Mol Genet Genomics (2013) 288:157–173
DOI 10.1007/s00438-013-0739-7

ORIGINAL PAPER

Functional analysis of the *carS* gene of *Fusarium fujikuroi*

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Received: 26 December 2012 / Accepted: 11 March 2013 / Published online: 30 March 2013

Abstract The ascomycete fungus *Fusarium fujikuroi* is a model system in the investigation of the biosynthesis of some secondary metabolites, such as gibberellins, bikaverin, and carotenoids. Carotenoid-overproducing mutants, generically called *carS*, are easily obtained in this fungus by standard mutagenesis procedures. Here we report the functional characterization of gene *carS*, responsible for this mutant phenotype. The identity of the gene was demonstrated through the finding of mutations in six independent *carS* mutants and by the complementation of one of them. The *F. fujikuroi carS* gene was able to restore the control of carotenogenesis in a similar deregulated mutant of *Fusarium oxysporum*, but only partially at the transcription level, indicating an unexpected complexity in the regulation of the pathway. Due to the pleiotropic characteristics of this mutation, which also modifies the production of other secondary metabolites, we did a screening for *carS*-regulated genes by subtracted cDNA hybridization. The results show that the *carS* mutation affects the regulation of numerous genes in addition to those of carotenogenesis. The expression of the identified genes was usually enhanced by light, a regulatory effect also exhibited by the *carS* gene. However, in most cases, their mRNA levels in *carS* mutants were similar to those of the wild type, suggesting a regulation that affects mRNA availability rather than mRNA synthesis.

Keywords Fusarium · RING finger protein · Carotenogenesis · Suppression subtractive hybridization · Light regulation

Communicated by S. Hohmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-013-0739-7) contains supplementary material, which is available to authorized users.

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Introduction

The ascomycete fungus *Fusarium fujikuroi*, the former mating population C of the *Gibberella fujikuroi* species complex, exhibits a rich secondary metabolism which includes the production of polyketides, as bikaverins and fusarins, and terpenoids, as the gibberellins and carotenoids (Avalos et al. 2007). The ability to produce gibberellins, growth-promoting plant hormones with agricultural applications (Rademacher 1997), is a landmark in this species. A vast amount of data has accumulated on the regulation and genetic basis of this biosynthetic pathway (Tudzynski 2005), which later extended to the synthesis of bikaverin (Limón et al. 2010), fusarins (Díaz-Sánchez et al. 2012), and other metabolites (Wiemann et al. 2010). The production of these compounds is modulated by environmental cues, with nitrogen availability being a key regulatory signal.

In recent years, *F. fujikuroi* has emerged as a model system in fungal carotenogenesis research. When grown in light, the colonies of this fungus exhibit a characteristic orange pigmentation due to the production of neurosporaxanthin (Avalos and Cerdá-Olmedo 1987), an acidic apocarotenoid that has been formerly identified in *Neurospora crassa* (Zalokar 1957). Five structural genes involved in the carotenoid metabolism in *Fusarium* have been described (Fig. 1). The first specific reaction for this

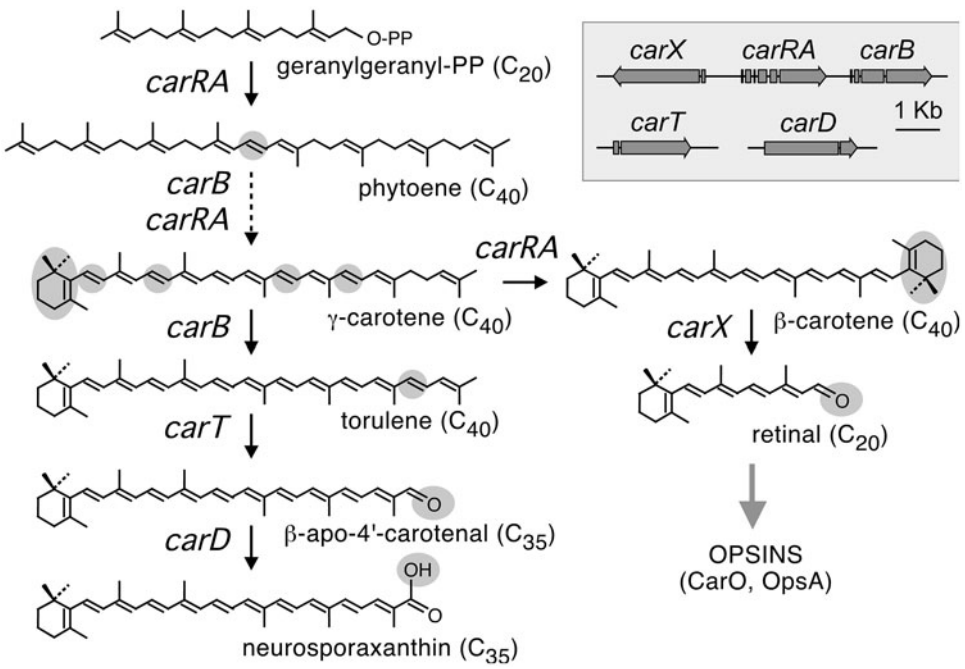

Fig. 1 Major enzymatic steps in the synthesis of neurosporaxanthin and retinal in *F. fujikuroi*. Geranylgeranyl pyrophosphate is also the precursor of gibberellin (GAs) biosynthesis. The genes whose products are responsible for each enzymatic reaction are indicated. *Numbers* of carbon atoms are shown in *parentheses*. *Shaded areas* indicate the location of enzymatic modifications. Genomic organization of the structural *car* genes is depicted in the *inner box*

pathway, the condensation of two geranylgeranyl pyrophosphate units to produce phytoene, is carried out by the carboxy domain of the bifunctional enzyme encoded by the *carRA* gene (Linnemannstöns et al. 2002). The *carB* gene product catalyzes up to five desaturations in the aliphatic backbone (Prado-Cabrero et al. 2009), resulting in conjugated double bonds that are responsible for the light-absorbing properties of carotenoids. The amino domain of CarRA presumably achieves the terminal cyclization, resulting in torulene. A final oxidative cleavage reaction carried out by an oxygenase [gene *carT,* (Prado-Cabrero et al. 2007)], and a subsequent oxidation by an aldehyde dehydrogenase [gene *carD*, (Díaz-Sánchez et al. 2011)], yield β-apo-4′-carotenal and the final neurosporaxanthin product (β-apo-4′-carotenoic acid), respectively. Torulene precursor γ-carotene may be subject to a second cyclization by CarRA to produce β-carotene, a side branch of the pathway that is prominent in a *carB* mutant defective in the fifth desaturation step (Prado-Cabrero et al. 2009). β-carotene is cleaved by a specific oxygenase [gene *carX*, (Prado-Cabrero et al. 2007)] to produce retinal, the light-absorbing prosthetic group of rhodopsins (Sharma et al. 2006). *F. fujikuroi* contains two genes for presumptive photoactive rhodopsins: *carO* (Prado et al. 2004) and *opsA* (Estrada and Avalos 2009). Genes *carRA*, *carB*, *carX,* and *carO* are clustered in the genome, while *carT*, *carD,* and *opsA* are unlinked (Fig. 1).

Stimulation of carotenoid biosynthesis by light (Avalos and Cerdá-Olmedo 1987; Avalos and Schrott 1990) is achieved at the transcription level, as evidenced by the rapid increase in the amounts of transcript for structural *car* genes following illumination (Avalos and Estrada 2010). Unlike findings in other fungi (Corrochano and Avalos 2010), the White Collar complex does not play a major photoreceptor role in the photoinduction of carotenogenesis in *Fusarium* (Estrada and Avalos 2008; Ruiz-Roldán et al. 2008), and the components of the responsible photoreceptor system remain to be identified. In addition to light, carotenoid biosynthesis is stimulated by nitrogen starvation (Rodríguez-Ortiz et al. 2009), a regulatory effect that is reminiscent of those observed for gibberellin (Tudzynski 2005) and bikaverin (Wiemann et al. 2009) production. The structural genes of the carotenoid pathway are controlled by a key negative regulator, evidenced by the easy isolation of deep-pigmented mutants with a high carotenoid content, generically called *carS* (Avalos and Cerdá-Olmedo 1987). The *carS* mutants accumulate large amounts of carotenoids, irrespective of illumination (Avalos and Cerdá-Olmedo 1987) or nitrogen availability (Rodríguez-Ortiz et al. 2009). Moreover, they contain very high mRNA levels for *car* genes (Prado et al. 2004; Thewes et al. 2005; Prado-Cabrero et al. 2007) and exhibit greater carotenogenic activity in cell-free systems (Avalos et al. 1988).

In addition to *F. fujikuroi*, *carS*-like carotenoid-overproducing mutants have been recently found and characterized in *Fusarium oxysporum*. The detection of such strains in a collection of *Agrobacterium*-mediated insertional mutants led to the identification of ORF FOX_09307, encoding a protein of the RING-finger (RF) family, as the gene *carS* in this species (Rodríguez-Ortiz et al. 2012). CarS has sequence similarity to CrgA from

Mucor circinelloides, a regulatory protein which contains two RF domains. In this fungus, the mutation of the *crgA* gene results in β-carotene overproduction (Navarro et al. 2001). RF domains have been associated with protein ubiquitylation, a process involved in the regulation of different cellular processes. CarS and CrgA also contain a LON domain, with similarity to a functional domain in ATP-dependent LON proteases. The mechanism of action of CrgA involves the ubiquitylation and subsequent inactivation of carotenogenesis regulator MCWC-1b, a protein of the White Collar family. The role of MCWC-1b is independent of activation by light, which is exerted by its paralog MCWC-1c. The RF and LON domains are essential for the regulatory role of CrgA in *M. circinelloides* carotenogenesis (Lorca-Pascual et al. 2004; Murcia-Flores et al. 2007).

