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Regulation of Carotenogenesis and Secondary Metabolism by Nitrogen in Wild-Type *Fusarium fujikuroi* and Carotenoid-Overproducing Mutants▽†

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The fungus *Fusarium fujikuroi* (*Gibberella fujikuroi* MP-C) produces metabolites of biotechnological interest, such as gibberellins, bikaverins, and carotenoids. Gibberellin and bikaverin productions are induced upon nitrogen exhaustion, while carotenoid accumulation is stimulated by light. We evaluated the effect of nitrogen availability on carotenogenesis in comparison with bikaverin and gibberellin production in the wild type and in carotenoid-overproducing mutants (*carS*). Nitrogen starvation increased carotenoid accumulation in all strains tested. In *carS* strains, gibberellin and bikaverin biosynthesis patterns differed from those of the wild type and paralleled the expression of key genes for both pathways, coding for geranylgeranyl pyrophosphate (GGPP) and kaurene synthases for the former and a polyketide synthase for the latter. These results suggest regulatory connections between carotenoid biosynthesis and nitrogen-controlled biosynthetic pathways in this fungus. Expression of gene *ggs1*, which encodes a second GGPP synthase, was also derepressed in the *carS* mutants, suggesting the participation of Ggs1 in carotenoid biosynthesis. The *carS* mutations did not affect genes for earlier steps of the terpenoid pathway, such as *fppS* or *hmgR*. Light induced carotenoid biosynthesis in the wild type and *carRA* and *carB* levels in the wild-type and *carS* strains irrespective of nitrogen availability.

Species in the genus *Fusarium* synthesize many secondary metabolites, including compounds of commercial importance because of their deleterious effects, e.g., mycotoxins, or because of their biotechnological applications. *Fusarium fujikuroi* (*Gibberella fujikuroi*, mating population C), the agent causing bakanae disease on rice, produces gibberellins (7, 43), plant hormones in the terpenoid family whose growth-promoting properties are used to improve agricultural yields (36). Depending on the growth conditions, this fungus also may produce other terpenoids, e.g., carotenoids, or polyketide mycotoxins, e.g., bikaverins and fusarins (6).

Carotenoids are fat-soluble pigments produced by all photosynthetic organisms and many heterotrophic bacteria and fungi (11). In plants they play an essential role as accessory pigments of the photosynthetic machinery (41). Animals metabolize dietary carotenoids to produce compounds such as retinal, the light-absorbing prosthetic group of visual opsins, or retinoic acid, a critical morphogen in vertebrate development (10). They also are used for pigmentation, e.g., astaxanthin in fish and flamingoes. *F. fujikuroi* produces an acidic apocarotenoid, neurosporaxanthin (Fig. 1) (3), first discovered in *Neurospora crassa* (1). Carotenoids have increasing commercial applications, and fungi are a preferred source for their biotechnological production (5).

The production of gibberellins and carotenoids (Fig. 1) begins with the synthesis of mevalonate from hydroxymethylglutaryl coenzyme A (HMG-CoA), a reaction mediated by HMG-CoA reductase, encoded by *hmgR* (49). This is followed by the synthesis of isopentenyl diphosphate (IPP) and several condensation steps, the latter mediated by a prenyl transferase presumably shared by both pathways and encoded by *fppS* (23), to yield farnesyl diphosphate (FPP). From this point, separate specific enzymes, encoded by genes *ggs1* (31) and *gibA* (originally *ggs2* [45]), are used to produce the common precursor geranylgeranyl diphosphate (GGPP) in different subcellular locations (15). The biosynthesis of gibberellins depends on seven genes organized in a cluster (44). A key reaction in the pathway is the conversion of GGPP to the tetracyclic precursor of the gibberellins, kaurene (Fig. 1), mediated by a bifunctional enzyme encoded by *gibB* (originally *cps/ks* [47]). The remaining reactions are carried out by four P450 monooxygenases and a desaturase (44).

Carotenoid biosynthesis branches out from GGPP through five different enzymatic reactions. Two GGPP molecules are condensed by the enzymatic product of *carRA* to produce phytoene, the first molecule with the typical C_{40} carotene structure (Fig. 1). Five desaturations, mediated by a dehydrogenase encoded by gene *carB* (28), and a cyclization, also mediated by the *carRA* product, yield torulene. This reddish carotene is the substrate for a carotenoid oxygenase encoded by *carT*, which carries out an oxidative cleavage reaction to produce C_{35} β-apo-4′-carotenal (34). The aldehyde group from this intermediate is further oxidized to an acid to yield the acidic end product (Fig. 1). A side branch of the pathway leads to β-carotene from γ-carotene through a second cyclization step and allows the synthesis of retinal by the symmetric fission of β-carotene, a reaction carried out by a different oxygenase encoded by *carX* (35, 42). The genes *carRA*, *carB*, and *carX* are clustered with a gene encoding an opsin-like protein (33), while *carT* is at another location in the *F. fujikuroi* genome (34).

