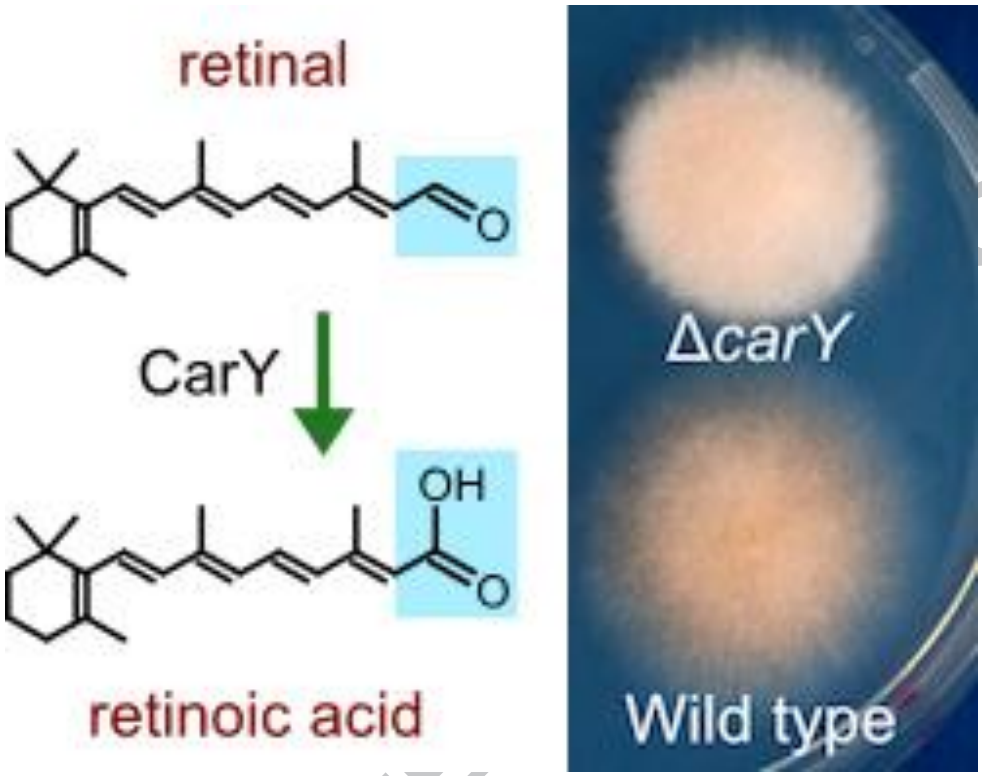


Table 1. Primer sets used in this study

Primer set (objective)	Forward primer (5'→3') Reverse primer (5'→3')
1 (FVEG_00585 cDNA cloning)	ATGGCTCCCCTCACTGTTGAG TCCGCTTTGCTTAGTACAGC
2 (FVEG_11973 cDNA cloning)	ATGAGTCTAGACGTTCAAC TTACAACTTCGCCGCAATG
3 (FVEG_07427 cDNA cloning)	ATGGCATCAACCACATCAATC GGCTATCTCGTATCACCCTC
4 (FVEG_10377 cDNA cloning)	ATGTCGGACCTGTTCGTCAAC CTACAGACGAGAACCAAGG
5 (FVEG_00151 cDNA cloning)	ATGGCTGCCTCAATTGAACTC AATGCAATCATCTACAACC
6 (*carY* cloning for gene disruption)	GAGAGCTCTGTTCTGCCCTT ATCCAAGGAAGTTCGGTGGC
7 (*carY* probe)	TGCTGGCATGGAAGATTGGC ATCCAAGGAAGTTCGGTGGC
8 (*carY* cloning for complementation)	GATACTCGATGCAAGTGGCGT CCTTATATCCTCTGCAAATGCC
9 (RT-PCR *carY*)	AGGTCGGCGATCCTTTCAA CGGTCGTACTGAAGCTGAGAGA
10 (RT-PCR *carRA*)	CGGCACTTTCGAGGGAAGA CAGCGGCTCGGCAGAA
11 (RT-PCR *carB*)	TGGGTGTCGAGTACCGTCTCT TGCCTTGCCGGTTGCTT
12 (RT-PCR *carO*)	CGCTGCCCTCGCATACAT ACGAGCTTCCCACGCGAGAACATAAA
13 (RT-PCR *carX*)	TGTTGCGAATACTGCTGTCTTGT CATGGGCGGTCCACTTTC
14 (RT-PCR *carT*)	CCCACACGCCGGTGAA GTAGACGTAGCGATAGGGTTTGC
15 (RT-PCR *carD*)	CCCAATGTCCCTTTGCAGTT AACGCCGAGTGGTTCGAA
16 (RT-PCR β-tubulin)	CCGGTGCTGGAAACAACTG CGAGGACCTGGTCGACAAGT

Graphical abstract



Highlights

RALDH enzymes convert retinal to the morphogen retinoic acid in chordates

The *Fusarium verticillioides* protein CarY (FVEG_00585) has RALDH activity *in vitro*

F. verticillioides mutants devoid of CarY (*ΔcarY*) exhibit developmental alterations

CarY is the first predicted retinoic acid-forming enzyme identified in fungi