The apparent functional conservation of CrgA-like regulatory proteins in fungal carotenogenesis is outstanding if we consider the very long taxonomic distance between *Mucor* and *Fusarium* from the Zygomycota and Ascomycota taxa, respectively (James et al. 2006; Hibbett et al. 2007). As a first step to understand its molecular mechanism of action, we report herein the functional characterization of the *carS* gene in *F. fujikuroi*. The identity of the gene was demonstrated through the sequencing of *carS* mutant alleles and by the complementation of a carotenoid-overproducing mutant. Furthermore, we found that the *carS* gene of *F. fujikuroi* restored the control of carotenogenesis in a *carS* mutant of *F. oxysporum*, although only partially at the transcription level of the structural *car* genes. Encouraged by the former observations of the effect of the *carS* mutation on other biosynthetic pathways, we carried out a screening for *carS*-regulated genes by subtracted cDNA hybridization to find that the *carS* mutation affects the regulation of a large set of genes. However, in most cases this regulation was not achieved at the transcript levels.

Materials and methods

Strains

Two wild-type strains from *Fusarium fujikuroi* (*G. fujikuroi* mating population C) were used: IMI58289 (Imperial Mycological Institute, Kew, UK) and FKMC1995 (Kansas State University Collection, Manhattan, KS, USA). The wild-type strain of *Fusarium oxysporum*, f. sp. *lycopersici*, used in the heterologous complementation experiment, was 4287 (FGSC9935, Fungal Genetic Stock Center, Kansas City, MO, USA). *F. fujikuroi* carotenoid-overproducing mutants SG1, SG22, SG36, and SG39 were obtained from IMI58289 by mutagenesis with *N*-methyl-*N*'nitro-*N*-nitrosoguanidine (Avalos and Cerdá-Olmedo 1987). SF134 was obtained from FKMC1995 by the same procedure (Díaz-Sánchez et al. 2011). SF1 and SF4 were sequentially obtained from FKMC1995, as described elsewhere (Prado-Cabrero et al. 2009). *F. oxysporum* carotenoid-overproducing mutant SX2 was obtained from *F. oxysporum* 4287 after exposure to *N*-methyl-*N*'nitro-*N*-nitrosoguanidine (Rodríguez-Ortiz et al. 2012). *F. oxysporum* strains SX8, SX9, SX10, SX12, SX13, and SX18 were obtained by transformation of SX2 protoplasts with plasmid pLR20.

Culture conditions

To obtain conidia from *F. fujikuroi*, strains were grown on conidiation agar (Avalos et al. 1985) for 7 days at 22 °C under continuous light. To obtain conidia from *F. oxysporum*, strains were grown on potato dextrose broth for 3 days at 30 °C. In both cases, conidia were collected from the culture and separated from mycelial debris by filtration, washed with water, and counted with a hemocytometer.

To analyze carotenoid production in surface colonies, strains were grown at 30 °C in darkness or under continuous illumination (5 Wm^{-2}) on minimal medium [DG agar, (Avalos et al. 1985)].

For the expression analysis, 10^8 spores were inoculated on Petri dishes with 25 ml of minimal medium and incubated at 30 °C in the dark. For illumination, plates were exposed to 5 Wm^{-2} white light (ca. 300 lux) for the indicated times. Mycelia were collected and washed with water, frozen in liquid nitrogen, and stored at −80 °C until use. The expression analyses in media without nitrogen were carried out with samples analyzed in a previous work (Rodríguez-Ortiz et al. 2009).

For the subtractive hybridization experiments, 100-ml samples of minimal medium were inoculated with 10^6 spores of *F. fujikuroi* wild-type IMI58289 or carotenoid-overproducing mutant SG36 and were incubated in 500-ml flasks for 3 days at 30 °C in the dark. Afterward, mycelia were filtered, washed with water, frozen in liquid nitrogen, and stored at −80 °C until use.

Expression analyses

Total RNA samples were extracted with the RNeasy mini Kit (Qiagen, Chatsworth, CA, USA) following the manufacturer's instructions for fungal samples and treated with RNAse-free DNAse I (USB, Affymetrix, Cleveland, OH, USA). RNA concentrations were estimated with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). The quality of the RNA samples was confirmed by their 260/280 nm absorbance ratios and by visualization in agarose gel electrophoresis. The cDNA samples for the subsequent real-time reversed-

transcribed PCR (abbreviated RT-PCR hereafter) analyses were obtained with the Transcriptor First-strand cDNA Synthesis Kit following the manufacturer's instructions (Roche, Mannheim, Germany). RT-PCR analyses were performed with cDNA as a template in a LightCycler® 480 Real-Time PCR System (Roche) using LightCycler® 480 SYBR Green I Master (Roche), which provides SYBR Green I dye and other reagents for amplification and detection. Dissociation curves were done to test amplification validity. Table 1 lists the genes investigated and their corresponding primer sets in the RT-PCR reactions. The β-tubulin gene was used as a control for constitutive expression. Primers were chosen with the Primer Express™ v2.0.0 software (Applied Biosystems, Branchburg, NJ, USA) from the exon sequences of each gene and were synthesized (HPLC grade) by StabVida (Oeiras, Portugal). Relative gene expression was quantified by the relative quantification method included in the software in

Table 1 Primer sets used for RT-PCR analyses and sequencing of the *carS* alleles of *F. fujikuroi*

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
carRA	CAGAAGCTGTTCCCGAAGACA	TGCGATGCCCATTTCTTGA
carB	TCGGTGTCGAGTACCGTCTCT	TGCCTTGCCGGTTGCTT
carO	TGGGCAACGCAGTGACAT	TGCGCAGACAGCCCAGTA
carS	GATACCCGGCGGAAAGGTTA	CTGACAGTCCATTTCAGCGC
β-tubulin[a]	CCGGTGCTGGAAACAACTG	CGAGGACCTGGTCGACAAGT
F. verticillioides ortholog (FVEG_#####) in subtractive hybridization analyses[b]		
02331 (a)	CCGTCCAGGGCCTTTCA	TTGTCGCCGCGTCTTCTT
09077 (b)	CGCAAGCCTGCTGATGCT	ACGACGTAGTTACCCTCATCTTCAT
03647 (c)	GTCGGAGGCGCTACAGTTG	CCGGATTGTCTCCCTGTTGT
09249 (d)	CATCGCCCATCGTCCAA	ACCAGGCGGCTTCAAAGATA
02620 (e)	AGCCCGAGCCGGTCTTC	TTCGAGAGATCCTCGTGGTCTT
08035 (f)	CGTCGTTCAGTCCATGTTCCT	GCCCCTGACACCCTCAAAC
07339 (g)	CGCGGACCTTGGGAAAC	TATCGCTCCTGCCATTGAGA
08384 (h)	GAAAAAGCAAAGGAACCAATGC	TTTGACTGCTGTTCTGCAAGGT
08105 (i)	GCGGGCATCATCATCTTTTT	ACGACGGCGAGAGAAATAGG
10856 (j)	TGGCCAAGATATCCTCGAGTTC	CAGGCGCGTGGAATATGAA
03646 (k)	CGCGCCCTGGCTCTTA	ACCCTGCCGATACCCTGTTA
05156 (l)	AGCTTGCCGACCGTGAAG	GTCCTTGTCCGGCTGTGTCT
05420 (m)	TTTCCAGTGAACAAAGTAATGCTAGTG	TTTCGCCCAAGTGTCAAGATAG
06908 (n)	TCATGCTTGGCCTCTCCAA	CGGCTGCTGATTTTTCTTGAA
11855 (o)	GATCGGGCCAGGGACAAG	TGTACCCGCAAAGGTTGATG
13742 (p)	GGTCGCAGCGAAGACTGAAC	GGCATGGAGCCCTTCAAGA
00996 (q)	ACTTGGGCAAAGCCATAGGA	CTGGCCTACCCCATCAACAG
01920 (r)	CGTGGATGTCAATCTCAATATCG	CGTGGATGTCAATCTCAATATCG
09913 (s)	GCCGTGGAACTCTCACGTCTA	GCCAGGTGGGTAGGAATGGT
09920 (t)	TCTCCCCTCAACTGTCATCGT	CGGCCATAGCAGTGGAGAAA
07988 (u)	CGGCCACCGACACCAA	CTGAGCTGTCCAAGCACATTG
08712 (v)	GGAAAGGTTCTTCGCCAAAGT	CCAGCGACTGTCCGATCCT
09919 (w)	CGTTATCCTCCATGCGAGAAA	TCTTCTTGCGTCGCAGACAA
01210 (x)	GACCAACGAGTCTGAGCTCCTT	GCCTCGGTGTCCTTGGAAA
03822 (y)	CCCTGCCCCCTATCGTTATC	AGTGTTTCGAGCACGCTTCA
Sequencing of *carS* alleles		
Segment 1	CGCACGCAATCTATAGACGT	ATCATCAGCATCAGGAGGTG
Segment 2	GAGGCCGATATATGCTACGA	TCCTCGAGGCTAACATCGTC
Segment 3	CCTTGACGGATACATCGTCG	CGAGAGATAGTAGGGCAAGC
Segment 4	CTGGTGTATGACGATCTCTA	AGGTTCGTGATCGGAATCGG

[a] Accession number U27303

[b] The number corresponds to the FVEG_##### of the genome annotation of this fungus. The small cap letter used to identify each gene in Table 3 and Fig. 6 is shown in parentheses

the RT-PCR equipment. Each RT-PCR analysis was done in duplicate to confirm repetitiveness.