The gibberellin and carotenoid biosynthetic pathways are

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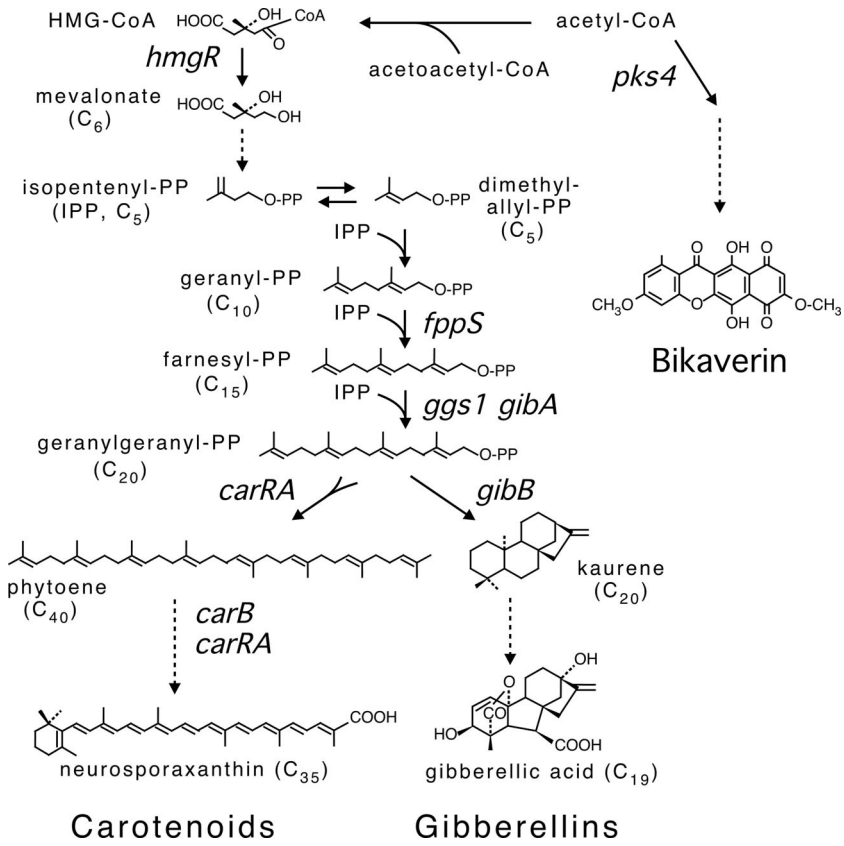

FIG. 1. Biosynthetic pathways for carotenoid, gibberellin, and bikaverin production in *F. fujikuroi*. The eight genes investigated in this work are indicated. Dotted arrows represent several reactions. Relevant numbers of carbon atoms are indicated. The bikaverin precursor produced by Pks4 is unknown.

subject to different types of regulation. Light is the major regulator of carotenoid biosynthesis (4, 9). The stimulation of the pathway by light is responsible for the orange pigmentation acquired on the surface of cultures of this fungus and is paralleled by a rapid increase in the mRNA levels of the structural *car* genes (33, 34, 42). In contrast, the key regulatory signal for gibberellin production is the exhaustion of the nitrogen source, a condition attained in media with a low N/C ratio (13, 38). This regulation is mediated by the nitrogen catabolite regulatory protein, AreA (32, 46). A similar dependence on nitrogen starvation has been described for bikaverin production (21), a regulation reflected in the expression of the gene *pks4* (29), coding for the polyketide synthase for this pathway (Fig. 1). The synthesis of bikaverin is also affected by other environmental factors, e.g., pH (21) and aeration (22).

Carotenoid-overproducing mutants, hereafter called *carS*, have a high carotenoid biosynthetic activity irrespective of illumination (4). These mutants contain high levels of mRNA for the *car* genes in the absence of light (33, 34, 42) and at least in one case also have higher levels of enzymatic activity (8). The gene or genes responsible for this phenotype have not been identified, but their phenotypic similarities (4) and their easy isolation in mutagenesis screenings (2) suggest that a functional key repressor of the carotenoid pathway has been altered. Previous studies found that *carS* mutants produced less gibberellins, an effect interpreted as a diversion of carbon substrates from the gibberellin to the carotenoid pathway (12).

An earlier work described that nitrogen availability affects carotenoid production in immobilized *G. fujikuroi* mycelia (19). To evaluate the relationship between carotenoid biosynthesis and the production of nitrogen-regulated secondary metabolites in *F. fujikuroi*, we analyzed the effect of nitrogen starvation on carotenogenesis in parallel to gibberellin and bikaverin production in the wild type and in *carS* mutants. Our data are consistent with negative regulation by nitrogen of the carotenoid pathway and suggest a connection between the gene product(s) affected in these mutants and nitrogen regulation of other secondary metabolites.

MATERIALS AND METHODS

Strains and culture conditions. The wild-type strain IMI58289 of *Fusarium fujikuroi*, formerly *Gibberella fujikuroi* mating population C (27, 37), was obtained from the Commonwealth Mycological Institute (Kew, United Kingdom). The carotenoid-overproducing mutants SG1, SG22, SG36, and SG39 were obtained from IMI58289 by chemical mutagenesis (4).

Cultures were incubated in 500-ml Erlenmeyer flasks containing 250 ml of culture medium, inoculated with 10^6 conidia and grown at 30°C in the dark on an orbital shaker at 150 rpm. To obtain conidia, the strains were grown on sporulation agar (2) for 1 week under light in a 22°C incubation chamber. Conidia were collected from the surface of the cultures in water, filtered through sterile Whatman paper, and counted with a hemocytometer.

For analysis of carotenoid, gibberellin, and bikaverin production, the strains

were grown in high-nitrogen medium: 80 g liter^{-1} D(+)-glucose, 4.8 g liter^{-1} NH_4NO_3, 5 g liter^{-1} KH_2PO_4, 1 g liter^{-1} $MgSO_4 \cdot 7H_2O$, and microelements, originally called ICI medium (20), or in low-nitrogen medium (the same but with 0.48 g liter^{-1} NH_4NO_3, also termed 10% ICI medium by the same authors) for 15 days.

For controlled nitrogen starvation, the strains were grown for 3 days in high-nitrogen medium, the mycelia were isolated by filtration through sterile Whatman paper, washed with sterile water, and resuspended in a 2% glucose solution supplemented with microelements ("nitrogen-free solution"). The glucose concentration was determined as described by Somogyi (39). When indicated (see the supplemental material), the mycelia were transferred to the same medium without nitrogen or to a 2% glucose solution supplemented with either 5 g liter^{-1} KH_2PO_4, 1 g liter^{-1} $MgSO_4 \cdot 7H_2O$, or 4.8 g liter^{-1} NH_4NO_3. These manipulations were done under a red safe light to avoid light induction of carotenoid biosynthesis. Samples (10 ml) were taken after 0, 6, 16, 48, and 144 h of incubation in the glucose solution, filtered through Whatman paper, and stored at −20°C until chemically analyzed. To check the effect of light, 16-h cultures in the glucose solution were filtered through 10-cm Ø circular Whatman paper to obtain wet mycelial pads that were placed in an empty petri dish of the same size and exposed for 24 h to 5 W m^2 of white light. Parallel experiments in high-nitrogen medium were done with the same incubation times (3 days plus 16 h) before filtering and transfer to illumination conditions. The same protocol, including the final incubations of the mycelial pads, was followed for control incubations with nonilluminated cultures. For analysis of the effect of light on gene expression, small mycelial samples were taken from the mycelial pads 1 h after light onset. As an alternative protocol, 10-ml mycelial suspensions were taken from the flasks at the same incubation times, transferred to 5-cm Ø petri dishes, and exposed to light.

RT-PCR. Mycelial samples obtained by filtration were washed with water, frozen in liquid nitrogen, and stored at −80°C until use. Total RNA samples were extracted with the Perfect RNA eukaryotic kit (Eppendorf, Hamburg, Germany). To ensure similar amounts of RNA in the samples, their concentrations were estimated with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Real-time PCR (RT-PCR) analyses were performed from RNA samples as a template with an ABI 7500 apparatus (Applied Biosystems, Norwalk, CT) using the optimized conditions of Estrada and Avalos (16). Dissociation curves were made to test amplification validity. The genes investigated, their database accession numbers (in parentheses), and their corresponding primer sets in the RT-PCRs were as follows: *carB* (AJ426418), 5′-TCGGTGTCGAGTACCGTCTCT-3′ and 5′-TGCCTTGCCGGTTGCTT-3′; *carRA* (AJ426417), 5′-CAGAAGCTGTTCCCGAAGACA-3′ and 5′-TGCGATGCCCATTTCTTGA-3′; *ggs1* (X96943), 5′-CCAGAAGGCGACTGGTTGA-3′ and 5′-AACCGGCGTTTGACATCTGT-3′; *gibA* (Y15280), 5′-ATCGTCGGTCACGATGCA-3′ and 5′-TTGCCTGACCCTGGAAGATT-3′; *gibB* (Y15013), 5′-CGCTGAGCGGAATGTGAA-3′ and 5′-GAGATTCTGTGACGTTCCGTTACA-3′; *pks4* (AJ278141), 5′-CTCGTCACCGACGCTCTAGTC-3′ and 5′-TGGGCATTGACCGGTATCA-3′; *ffpS* (X96940), 5′-TGGCCAGCCCTGTTGGTA-3′ and 5′-CATGAAAGCGTCGTTGATAGCT-3′; and *hmgR* (X94307), 5′-GAGCACAACATCGACCGAAAC-3′ and 5′-CGGAGCCGGTGTCGTTAA-3′. The β-tubulin gene from *F. fujikuroi*, amplified with primers 5′-CCGGTGCTGGAAACAACTG-3′ and 5′-CGAGGACCTGGTCGACAAGT-3′, (16) was used as a control for constitutive expression. The primers were chosen with the software Primer Express v2.0.0 (Applied Biosystems) from exon sequences of each gene and synthesized (high-performance liquid chromatography [HPLC] grade) by StabVida (Oeiras, Portugal). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ (cycle threshold) method with Sequence Detection Software v1.2.2 (Applied Biosystems). Each RT-PCR analysis was run twice to test consistency.

Chemical determinations. For gibberellic acid (GA_3) determinations, 1-ml samples were acidified to pH 2.5 with 10 M HCl, cleaned by filtration through 0.22-μm-pore Millipore filters, and run in an HPLC device (Agilent Technologies, Santa Clara, CA) with 30% methanol and 10 mM H_3PO_4 (pH 3) (14). Concentrations were obtained from the peaks at 206 nm of commercial GA_3 (Sigma, Saint Louis, MO) used as a standard.

Two methods were used for bikaverin determination. In case of low concentrations (less than 50 mg liter^{-1}), the sample was acidified to pH 2.5 with 10 M HCl and extracted with 1 volume of chloroform until the sample was colorless. The solvent fraction was vacuum dried and resuspended in an appropriate volume for spectrophotometric determination (ε_{521} = 8,400 cm^{-1} M^{-1}). The partition method was inefficient at high bikaverin concentrations because the compound crystallizes. In this case, a 0.1-ml sample was added to 1.4 ml acetone and measured spectrophotometrically at 500 nm. If required, the samples were diluted with additional 14:1 acetone-water until reliable spectrophotometric measures were obtained. To determine the bikaverin concentration, a calibration curve was constructed with parallel measures with known bikaverin amounts in acetone before addition of water (ε_{500} = 4,713 cm^{-1} M^{-1} in 14:1 acetone-water).

Carotenoids were extracted as previously described (50), by using a Fastprep-24 device (MP Biomedicals, Irvine, CA) to break the cells. Total carotenoids were estimated from maximal absorption spectra in hexane assuming an average maximal E (1 mg liter^{-1}, 1 cm) of 200.