Complementation and targeted gene disruption of the *carS* gene

For the complementation experiments, protoplasts were obtained from the carotenoid-overproducing mutants SG39 of *F. fujikuroi* and SX2 of *F. oxysporum,* as previously described (Prado-Cabrero et al. 2007). Approximately 10^8 protoplasts of each strain were transformed with ca. 5–10 μg of plasmid pLR20. Transformations were done following a published procedure (Proctor et al. 1997). Transformants were selected on hygromycin B-supplemented medium (250 mg l^{-1}).

For pLR20 construction, the ORF *carS* (orthologous to FOXG_09307) was obtained by PCR with primers 5′-CG CACGCAATCTATAGACGT-3′ and 5′-CGAGAGATAG TAGGGCAAGC-3′ from *F. fujikuroi* IMI58289 and was cloned into the pGEM-T easy vector (Promega, Mannheim, Germany), resulting in plasmid pLR14. Plasmid pLR20 was obtained from pLR14 after removing the entire *carS* gene from the polylinker sequence by digestion with *Not*I and by subsequently cloning into the single *Not*I restriction site of plasmid pHJA2 (Fernández-Martín et al. 2000), which contains a hygromycin resistance (hygR) cassette. The carS *F. fujikuroi* sequence was obtained from the data generated by a whole sequencing approach, as described in a later section.

Determination of *carS* allelic sequences

carS alleles were sequenced from the two wild-type strains of *F. fujikuroi* (IMI58289 and FKMC1995) and six carotenoid-overproducing mutants (SG1, SG22, SG36, SG39, SF4, and SF134). *carS* ORFs were amplified from each strain by PCR with primers 5′-CTGGTGTATGACGAT CTCTA-3′ and 5′-CGAGAGATAGTAGGGCAAGC-3′. The resulting 2.7 kb PCR product was cloned into the pGEM-T easy vector (Promega, Mannheim, Germany). Four overlapping DNA segments were obtained from each clone with the primer sets indicated in Table 1 (*carS* sequencing primers). Then 50 μl of each pGEM-T easy derived vector (0.2 μg μl^{-1}) were obtained with the Wizard Plus SV Minipreps DNA Purification System (Promega) and were sent to the CNIO (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain) sequencing service.

Carotenoid analysis

Carotenoids were extracted as described elsewhere (Youssar and Avalos 2007) using a Fastprep®-24 device (MP biomedicals, USA) for cell disruption. Total carotenoids were estimated from the maximal absorption spectra in hexane by assuming an average maximal E (1 mg l^{-1}, 1 cm) = 200.

Subtractive hybridization

Total RNA samples were extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for the suppression subtractive hybridization (SSH) method (Diatchenko et al. 1996). RNA samples were used with the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories Inc.) following the manufacturer's instructions. Final products were amplified by PCR with Expand High Fidelity Polymerase (Roche, Sant Cugat del Vallés, Spain). PCR products were cloned into the pGEM-T easy vector (Promega) and the derived vectors were sent to the CNIO for sequence determinations. Sequences were checked with the Blast tool against the *Fusarium* group genome database (Broad Institute, www.broadinstitute.org) to determine the up-regulated or down-regulated genes in the *carS* mutant, depending on each case.

Sequence analyses

Sequences of the *F. fujikuroi* genes were obtained from the data generated by a whole sequencing approach, achieved with the genomic DNA of wild-type strain IMI58289 with a Roche 454 Genome Sequencing System (454 Life Sciences Corporation, Branford, CT, USA) by the Unitat de Genòmica (CCiT, Universitat de Barcelona, Spain). Genome assembly was done by S. Beltrán (Unitat de Bioinformatica, Serveis Cientificotecnics de la Universitat de Barcelona, Spain) based on 339 Mb of total reads, with an average read size of 287 bp.

The DNA sequences obtained from the CNIO sequencing service were analyzed with the 4Peaks software (http://nucleobytes.com) and comparisons between sequences were made with the ClustalX 1.83 program (Thompson et al. 1997).

The protein domains of CarS and related proteins were analyzed with the simple modular architecture research tool (SMART) via its online tool [http://smart.embl-heidelberg.de/ (Letunic et al. 2012)].

Results

Carotenoid-overproducing strains contain mutations in the *carS* gene

We formerly reported the identification of the *carS* gene of *F. oxysporum* (Rodríguez-Ortiz et al. 2012), which is

responsible for the mutant carotenoid overproduction phenotype, and the occurrence of an orthologous gene in *F. fujikuroi* (accession No. HF568953). The *F. fujikuroi carS* gene coincides with its *F. oxysporum* counterpart at the 93 and 95 % positions at the DNA and protein level, respectively, and it maintains all its sequence features. Similar genes are found in the genomes of other filamentous fungi. They coincide in that the LON domain is present in the carboxy region, but differ in the organization of the RING finger domains in the amino region and, in some cases, in the presence of other protein domains (Fig. 2a). Detailed protein comparisons are shown in Online Resource S1.

To establish the relation between carotenoid overproduction and the *carS* gene in *F. fujikuroi*, we determined the sequences of the *carS* alleles in four independent carotenoid-overproducing mutants obtained by chemical mutagenesis: SG1, SG22, SG36, and SG39 (Avalos and Cerdá-Olmedo 1987; Rodríguez-Ortiz et al. 2009). They all contained transition mutations affecting the encoded polypeptide sequence (Fig. 2b). The SG1 allele exhibited a premature stop mutation ($G_{506} \rightarrow A$, $Trp_{169} \rightarrow$ amber), predicted to generate a truncated polypeptide with only 168 residues of the 547 residues of the wild-type protein. The *carS* alleles of the other three mutants contained different mutations, leading to relevant changes in the coding sequence. SG22 contained three mutations: $A_{698} \rightarrow G$ ($Asp_{233} \rightarrow Gly$), $T_{849} \rightarrow C$ (synonymous substitution), and $G_{1190} \rightarrow A$ ($Gly_{330} \rightarrow Asp$). SG36 and SG39 contained a single mutation, $G_{1190} \rightarrow A$ ($Gly_{330} \rightarrow Asp$) in SG36, and $G_{749} \rightarrow A$ ($Cys_{250} \rightarrow Tyr$) in SG39.

As a further confirmation, the analysis was extended to two other carotenoid-overproducing mutants, SF4 and SF134, obtained from a different wild-type strain, FKMC1995. The sequence of the *carS* gene of FKMC1995 was identical to that of IMI58289 at the DNA and protein levels, and the 594-bp upstream regulatory sequence

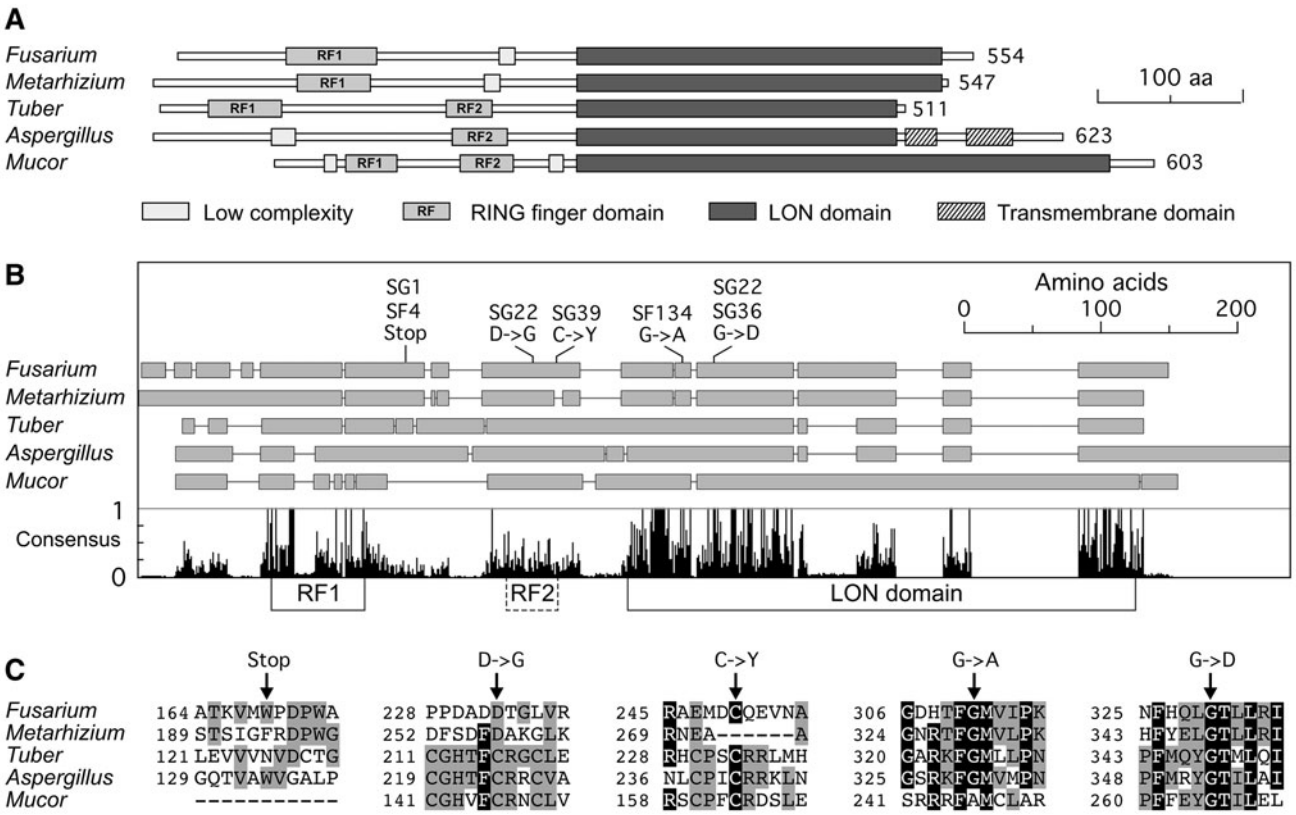

Fig. 2 Schematic representation of domain organization of the CarS protein and the location of identified mutations. **a** Relative locations of the protein domains in CarS from *F. fujikuroi* and in the proteins from the same family of *Metarhizium acridum* (Accession No. EFY88926), *Tuber melanosporum* (CAZ80137), *Aspergillus nidulans* (XP_663565), and *Mucor circinelloides* (CrgA, CAB61339). Domains were identified with the Simple Modular Architecture Research Tool (SMART). Proteins were arbitrarily aligned at the start of the LON domain. **b** Simplified representation of the Clustal alignment of the proteins displayed in the *upper* scheme. The locations of the amino acid changes in the indicated *F. fujikuroi carS* mutants are displayed above. The *lower* histogram summarizes the presence of consensus residues in relation to the CarS domains. The *dotted box* for RF2 indicates the location of the second RF domain in other proteins. **c** Alignments of the protein segments containing the mutations displayed in Scheme (**b**). The amino acids present at the same position in at least two proteins are highlighted in *gray*, and those found in at least four proteins are highlighted with a *black background*