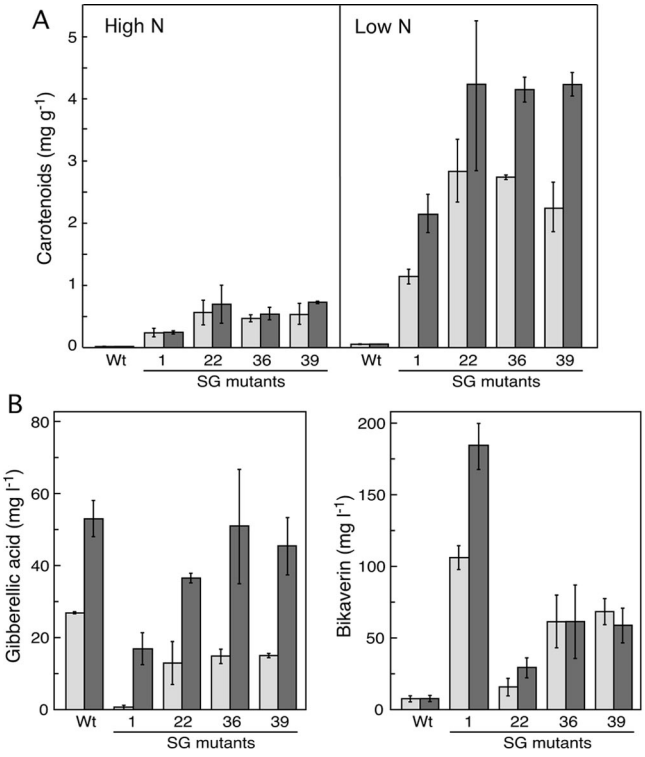

FIG. 2. Secondary metabolite production by the wild type (Wt) and four *carS* mutants. (A) Carotenoid content in the mycelia grown in high- and low-nitrogen medium. (B) GA_3 and bikaverin in the filtrates of cultures grown on low-nitrogen medium. The data show average amounts and standard deviations of the metabolites produced after 9 days (light bars) and 15 days (dark bars) in two independent experiments.

RESULTS

Metabolite production in high- and low-nitrogen medium. As predicted by their phenotypes on minimal agar and the lack of illumination in the experiment, carotenoids were produced in very small amounts by the wild type and in significant amounts by the *carS* strains on high- or low-nitrogen media (Fig. 2A). Unexpectedly, the concentration of these pigments in the mutants was about five times higher in the low-nitrogen medium. A similar stimulation also occurred in the wild type (48 μg g^{-1} in low-N medium versus 10 μg g^{-1} in high-N medium after 15 days), suggesting that high nitrogen repressed carotenoid biosynthesis. The amounts of carotenoids accumulated by the *carS* strain SG1 were about half that of the other three mutants tested, a difference not observed in earlier analyses from surface cultures (4).

No gibberelllins or bikaverins were found in the cultures of any of the five strains grown in high-nitrogen medium, indicating that the mutations in the *carS* strains do not alter the

nitrogen repression of these pathways. As expected, these metabolites were produced under nitrogen-limiting conditions (Fig. 2B). Interestingly, although to different degrees, the *carS* mutants produced more bikaverin than did the control strain. This difference was particularly marked in the SG1 mutant, which produced 20-fold more bikaverin than did the wild type. However, GA_3 was produced in similar amounts by the wild type and three of the *carS* mutants and in a smaller amount by one of them. Interestingly, this was SG1 (i.e., the mutant that produced higher levels of bikaverin).

Based on these results, we decided to evaluate in more detail the effect of nitrogen starvation on carotenoid biosynthesis and the effect of the *carS* mutations on the regulation of gibberellin and bikaverin production. To this end, nitrogen was removed from early cultures in high-nitrogen medium by washing and then transferring the mycelia to a glucose solution. The amounts of the three metabolites and the expression of key genes for these pathways were followed in time course experiments.

Carotenoid biosynthesis under nitrogen starvation. Consistent with the initial results from 9 and 15 days, wild-type cultures grown in the dark for 3 days in high-nitrogen medium contained small amounts of carotenoids: about 0.02 mg g^{-1}. Transfer to a nitrogen-free solution resulted in the slow accumulation of carotenoids, reaching 0.15 mg g^{-1} after 144 h (Fig. 3A). This result was accompanied by a significant induction of the mRNA for the structural genes *carRA* and *carB* during the first 24 h in the glucose solution (Fig. 3B). Carotenoid biosynthesis was equally induced if the solution contained phosphate or sulfate, but not if it contained nitrogen (see the supplemental material), indicating a specific effect by nitrogen starvation.

As expected, the four *carS* mutants contained higher levels of carotenoids than did the wild type at the time of transfer (Fig. 3A). However, significant differences were found between them: SG36 and SG39 accumulated more carotenoids than did SG1 and SG22. The smallest amount, ~0.13 mg g^{-1} accumulated by SG22, was considerably larger than the 0.02 mg g^{-1} accumulated by the wild type. The four *carS* mutants increased their carotenoid content during the incubation in the nitrogen-free solution by 5- to 10-fold, an increase comparable to that observed in the wild type.

The higher carotenoid biosynthetic capacity of the mutants compared to the wild type in the cultures before transfer correlated with larger mRNA amounts for the structural genes *carRA* and *carB* (time zero in Fig. 3B). The transfer to the glucose solution resulted in a 10-fold stimulation of the mRNA levels in three mutants during the first 24 h, followed by a decrease in the following days (Fig. 3B). A more modest stimulation occurred with SG39, although in this mutant the mRNA levels were more stable throughout the 144-h incubation and the carotenoid content increased continuously during this time (Fig. 3).

Gibberellin and bikaverin production under nitrogen starvation. As expected, no GA_3 or bikaverin was found in the 3-day-old cultures in high-nitrogen medium, and both biosynthetic pathways were induced upon transfer to the nitrogen-free solution. Significant amounts of bikaverin were detected in the nitrogen-free cultures in less than 24 h, but the formation of GA_3, the final product of the GA pathway, required a longer incubation time (Fig. 4). After 6 days in the glucose solution, the amount of bikaverin was five times higher than that of GA_3, despite the lower accumulation rate of bikaverin between days 2 and 6.

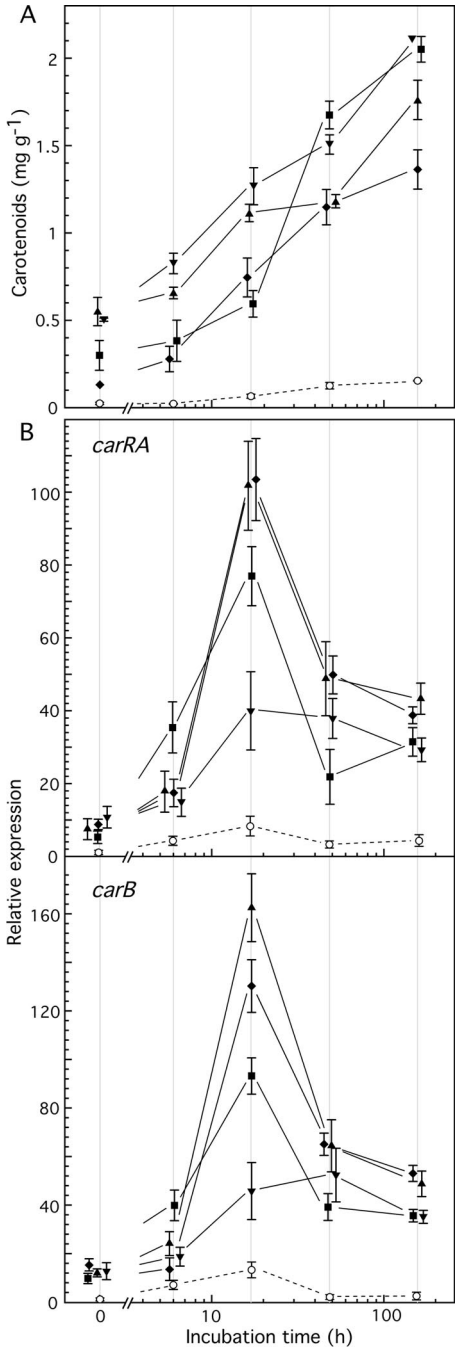

FIG. 3. Effect of nitrogen starvation on carotenoid production (A) and mRNA levels of genes *carRA* and *carB* (B) in the wild type (open circles with dashed lines) and four *carS* mutants (closed symbols with continuous lines: ■, SG1; ◆, SG22; ▲, SG36; ▼, SG39). The strains were grown in high-nitrogen medium for 3 days and incubated afterwards in 2 g $liter^{-1}$ glucose for the times indicated in the abscissas. The data show the average and standard deviation from four determinations and two independent experiments. Overlapping symbols for each time (0, 6, 16, 48, and 144 h, vertical gray lines) were separated for better visualization.

The *carS* mutants differed in their abilities to produce GA_3

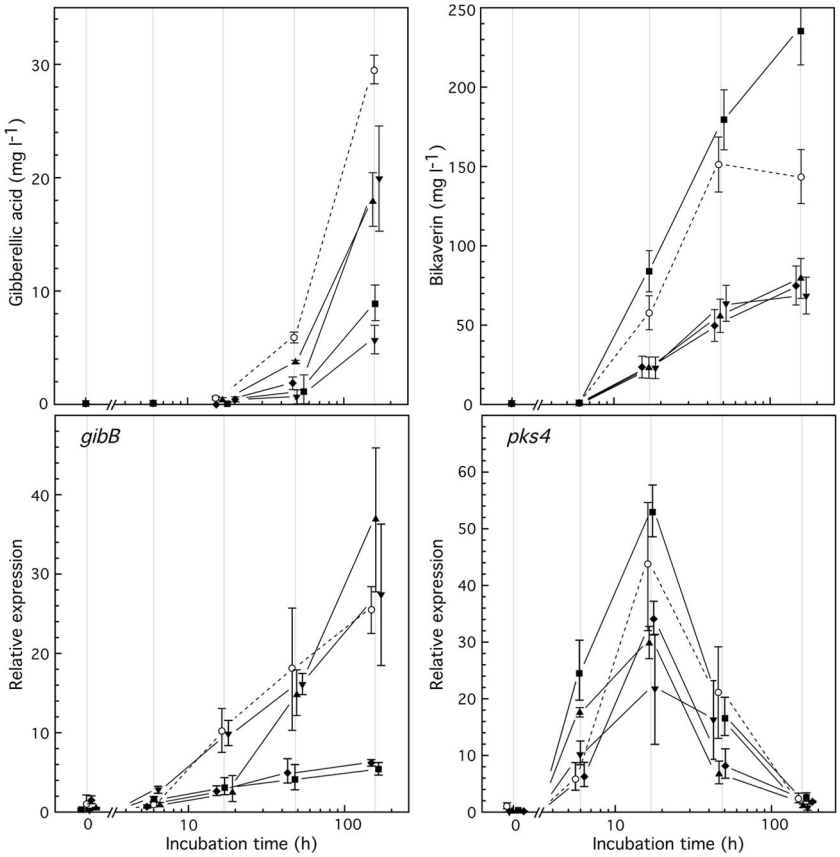

FIG. 4. Effect of nitrogen starvation on bikaverin and GA_3 production and mRNA levels of genes *gibB* and *pks4* in the wild type (open circles with dashed lines) and four *carS* mutants (closed symbols with continuous lines: ■, SG1; ◆, SG22; ▲, SG36; and ▼, SG39). The strains were grown in high-nitrogen medium for 3 days and incubated afterwards in 2 g liter^{-1} glucose for the times indicated in the abscissas. The data show average and standard deviation from four determinations and two independent experiments. Relative RNA levels were referred to the value of the wild-type control (time zero). Overlapping symbols for each time (0, 6, 16, 48, and 144 h [vertical gray lines]) were separated for better visualization.