differed only in two base pairs (99.6 % identity). The SF4 *carS* allele contained a premature stop codon at the same position as SG1, which resulted from a different point mutation ($G_{507} \rightarrow A$, $Trp_{169} \rightarrow$ opal), whereas the SF134 *carS* allele contained a $G_{1133} \rightarrow A$ transition ($Gly_{311} \rightarrow$ Ala). The amino acid changes observed in the CarS polypeptides predicted for SG22, SG36, SG39, and SF134 affected highly conserved amino acids (Fig. 2b). This fact and the occurrence of premature stop codons in the other two mutants strongly suggest that an alteration of this protein is responsible for the carotenoid-overproducing phenotype of *F. fujikuroi*.

The carotenoid-overproducing phenotype is complemented by the *carS* gene

To confirm the correlation between *carS* mutation and carotenoid overproduction, one of the mutants was chosen for a complementation experiment. Protoplasts of the mutant SG39 were transformed with plasmid pLR20, holding a wild-type *carS* allele and a hygR resistance cassette. An albino hygromycin-resistant transformant, called SG39C, was isolated and passed through microconidia to ensure homokaryosis. The carotenoid content of this strain in the dark was as low as that of the wild type. However, when grown in the absence of hygromycin, it developed deep-orange sectors (Fig. 3a, left dish). Microconidia were collected from a non pigmented sector and were inoculated on minimal medium in the dark. As a result, a mixture of pigmented and non pigmented colonies was obtained in which the later predominated (Fig. 3a, right dish). One hundred random colonies were transferred to hygromycin-supplemented medium. The albino strains were able to grow in the presence of the antibiotic, but most of the orange colonies did not grow or exhibited only marginal albino mycelia growth (Fig. 3b), indicating that the transforming plasmid may be spontaneously lost and that the *carS* mutation is recessive. In one case, an orange colony was formed in the presence of the antibiotic (blue arrowhead in Fig. 3b), which may be explained by a gene conversion event, as formerly described in some transformants of this fungus (Fernández-Martín et al. 2000).

The correlation between hygromycin resistance and restoration of the wild-type phenotype in our experiment strongly supports that the wild-type *carS* allele complements the mutant phenotype. To confirm this hypothesis, it is necessary to prove the presence of the wild-type *carS* allele in the complementing SG39C transformants. The SG39 allele holds a point *carS* mutation than does not affect any known restriction enzyme target. Therefore, we analyzed the alleles present in the transformant by sequencing random clones of the relevant *carS* segment. For this purpose, a 700-bp fragment containing the

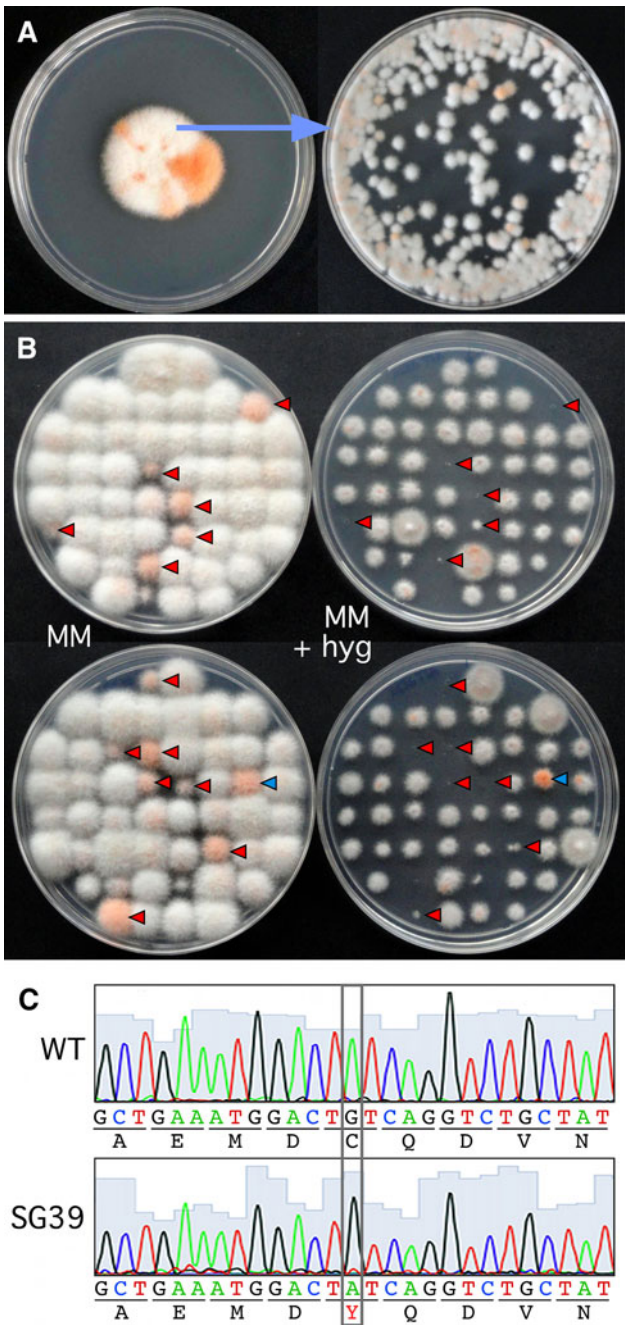

Fig. 3 Complementation of the *carS* phenotype of *F. fujikuroi*. **a** Formation of *deep-orange sectors* by a *carS* complementing strain obtained from mutant SG39. The colony was grown on minimal medium (*MM*) without selection. The Petri dish on the *right* shows the colonies generated by the conidia obtained from the albino sector of the colony displayed on the *left*. **b** Growth of 100 individual colonies from the *upper* picture (*right plate*) on either MM or MM supplemented with hygromycin (+hyg). *Red arrowheads* indicate the colonies that recovered the SG39 *deep orange* phenotype, which were unable to grow properly on hygromycin-supplemented medium. *Blue arrowheads* indicate a carotenoid over-producing colony that retains the hygromycin resistance trait. **c** Representative examples of partial sequence chromatograms from the gene segment containing the wild-type allele (*above*) or the SG39 mutation (*below*) in independent DNA clones

sequence of the *carS* gene holding the SG39 mutation was amplified by PCR from two genomic DNA samples obtained from regions in the mycelium of SG39C incubated in the absence of selection. A sample was obtained from an albino mycelium region, and another sample was obtained from an orange sector. Ten independent clones were obtained and their sequences determined. The results showed the presence of the mutant allele in all the clones from the deep orange mycelium; however, nine of the ten clones of the albino mycelium corresponded to the wild-type allele and only one to the mutant allele. Relevant segments of a sequence of each type are displayed in Fig. 3c. The bias toward wild-type alleles in the albino mycelium, for which we predicted a 50 % probability for each allele, may be due to the existence of pure albino sectors in the mycelial region used in the experiment; i.e., the mycelia that lost the mutant allele via the opposite mechanism, which generated orange sectors. The occurrence of wild-type and mutant alleles in the albino mycelium, but the detection of only mutant alleles in the orange sector, confirm the complementation of the carotenoid-overproducing phenotype by the wild-type *carS* allele in the transformant.

The *F. fujikuroi carS* gene partially replaces the *carS* ortholog of *F. oxysporum*

The data we obtained from the functional identification of the *carS* of *F. fujikuroi* are consistent with those formerly reported for the orthologous *carS* gene in *F. oxysporum*, whose mutation leads to a similar phenotype (Rodríguez-Ortiz et al. 2012). To check whether the *F. oxysporum* and *F. fujikuroi* CarS proteins are functionally interchangeable, transformation experiments were carried out to introduce the wild-type *F. fujikuroi carS* allele into *F. oxysporum* mutant SX2, which holds a premature stop mutation in the *carS* gene (Rodríguez-Ortiz et al. 2012). After incubating the SX2 protoplasts with plasmid pLR20, 13 transformants were obtained under selective conditions, five of which were albino, and eight exhibited the SX2 deep orange pigmentation. Strains were purified by passage through single uninucleate microconidia before performing further molecular analyses. The minor differences between the *F. fujikuroi* and *F. oxysporum carS* sequences allowed their detection by PCR. All the tested strains contained the *F. oxysporum carS* gene, but only the albino strains contained the *F. fujikuroi carS* gene (representative examples shown in Fig. 4a), indicating that this gene replaces the function of the mutated *F. oxysporum* counterpart.

A further subculture of the complementing transformants revealed differences in the stability of the albino phenotype. Transformant #9, and to a lesser extent #18, developed some orange sectors, while these sectors less frequently formed in transformants #8, 10, and 13. Transformants #8, 10, 13, and 18 were chosen for a more detailed phenotypic analysis. The four strains contained higher levels of *carS* transcripts, as expected from the presence of at least one additional copy of the *carS* gene (Fig. 4b), but much smaller amounts of carotenoids as compared with its predecessor strain, SX2. Regarding the effect of light, they exhibited photoinduction of carotenogenesis, but accumulated more carotenoids in the dark than the wild type, be it still at insufficient levels to confer apparent pigmentation. However, the analysis of their *carRA* and *carB* mRNA contents yielded unexpected results. In the wild type and the *carS* mutant SX2, the *carRA*/*carB* transcript levels correlated with their carotenoid content either in the dark or the light. This correlation was not apparent in the strains harboring the *F. fujikuroi carS* gene: (1) despite the four transformants produced more carotenoids in the light than in the dark, their *carRA* and *carB* mRNA levels showed an opposite pattern; (2) transformants #10, 13, and 18 contained about tenfold more *carRA* and *carB* mRNA in the dark than the wild type in the light, but their carotenoid content was at least fivefold lower. Taken together, these results indicate that the CarS protein of *F. fujikuroi* does not totally replace the function of the *F. oxysporum* counterpart in the control of mRNA levels of the *car* genes. The lack of correlation between the *car* transcripts and the in vivo carotenogenesis in the *F. oxysporum* transformants suggests that CarS participates at other regulatory levels in a species-specific manner.

The *carS* mRNA levels of *F. fujikuroi* are affected by light

The *carS* gene expression was investigated in both the wild type and the *carS* mutants. The *carS* mRNA levels increased by about fivefold after 1 h of illumination in the wild type, which is a modest induction if compared with that of enzymatic genes *carRA* and *carB* (Fig. 5a). Similar mRNA levels were found in the dark and after illumination in the *carS* mutants (Fig. 5b). This result was similar to the fourfold induction produced by light on the *carS* mRNA content in *F. oxysporum*, irrespective, of the occurrence of mutations in the corresponding *carS* alleles (Rodríguez-Ortiz et al. 2012). Lack of significant effects of the *carS* mutations on the *carS* mRNA levels in either *F. fujikuroi* or *F. oxysporum* indicates that CarS does not participate in the control of its own expression. Additionally, the transient induction produced by nitrogen starvation on the enzymatic genes (Rodríguez-Ortiz et al. 2009) was not as marked in *carS* mRNA (Fig. 5c).

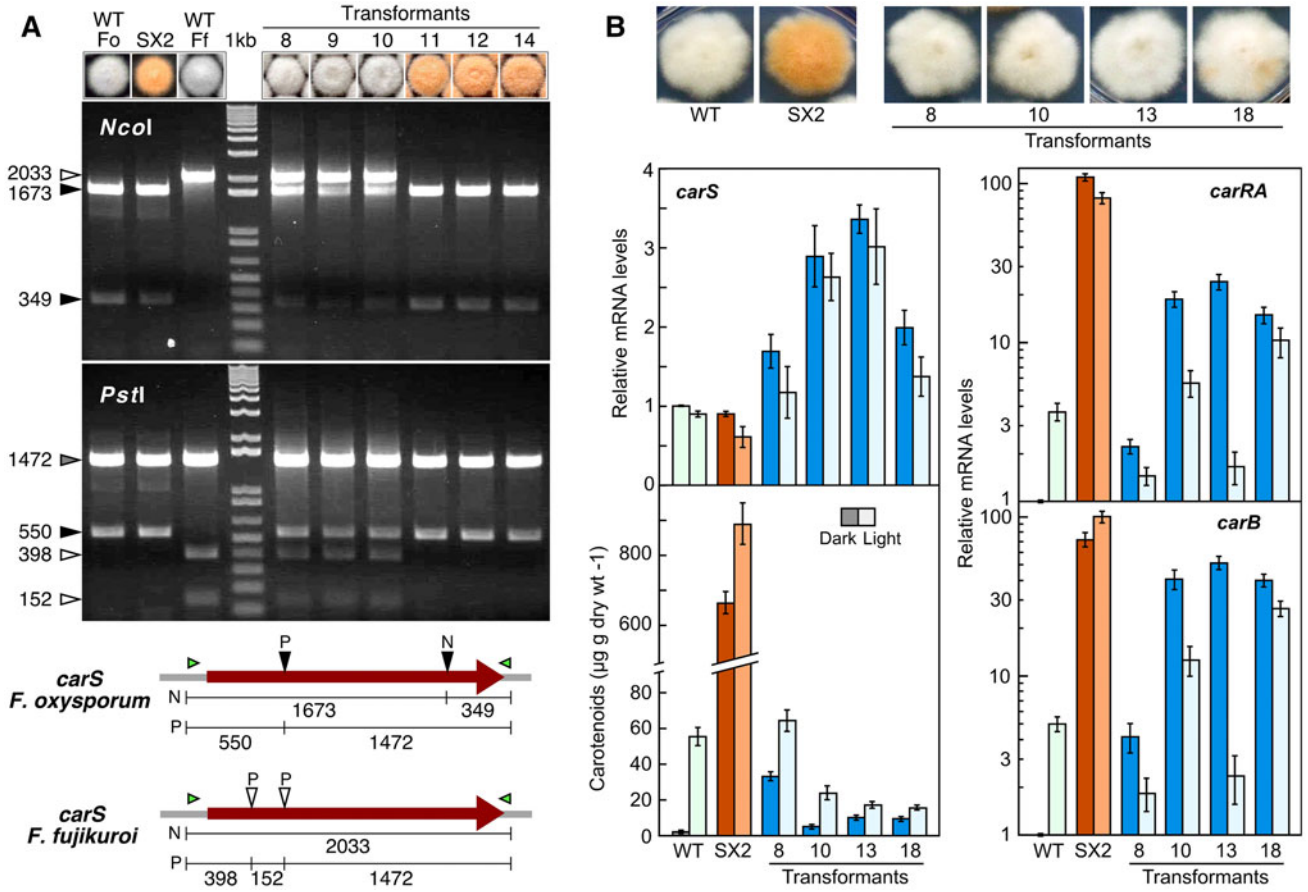

Fig. 4 Molecular characterization of the strains derived from the *carS* mutant SX2 of *F. oxysporum* after transformation with the *F. fujikuroi carS* gene. **a** Gel electrophoresis to differentiate the *carS* alleles of *F. oxysporum* and *F. fujikuroi*. The above pictures show the pigmentation of the colonies from the wild-type strains of two species (WT Fo: *F. oxysporum*, WT Ff: *F. fujikuroi*), mutant SX2 and six representative transformants incubated in the dark. The electrophoresis pictures show the PCR products obtained from the DNA of the strains displayed above after digestion with either *Nco*I (*upper picture*) or *Pst*I (*lower picture*). The scheme given below shows the targets for restriction enzymes *Nco*I (N) and *Pst*I (P) on the maps for the *carS* genes of *F. oxysporum* (*black arrowheads*) and *F. fujikuroi* (*white arrowheads*). The sizes of the respective DNA digestion products are indicated in bp. **b** *Above* aspect of 7-day-old colonies of wild-type *F. oxysporum*, mutant SX2 and four albino transformants. *Below* Carotenoid content in the dark (*dark-colored bars*) and under continuous illumination (*light-colored bars*), and the mRNA levels for genes *carS*, *carRA* and *carB* in the same strains in the dark (*dark-colored bars*) or after 1 h of illumination (*light-colored bars*). See "Materials and methods" for the growth conditions. Data show the average and standard deviations of two independent experiments

carS mutations produce wide-range effects on gene expression

Former data have revealed that the *carS* mutation does not only affect the carotenoid pathway, but also the production of other metabolites (Rodríguez-Ortiz et al. 2009). As a preliminary insight into the impact of the *carS* mutation on the *F. fujikuroi* transcriptome, cDNA samples were obtained from the wild type and the *carS* mutant SG22, and were used for differential RAPD display analyses. As expected, the wild-type mycelia used in this experiment were albino and those of SG22 were deeply pigmented due to the accumulation of large amounts of carotenoids, indicating low and high expression of the *car* genes, respectively (see, e.g., Díaz-Sánchez et al. 2011). After 81 PCR reactions with different arbitrary primers, ca. 500 bands were detected. Most were similarly found in the wild-type and *carS* samples, but a small proportion of the bands were unevenly amplified. After eliminating false positives by repeating the same PCR reactions with two other *carS* mutants, SG1 and SG36, nine bands were confirmed as being differentially amplified from either wild-type or *carS* cDNAs. From this pool, seven bands were preferentially amplified from *carS* mutant cDNA and two bands were preferentially amplified from wild-type cDNA (examples shown in Online Resource S2). Apart from the method's limitations, e.g., bias in favor of larger transcripts, these data suggest that the mRNA levels of about 2 % of genes significantly changed in the *carS* mutants.