and bikaverin (Fig. 4). As before, SG1 produced the most bikaverin. However, the wild type produced more bikaverin than under the culture conditions used in Fig. 2, and in this case, the other three *carS* mutants produced less bikaverin than did the control strain. In contrast, the four *carS* strains—especially SG1 and SG22—produced less GA_3 than the wild type (Fig. 4). Disparities with the data shown in Fig. 2 are probably due to differences in compositions of the media: both experiments differ in phosphate, sulfate, and glucose availability during the production phase. These nutrients are in excess under the nitrogen-limiting conditions of the experiment shown in Fig. 2. Thus, more than 40 g liter^{-1} glucose remains in the culture after 15 days of incubation (12). However, here the mycelia were transferred to a solution that lacks phosphate or sulfate and only contains 20 g liter^{-1} glucose, which was consumed during the 6-day incubation. If the mycelia were instead transferred to fresh medium without nitrogen, production of GA_3 and bikaverin was reduced compared to the levels obtained in the former experiments (see the supplemental material). However, as found in low-nitrogen medium (Fig. 2), the wild type produced less bikaverin than the mutants SG1, SG36, and SG39 and amounts similar to those produced by SG22. The data from the three experiments suggest different sensitivities of bikaverin production to glucose or salt availabilities in the wild type and *carS* mutants. Experiments to evaluate the role of carbon source and salts on the regulation of GA_3 and bikaverin production are under way.

The high GA_3 and bikaverin production levels following transfer to the nitrogen-free solution make these conditions suitable for gene expression analyses. The production levels of these metabolites roughly correlated with differences in the mRNA levels for the key genes of both pathways, *gibB* and *pks4* for GA_3 and bikaverin biosynthesis, respectively (Fig. 4). Two exceptions stand out: *gibB* mRNA levels in mutants SG36 and SG39 were similar to those in the wild type, while those in SG1 and SG22 were about four times lower, indicating differences in the effects of the carotenoid-deregulating mutations between the pairs of *carS* strains.

Two noticeable differences were found in the nitrogen regulation of genes *gibB* and *pks4*. As expected, induction of the gibberellin and bikaverin pathways was mediated through transcriptional activation of their structural genes. However, *pks4* mRNA appeared earlier than did *gibB* mRNA, a result that could explain the more rapid formation of bikaverin in the culture. More importantly, the *pks4* induction was transient, while the *gibB* induction was sustained throughout the 6-day incubation. In agreement with the *gibB* mRNA patterns, higher levels of kaurene biosynthetic activity were maintained for the

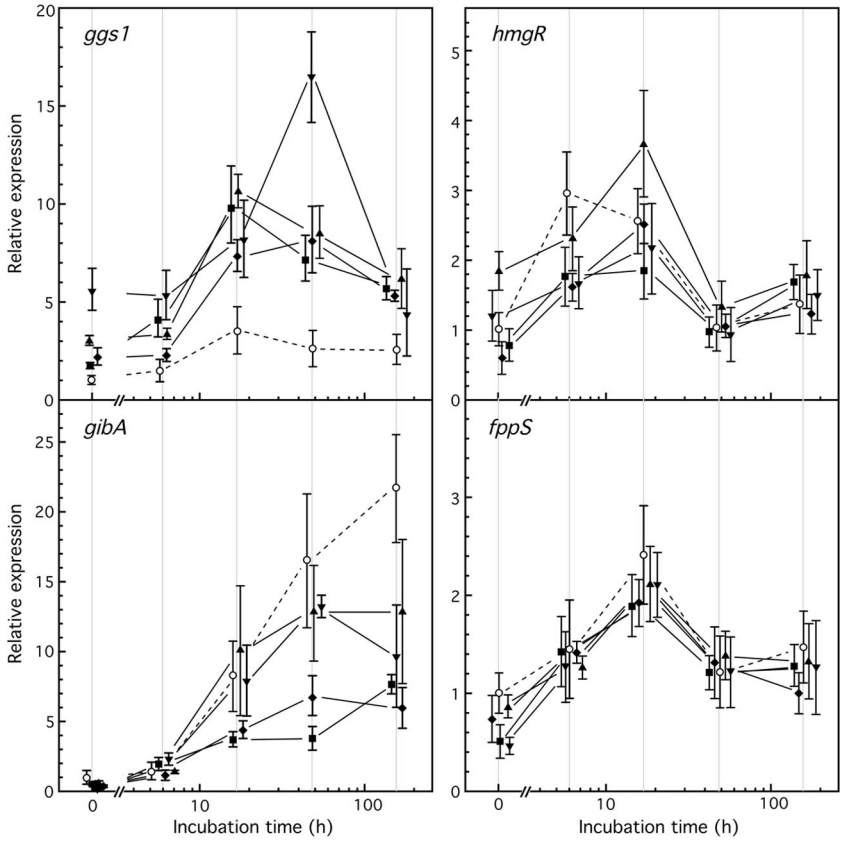

FIG. 5. Effect of nitrogen starvation on mRNA levels of genes *ggs1*, *gibA*, *hmgR*, and *fppS* in the wild type (open circles with dashed lines) and four *carS* mutants (closed symbols with continuous lines: ■, SG1; ◆, SG22; ▲, SG36; ▼, SG39). The strains were grown in high-nitrogen medium for 3 days and incubated afterwards in 2 g liter^{-1} glucose for the times indicated in the abscissas. The data show the average and standard deviation from four determinations and two independent experiments. Relative RNA levels were referred to the value of the wild-type control (time zero). Overlapping symbols for each time (0, 6, 16, 48, and 144 h [vertical gray lines]) were separated for better visualization.

same days under similar experimental conditions (18). The regulatory differences between bikaverin and gibberellin induction also were observed in the *carS* strains.