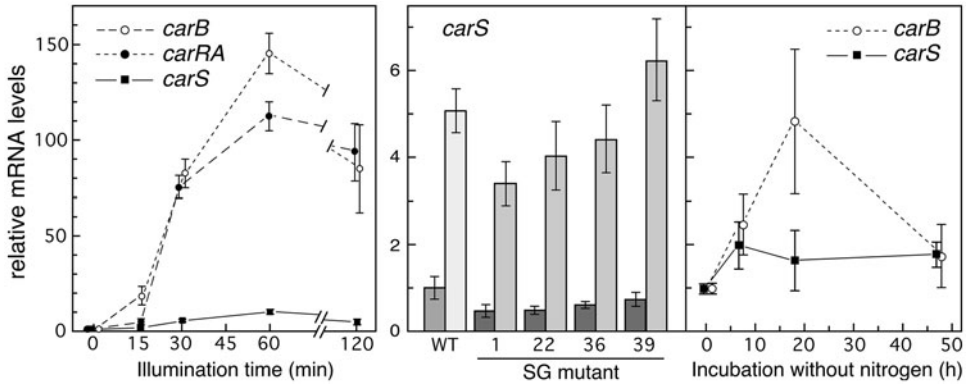

Fig. 5 Transcriptional regulation of the *carS* gene. **a** Effect of illumination on the relative mRNA levels for genes *carB*, *carRA* and *carS* in a dark-grown culture of the wild-type strain of *F. fujikuroi*. **b** Relative *carS* mRNA levels in the wild-type strain (*WT*) or in *carS* mutants SG1, SG22, SG36 and SG39 in the dark (*dark bars*) and after 1 h of illumination (*light bars*). **c** Effect of nitrogen starvation on the relative mRNA levels for genes *carB* and *carS* in a dark-grown culture of the wild-type strain. Mycelia were transferred to a nitrogen-free solution for the indicated *times*. In all the experiments, the data were obtained by RT-PCR analyses from RNA samples of the indicated strains and refer to the wild-type control (zero time or dark control). *Bars* represent the standard deviation for four determinations of two independent biological replicates

Table 2 Summary of the clones investigated in the SSH analysis

	Up-regulated in the *carS* mutant	Down-regulated in the *carS* mutant
Total number of sequenced clones	181	166
Valid sequences[a]	155	92
Total number of genes	37	29
Genes repeated in more than one clone	23 (62 %)	11 (38 %)

[a] After removal of rRNA sequences and sequences without similarity to *F. verticillioides* ORFs

In order to obtain broader knowledge of the impact of the *carS* mutation on the *F. fujikuroi* transcriptome, we followed the SSH method to identify the genes which were differentially expressed in a *carS* mutant. The SSH method allows a comparison of two populations of mRNA molecules and the selection of those that abound more in one population if compared with the other. The analysis was carried out in parallel in both directions, i.e., over-represented or under-represented mRNAs, in the *carS* mutant cDNA in relation to wild-type cDNA. Mutant SG36 was arbitrarily chosen for this analysis. Two libraries containing 181 independent up-regulated and 166 down-regulated expressed sequence tags (ESTs), defined according to the effect of the *carS* mutation on their presumed mRNA abundance, were obtained and sequenced. After discarding the clones containing rRNA sequences, we obtained two sets of 155 and 92 differentially expressed candidate genes (Table 2). To establish gene identities, both libraries of EST sequences were analyzed by BLAST against the annotated *Fusarium verticillioides* genome, the closer *F. fujikuroi* relative with a publicly available genome database. To determine the putative gene functions, the identified *F. verticillioides* predicted proteins were checked against the SwissProt database. Table 3 lists the genes which were found at least twice in the EST libraries, their ORF sizes and conserved domains, and the presumed functions of the best matches in the *F. verticillioides* genome. Online Resource T1 provides the same data for the clones that appeared only once in the EST libraries. The *F. verticillioides* orthologs of the genes identified in the SSH screenings are randomly distributed in the genome of this fungus (Online Resource S3), whose gene distribution is mostly coincident with that of *F. fujikuroi* in the investigated cases (unpublished observations).

Some genes are overrepresented in the differentially expressed EST libraries

The set of 155 predicted up-regulated ESTs contained sequences for 37 different genes, 23 of which were found at least twice. The sample included 9 ESTS for the *car* genes (five for *carRA*, and two for *carB* and *carO*), previously known for their up-regulation in the *carS* mutants (Fernández-Martín et al. 2000; Linnemannstöns et al. 2002; Prado et al. 2004). The presence of these genes constitutes an internal control of the correct functioning of the subtractive hybridization protocol. However, no ESTs were found for genes *carX* and *carT*, indicating that the EST library is not complete.

Two genes orthologous to FVEG_09077 and FVEG_02331 were especially abundant in the up-regulated ESTs libraries (29 % of the 139 EST sequences). FVEG_09077 encodes a protein involved in the synthesis

Table 3 The clones identified at least twice in the SSH analysis for the up-regulated and down-regulated genes in the *carS* mutant

FVEG_#[a]	Clones	ORF size[b]	Conserved domains[c]	Best match (species and accession number)[d]	e-value[e]
Up-regulated					
02331 (a)	24	177	None	None	–
09077 (b)	21	321	Putative thiazole synthesis (PLN02661)	Thiamine thiazole synthase (*F. oxysporum*, P23618)	0
04512	9	80	None	None	–
03647 (c)	7	67	None	None	–
06468	6	268	None	None	–
09249 (d)	6	205	None	None	–
02620 (e)	5	128	None	Translation machinery-associated protein 10 (*Saccharomyces cerevisiae*, Q06177)	8e-06
08035 (f)	5	210	None	None	–
03920	4	282	GPR1/FUN34/yaaH (pfam01184)	Ammonia transport outward protein 2 (*S. cerevisiae*, P32907)	9e-63
07339 (g)	4	282	Transcription repressor MYB5; provisional (PLN03212)	Myb-like DNA-binding protein myb-1 (*N. crassa*, O13493)	1e-40
08384 (h)	4	330	Formate/nitrite transporter (pfam01226)	Probable formate transporter (*Methanobacterium formicicum*, P35839)	1e-46
08105 (i)	3	169	None	None	–
10856 (j)	3	177	None	None	–
03646 (k)	2	316	Mitochondrial carrier protein (pfam00153)	Mitochondrial phos-phate carrier protein (*S. cerevisiae*, P23641)	3e-91
05156 (l)	2	343	None	None	–
05420 (m)	2	283	None	DNA damage-responsive protein 48 (*S. cerevisiae* P18899)	8e-31
06908 (n)	2	143	None	None	–
11855 (o)	2	520	Cytochrome P450 (pfam00067)	Probable sterigmatocystin biosynthesis P450 monooxygenase STCB (*Emericella nidulans*, Q12608)	1e-31
11964	2	309	GH16 laminarinase like (cd08023)	Glucan endo-1,3-β-glucosidase A1 (*Bacillus circulans*, P23903)	1e-15
13742 (p)	2	337	Retinol dehydrogenase, light dependent protochlorophyllide oxido reductase and related proteins, classical SDRs (cd05233)	Putative oxidoreductase bli-4 (*N. crassa*, Q92247)	2e-147
The genes formerly known to be up-regulated in the *carS* mutants					
10718	5	612	*carRA* (bifunctional lycopene cyclase/phytoene synthase)		
10717	2	570	*carB* (phytoene desaturase)		
10716	2	307	*carO* (opsin-like protein)		
Down-regulated					
00996 (q)	5	610	Major facilitator superfamily (cd06174)	Siderophore iron transporter mirB (*Emericella nidulans*, Q870L2)	0
00015	4	468	Uncharacterized protein conserved in bacteria DUF2236 (pfam09995)	Latex clearing protein (*Streptomyces sp.*, Q3L8N0)	2e-04
01920 (r)	4	402	Hex1, S1-like RNA-binding domain (cd04469)	Woronin body major protein (*N. crassa*, P87252)	1e-88
09913 (s)	4	1,246	None	None	–
09920 (t)	4	264	None	None	–

Table 3 continued

FVEG_#[a]	Clones	ORF size[b]	Conserved domains[c]	Best match (species and accession number)[d]	e-value[e]
07988 (u)	3	457	NADP-specific glutamate dehydrogenase; provisional (PTZ00079)	NADP-specific gluta-mate dehydrogenase (*G. fujikuroi*, Q96VJ7)	0
08712 (v)	3	576	Major facilitator superfamily (cd06174)	Siderophore iron transporter mirB (*Emericella nidulans*, Q870L2)	1e-129
09919 (w)	3	282	None	Probable inactive receptor kinase (*Arabidopsis thaliana*, Q9LP77)	2e-04
01210 (x)	2	553	F0F1 ATP synthase subunit α (PRK09281)	ATP synthase subunit α, mitochondrial (*N. crassa*, P37211)	0
03822 (y)	2	234	None	Cross-pathway control protein 1 (*N. crassa*, P11115)	7e-68
*09077	2	321	Putative thiazole synthesis (PLN02661)	Thiamine thiazole synthase (*F. oxysporum*, P23618)	0

[a] Gene number from code FVEG_NNNNN

[b] ORF size (aa number) as deduced from the annotated gene in the *F. verticillioides* genome database (www.broad.mit.edu/annotation/fungi/neurospora/index.html)

[c] Conserved domains (specific hits), multidomain or protein superfamily found with the lower e-value by the NCBI BLAST server

[d] Best BlastP match against the SwissProt database. If the best match was not significant (e-value exceeding 1e-03), the best match against the translated nucleotide databases (GenBank + EMBL + DDBJ + PDB) is shown instead and is indicated with an asterisk

[e] e-value for the best match with a predicted function in November 2012, shown in the neighbor column. Only e-values under 1e-03 were considered

of thiazol, a precursor of thiamine. The orthologous gene has been investigated in detail in *F. oxysporum*, where it is encoded by gene *sti35* (Ruiz-Roldán et al. 2008), formerly identified as a stress-responsive gene (Choi et al. 1990). FVEG_02331 encodes a small protein with an unpredicted function, formerly described in *F. fujikuroi* (strain M567, Accession No. CAG28692). The gene was identified in a research work on the regulatory role of glutamine synthetase in *F. fujikuroi* secondary metabolism (Teichert et al. 2004) based on differential cDNA or macroarray screenings for genes whose transcription was altered in a null *glnA* mutant, devoid of this enzymatic function.

In addition to FVEG_02331, the library of the up-regulated ESTs was enriched in genes encoding small proteins, with sizes ranging from 68 to 268 amino acids, and most with no predicted function. One of these proteins was the ortholog for FVEG_06908, a neighbor to *carS*. This gene was formerly investigated in *F. oxysporum* (FOXG_09306), where its mRNA levels were moderately enhanced (2–8-fold depending on the strain) in T-DNA insertional mutants with carotenoid-overproducing phenotypes (Rodríguez-Ortiz et al. 2012).

The proportion of valid sequences and the total number of repeated genes were lower in the library with the predicted down-regulated ESTs (Table 2). Fifty clones of this set corresponded to an internal segment of the 28S ribosomal RNA sequence, which was wrongly identified as a presumptive 148 aa encoding ORF in the annotation of the *F. verticillioides* genome, called FGEV_14158. The remaining 92 sequences corresponded to 29 genes, 11 of which were found at least twice, encoding proteins of diverse functions. One of them was NADP-dependent glutamate dehydrogenase, whose sequence was formerly reported in *Gibberella fujikuroi* (Accession No. Q96VJ7). Interestingly, the set included two predicted siderophore iron transporters, which shared about 40 % of identity. Taken together, these siderophores corresponded to 9 % of the genes predicted to be down-regulated in the *carS* mutant.

The predicted up- and down-regulations of SSH-identified genes in *carS* mutants do not correlate with real transcript levels

To verify the relationship between the *carS* mutations and the actual mRNA levels for the identified genes, RT-PCR assays were conducted for the genes described in Table 3. To confirm the effect of the *carS* mutation, the assay was extended to three different *carS* mutants: SG36, SG1, and SG22 (Fig. 6). The results show the expected increase in the *carRA*, *carB,* and *carO* mRNAs in SG36 and in the other *carS* mutants in the dark (Fig. 6a). This increase was

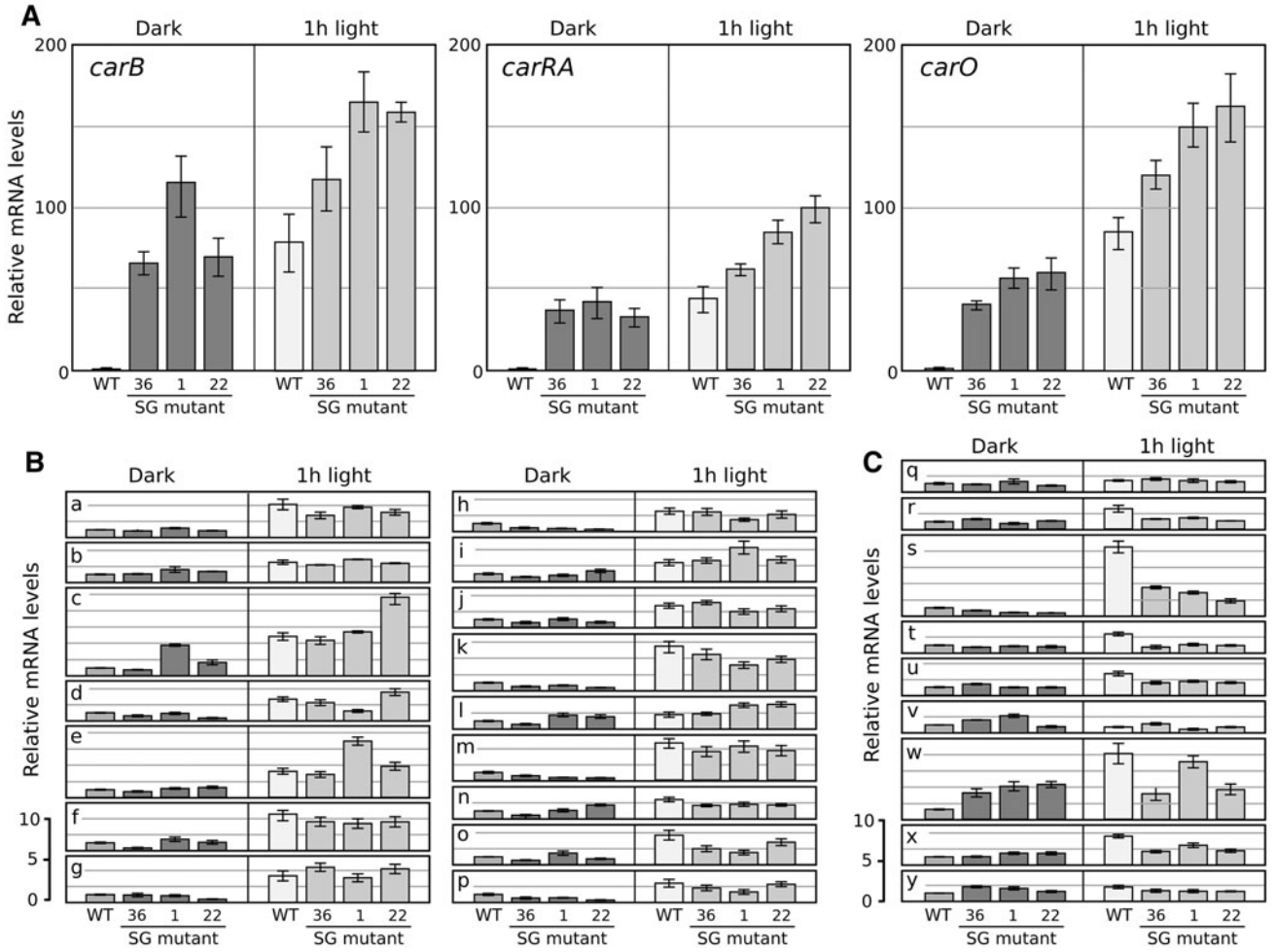

Fig. 6 Effect of the *carS* mutations on the mRNA levels for those genes identified in the subtractive hybridization assays. **a** Relative mRNA levels for genes *carB*, *carRA* and *carO* in the wild type and in *carS* mutants SG36, SG1 and SG22 grown in the dark or after 1 h of illumination. **b** Relative mRNA levels in the same RNA samples for the alleged up-regulated genes in the *carS* mutants. **c** The same analysis for the down-regulated genes. Each gene is identified with a *small cap letter*, indicated in Table 3. *Bars* represent the standard deviation for four determinations of two independent biological replicates

not apparent in SG36 for the remaining putative up-regulated genes assayed (Fig. 6b) and, in some cases, the mRNA levels were even lower in the three *carS* mutants tested (e.g., see clones h, k, m and p). As expected, a patent photoinduction was observed for the *car* genes in the wild-type strain, and was also manifested in the *carS* mutants, but to a lesser extent (Fig. 6a). It is noteworthy that most of the putative up-regulated clones exhibited a mild photoinduction in either the wild type or the *carS* mutants.

Similar results were obtained for the expressions of those genes identified as being presumably repressed in the *carS* mutants: their mRNA levels were similar, or even higher, in these strains (Fig. 6c). The only exception was the ortholog for FVEG09913 (clone s), which encoded a small protein of unknown function. The mRNA levels for this gene were lower in the three *carS* mutants in either the dark or after illumination. However, the difference was not remarkable.

Discussion

We have identified and characterized the *carS* gene of *F. fujikuroi*, responsible for the carotenoid overproducing phenotype in some mutants of this fungus (Avalos and Cerdá-Olmedo 1987; Rodríguez-Ortiz et al. 2009). The identification, based on its similarity to its counterpart in *F. oxysporum*, was supported by the finding of mutant *carS* alleles in different carotenoid-overproducing strains and also by the ability of *carS* to complement the mutant phenotype of one of them. Furthermore, the *F. fujikuroi carS* gene was able to replace, at least in part, the function

of its ortholog in *F. oxysporum*. Taken together, our results strongly support the identity of the *carS* gene in *Fusarium* and its participation as a major negative regulator of carotenogenesis. Furthermore, the occurrence of *carS* mutations in six independent mutants suggests that *carS* is the only gene whose functional loss results in deregulated carotenoid overproduction in *F. fujikuroi*.

As formerly found in *F. oxysporum*, the *carS* mRNA levels in *F. fujikuroi* increased after 1 h of illumination, but the photoinduction was quantitatively much lower than those exhibited by structural carotenogenesis genes, represented in our assays by *carRA* or *carB*. The lower response of *carS* to light may relate to a higher mRNA content in the dark, as suggested by the Ct values observed in the RT-PCR experiments. The regulation of *carS* is reminiscent of that of its orthologous gene *crgA* in zygomycetes *M. circinelloides* (Navarro et al. 2001) and *Blakeslea trispora* (Quiles-Rosillo et al. 2005). In both cases, the *crgA* levels increase in response to light. However, the mRNA amounts in these fungi in the dark are apparently lower and photoinduction is faster. It is also interesting to note that CarS does not participate in its own regulation, at least not at the mRNA content level, and that nitrogen starvation barely affects this regulation. Actually, CarS is not involved in the nitrogen regulation of *car* genes, as indicated by the persistent induction by nitrogen starvation of the *carRA* and *carB* mRNA levels in the *carS* mutants (Rodríguez-Ortiz et al. 2009).