Regulation of genes for early reactions in the terpenoid pathway. The *F. fujikuroi* genome contains two paralogous genes that each encode a prenyl transferase responsible for the synthesis of GGPP (31, 45). The mRNA levels for these two genes, *ggs1* and *gibA*, were measured in the same samples from the transfer experiments (Fig. 5). *gibA*, originally termed *ggs2*, is located in the gene cluster for the GA pathway and is activated following nitrogen exhaustion (32, 47). Accordingly, its mRNA levels increased upon nitrogen starvation in a manner similar to *gibB* (Fig. 4). In this case, all four *carS* mutants contained lower levels of *gibA* mRNA than did the wild-type strain. As found for *gibB*, this decrease was more pronounced for SG1 and SG22 than for SG36 and SG39.

In contrast to *gibA*, the amounts of *ggs1* mRNA were larger in the *carS* mutants than in the wild type (Fig. 5), indicating a coregulation with the structural genes for the carotenoid pathway. In all of the strains, *ggs1* induction following transfer to the nitrogen-free solution resembled those for *carB* and *carRA* (Fig. 3B) and *pks4* (Fig. 4), but with a less prominent induction peak.

We extended the analysis to the genes for earlier reactions in the terpenoid pathway, *hmgR* (49) and *fppS* (23) (Fig. 1). In contrast to *carRA*, *carB* (Fig. 3B), and *ggs1*, the mRNA levels for *hmgR* and *fppS* were similar in the wild-type and *carS* strains (Fig. 5), indicating that the CarS protein plays no role in the regulation of the steps preceding the synthesis of GGPP (Fig. 1). However, both genes were induced by nitrogen starvation at similar levels to *ggs1*.

Effect of light under nitrogen starvation. To evaluate the relationship between the activation of carotenogenesis by nitrogen starvation and that produced by light, cultures incubated for 3 days in high-nitrogen medium were transferred to a nitrogen-free solution for 16 h and illuminated subsequently for 24 h, a time sufficient for full photoresponse of carotenoid biosynthesis (9). Parallel experiments were done with samples incubated all the time in high-nitrogen medium and with control samples kept under the same conditions in the dark. For efficient mycelial irradiation, illumination was done on submerged mycelial suspensions in petri dishes and on air-exposed mycelial pads. No photoinduction was detected after illumination of the mycelial suspensions (result not shown), indicating the need for appropriate aeration for efficient carotenoid photoinduction. In contrast, illumination of mycelial pads led to a significant increase in the carotenoid content in the wild

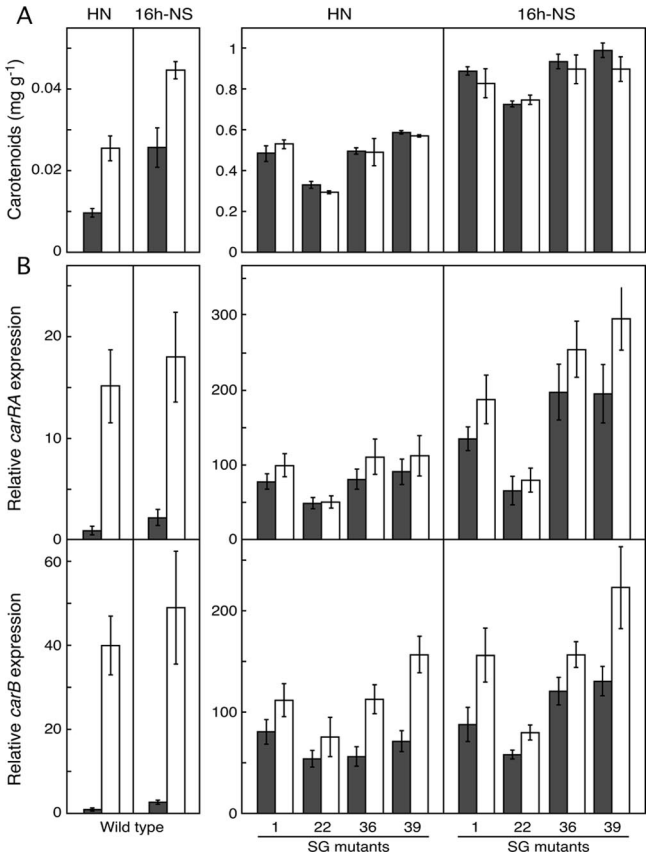

FIG. 6. Effect of light and nitrogen starvation on carotenoid production (A) and mRNA levels of genes *carRA* and *carB* (B) in the wild type (left graphs) and four *carS* mutants (right graphs). The strains were grown for 3 days in high-nitrogen medium (HN) or incubated afterwards for 16 h in 2 g liter^{-1} glucose (16 h-NS), filtered, and exposed to air as mycelial pads under white light (white bars) or in the dark (dark bars). The mycelial pads were incubated for 1 h for mRNA analyses and 24 h for carotenoid analyses. Relative RNA levels were referred to the value of the wild-type control incubated in the dark. The data show the average and standard deviation from four determinations and two independent experiments.

type (Fig. 6A). No light induction of the carotenoid content was detected in the *carS* mutants. As expected, the five strains accumulated more carotenoids following 16 h of incubation under nitrogen starvation. In the wild type, this increase was additive with that produced by light.