The regulatory effects resulting from the expression of the *F. fujikuroi carS* gene in a *carS* mutant of *F. oxysporum* offer interesting clues as to the regulation of carotenogenesis at the molecular level. The *F. oxysporum* and *F. fujikuroi* CarS proteins have an identical RF domain and differ only in 13 of the 252 amino acids of the LON domain. However, the complementing *F. oxysporum* transformants contain more carotenoids in the dark than the wild-type control, especially in the transformant with the lowest expression for the heterologous *carS* gene. We conclude that the *F. fujikuroi* CarS protein does not fully replace the *F. oxysporum* CarS function, suggesting high specificity in its regulatory activity. This is particularly noteworthy in the case of the unexpectedly high mRNA levels noted for structural genes *carRA* and *carB* found in the transformants. The striking contrast between mRNA levels and actual carotenoid content in these strains reveals two levels of regulation, transcriptional and post-transcriptional, which are poorly and efficiently replaced with the *F. fujikuroi* CarS protein, respectively. Similar experiments carried out in zygomycetes have produced divergent results: the *crgA* mutant phenotype of *M. circinelloides* is complemented by the CrgA ortholog of *Blakeslea trispora* (Quiles-Rosillo et al. 2005), but complementation could not be achieved with the *Phycomyces blakesleeanus* counterpart (V. Tagua; personal communication).

The pleiotropic phenotype of the *carS* mutants, (Candau et al. 1991; Rodríguez-Ortiz et al. 2009) suggests that CarS plays a more general regulatory role in *F. fujikuroi*. For the purpose of identifying genes whose mRNA levels are affected by the mutations of the gene *carS*, we carried out scrutinies for differential expression by two different techniques: differential RAPD display and subtractive hybridization. The RAPD method was used as a low-cost preliminary approach to explore the occurrence of differential expression. The RAPD analysis gave positive results in other organisms, such as *P. blakesleeanus*, where it allowed the identification of genes induced in the photomorphogenesis process (Corrochano 2002). The finding of differentially expressed RAPD clones encouraged us to follow the subtractive hybridization method, which revealed more than 60 putative *carS*-regulated genes. More than half the clones appeared at least twice, indicating a partial saturation of the genes that meet scrutiny requirements. According to the statistics data on the available *Fusarium* genome sequences (Broad Institute website), annotation processes predict 14,169, 17,708 and 13,321 genes in the genomes of *F. verticillioides*, *F. oxysporum,* and *F. graminearum*, respectively. We can estimate, therefore, that the *F. fujikuroi* genome contains information for at least 13,000 genes. Based on these numbers, the repetition of many clones in the sample, and the presence among them of three *car* genes whose regulation by *carS* was already known, are strong evidence that the method worked effectively. The presence of two independent ESTs for *carS* neighbor gene FVEG_06908, encoding only 143 amino acids, is particularly informative of the singularity of the identified SSH clones, and whose loss of function produces no apparent phenotype in *F. oxysporum* (Rodríguez-Ortiz et al. 2012). The close linkage of this gene to *carS* strongly suggests a regulatory connection.

The RAPD and SSH screenings were achieved with a *carS* mutant, which probably holds further mutations. This is also valid for the other *carS* strains, which contain different *carS* alleles (Fig. 2b) and exhibit differences in their abilities to produce other secondary metabolites (Rodríguez-Ortiz et al. 2009). To rule out the possible undesired effects resulting from differences in either *carS* alleles or secondary random mutations, the expression of the genes identified at least twice in the library was investigated in three independent *carS* mutants and compared with the wild type. Our predictions were not fulfilled, except for three *car* genes, as no significant changes were seen in the majority of the identified genes at their mRNA levels in the *carS* mutants. However, a detailed analysis of these genes reveals interesting features that are potentially related to carotenoid metabolism; e.g., the mRNA levels of the

majority of the genes increased at 1 h of illumination. The Ct values in the RT-PCR reactions showed no marked differences between the light and dark samples at the mRNA levels for the β-tubulin gene, which was used as a reference in the relative mRNA content calculations. Despite the photoinduction levels being modest, it is intriguing that they did not differ much from that exhibited by the *carS* gene itself. Interestingly, the list includes genes closely linked to not only the *wc-1* ortholog *wcoA* [FVEG_12138, (Estrada and Avalos 2008)], but also the *wc-2* and *vvd* orthologs (FVEG_00478, FVEG_02125, respectively), all of which are presumably related to regulation by light (Avalos and Estrada 2010).

Another interesting characteristic is that some of the genes identified during the SSH screening are related to either nitrogen metabolism or stress, both of which are associated with secondary metabolism (Yu and Keller 2005). This group includes the FVEG_02331, FVEG_04512, and FVEG_03647 orthologs, which are three genes identified in a differential expression analysis between a glutamine synthase mutant of *F. fujikuroi* and the wild-type strain (Teichert et al. 2004). The FVEG_08384 and FVEG_03920 orthologs are also related to nitrogen and are presumably involved in nitrite and ammonia transport, respectively. A possible connection of their presence with carotenogenesis regulation by nitrogen starvation is suggestive, as recently reported in *F. fujikuroi* (Rodríguez-Ortiz et al. 2009), already mentioned. Carotenoids may also be related to oxidative stress (e.g., see Michán et al. 2003; Liu and Wu 2006). The null mutants of the FVEG_09077 ortholog in *F. oxysporum*, *sti35,* are auxotrophic for thiamine and they exhibit reduced tolerance to menadione, a superoxide-generating agent, indicating that the Sti35 protein is also involved in the oxidative stress response (Ruiz-Roldán et al. 2008). The possible role of neurosporaxanthin, the major *Fusarium* carotenoid product, in stress protection is currently being investigated.

The combination of these characteristics, together with the repetition of the clones in the two subtraction libraries, suggest a molecular basis for their isolation by the SSH approach. However, the minor differences found in the mRNA levels for most of the genes between the wild type and the *carS* mutants demand an unusual explanation. We propose that the mRNAs for the identified genes are less accessible in the cDNA synthesis step of the SSH protocol, probably because of the interaction with other regulatory molecules, such as non coding RNAs (ncRNAs). The possible candidates in this regulation are miRNA, whose mechanisms of action on target genes are currently being actively investigated (Zhao and Liu 2009). Known mechanisms include inhibition of initiation or elongation of mRNA translation, or mRNA sequestering, which involve the interactions between miRNA and mRNA targets and do not necessarily imply changes in the actual mRNA levels in the cell. Alternative candidates are antisense RNAs or lncRNAs, which are of emerging relevance in higher eukaryotes (Guil and Esteller 2012; Rinn and Chang 2012), where there is increasing evidence of their participation in the formation of the ribonucleic-protein complexes involved in chromatin remodeling (Magistri et al. 2012). In *N. crassa*, a *frq* antisense RNA participates in the regulation of the circadian clock (Kramer et al. 2003), and more examples of low eukaryotic lncRNAs have become available in recent years (see the lncRNA database, http://www.lncrnadb.org). If regulatory miRNAs or lncRNAs form stable complexes with specific mRNA targets, they can affect their accessibility for cDNA synthesis. According to this hypothesis, the list of genes identified by the SSH technique can be enriched in genes controlled by CarS-dependent ncRNA mechanisms.

The post-transcriptional regulation of genes or enzymes of the terpenoid metabolism has been demonstrated in some fungal species, but the underlying molecular mechanisms remain unknown. Two *Mucor mucedo* genes participating in the synthesis of trisporic acids, carotene-derived sexual hormones of zygomycetes, are regulated at either the post-transcriptional (*tsp1*) or the post-translational levels (*tsp1* and *tsp2*, Schimek et al. 2005; Wetzel et al. 2009). Similar mechanisms are possibly involved in the carotene overproduction exhibited by some regulatory mutants of *P. blakesleeanus,* which is not accompanied by concomitant increases in the mRNA levels of the structural genes for either carotenogenesis or early terpenoid biosynthesis steps (Almeida and Cerda-Olmedo 2008). In *F. fujikuroi*, there is no evidence for the post-transcriptional control of terpenoid genes, although regulation is needed, at least at the level of the enzymes targeting appropriate cell compartments (Domenech et al. 1996). Overall, available information suggests that transcription is a major regulatory step for the synthesis of gibberellins and carotenoids (Tudzynski 2005; Avalos and Estrada 2010; Díaz-Sánchez et al. 2011). In our SSH libraries, the only genes exhibiting large differences in mRNA levels between wild type and *carS* mutants are *carRA*, *carB,* and *carO,* suggesting that CarS is a specific transcriptional regulator of the *car* genes in *F. fujikuroi*. The absence in the up-regulated library of *carX* and *carT*, which are also derepressed in *carS* mutants (Thewes et al. 2005; Prado-Cabrero et al. 2007), can be explained by lack of screening saturation. The similar deregulation of the structural *car* genes in *carS* mutants suggests the mediation of a common repressing mechanism. This might be explained by either a direct repressing role of CarS in the transcription of *car* genes or the participation of another CarS-regulated transcription factor. To support the latter hypothesis, RF domains have been associated with protein–protein

interactions (Matthews and Sunde 2002), but not with DNA binding.

The taxonomic distance between zygomycete and ascomycete fungi (James et al. 2006; Hibbett et al. 2007) suggests profound differences between the mechanisms of action of CrgA in *M. circinelloides* and *B. trispora* and of CarS in *Fusarium*. As shown in Fig. 2a, despite major protein domains being conserved, there has been considerable diversification between RF-LON proteins in fungi, and they are not present in all species. An excellent example is found in the fungus *N. crassa*, which shares the same carotenoid pathway with *F. fujikuroi* (Avalos and Corrochano 2013). The *N. crassa* genome lacks a putative *carS* ortholog, and no *carS*-like carotenoid overproducing mutants have been described in this fungus. The investigation of the mechanism of action of CarS will open up new perspectives in the researching of the regulatory mechanisms governing fungal carotenogenesis and will provide new insights into the biological function of this family of fungal proteins.

Acknowledgments This research has been supported by grants from the Spanish Government (Ministry of Science and Technology, Projects BIO2006-01323 and BIO2009-11131), and from the Regional Andalusian Government (Project P07-CVI-02813). Spanish grants include support from the European Union (European Regional Development Fund, ERDF). L.R. Rodríguez-Ortiz has been supported by a CONACYT fellowship from the Government of Mexico.

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