To evaluate the effect of light on *carRA* and *carB* mRNA levels, mycelial samples were collected from 1-h-illuminated pads, an optimal time for light response of these genes (33). As expected, light produced a strong induction of *carB* and *carRA* in the wild type (Fig. 6B). Moderate increases also were found in the *carS* mutants, but differences were not statistically significant in several cases. However, all measures from illuminated samples exceeded those from parallel nonilluminated samples, indicating that the *carS* strains are still responsive to light. Under these experimental conditions, only a mild effect of nitrogen starvation was visible on *carRA* and *carB* mRNAs, more apparent in the wild type than in the *carS* mutants.

DISCUSSION

The rich secondary metabolism of *F. fujikuroi* is controlled by complex regulatory networks governing the synthesis of different metabolites in response to environmental signals, such as nutrients, pH, or light (6). Nitrogen availability is a major determining agent in the regulation of gibberellin and bikaverin biosynthesis (13, 21), but the role of this nutrient in the production of other secondary metabolites by this fungus has not previously been extensively investigated. Carotenoids accumulate in response to light (4, 9) through the transcriptional induction of at least three structural genes, *carRA*, *carB*, and *carT* (33, 34). In the absence of light, the amount of carotenoids is affected by temperature (4), medium composition (9), and the presence of some alcohols (3), but limited information is available on the effect of other environmental signals, such as nitrogen.

We found that nitrogen represses the carotenoid pathway of *F. fujikuroi*. This conclusion is supported by the higher levels of carotenoids produced in low-nitrogen medium than in the same medium with excess nitrogen. In the low-nitrogen medium, the nitrogen source is exhausted in the first days of incubation (13), while in the high-nitrogen medium it is present throughout the incubation. A similar result was found in the carotenoid-overproducing mutants, although scaled 10-fold in this case due to the derepression of the pathway. The negative effect of nitrogen on the carotenoid pathway was confirmed for the same strains following transfer of mycelia grown under nitrogen excess conditions to a nitrogen-free solution. This two-phase experiment is similar to the incubation in low-nitrogen medium in that a nitrogen excess phase is followed by a nitrogen starvation phase. However, the two-phase approach allows a precise time course analysis of the events following nitrogen starvation. Nitrogen removal increased carotenoid biosynthesis in both the wild-type and *carS* strains. In the case of the wild type, the carotenoid levels after 6 days in the nitrogen-free solution were comparable to those reached following continuous illumination of surface cultures (4) and exceeded those reached following 24 h of illumination of mycelial pads (Fig. 6A).

Our results fit earlier observations in *Neurospora crassa*. Incubation of this fungus under nitrogen starvation increased the levels of the *carB* and *carRA* orthologues, *al-1* and *al-2* (38). As in *F. fujikuroi*, this activation was independent of that produced by light, as indicated by the similar result obtained with wc mutants, totally devoid of photocarotenogenesis. A different approach led to similar conclusions in *G. fujikuroi* (19). Immobilized mycelia incubated under nitrogen starvation in the dark increased their carotenoid content for several days, but addition of nitrogen resulted in cessation of the synthesis. Furthermore, exchange of immobilized mycelia between media with different nitrogen contents resulted in induction or cessation of carotene synthesis, depending on the absence or presence of nitrogen, respectively. The mRNA levels for the structural genes were not included in this investigation, but the data suggest a repressor effect of nitrogen on carotenogenesis. This is not the case in other fungi: e.g., astaxanthin production by *Phaffia rhodozyma* was hardly affected by large variations in nitrogen concentration in the medium (25).

As expected, gibberellins and bikaverins were produced by

F. fujikuroi only in low-nitrogen medium or after nitrogen removal. Expression of the structural genes for both biosynthetic pathways, represented in this study by *gibA* and *gibB* in one case and *pks4* in the other, was induced after nitrogen starvation. However, they exhibited like responses, indicating the mediation of different regulatory mechanisms. First, the induction of *gibA* and *gibB* occurred later than did the induction of *pks4* and, accordingly, the accumulation of GA_3 in the medium began later than did bikaverin accumulation. Second, the induction of *pks4* was transient, with the maximum mRNA levels occurring in the first 24 h, while the mRNA levels for *gibA* and *gibB* increased continuously throughout the 6-day incubation, indicating sustained activation in the absence of nitrogen. The different regulatory pattern of *pks4* is consistent with previous observations of regulatory differences between both biosynthetic pathways: despite the common regulation by nitrogen, *areA* mutation has different effects on *pks4* and *gibB* expression (29), and bikaverin production is drastically affected by other factors, e.g., pH (21). The phenotype resulting from the mutation of *wcoA*, the putative *wc-1* photoreceptor gene in *Fusarium*, also exemplifies this pattern. Unexpectedly, the *wcoA* mutants exhibited changes in gibberellin and bikaverin production, but the changes were in the opposite directions (16).

As for *pks4*, nitrogen removal produced a transient increase of the mRNA amounts for specific structural genes of the carotenoid pathway, *carRA* and *carB*, in all of the strains tested. We conclude that the rapid increase observed in the carotenoid content in the *carS* strains is at least partly due to the increased levels of these mRNAs. The transient response is similar to the one produced in response to light by *carRA* and *carB* (16, 33), which exhibit a characteristic postinduction decrease known as light adaptation. However, the response to light is much faster: mRNA levels of the *car* genes reach a maximum 1 h after illumination and decrease rapidly afterwards (16, 33), while the peak induced by nitrogen starvation requires several hours of incubation. The similar responses of *carRA*/*carB* and *pks4* mRNAs to nitrogen starvation suggest that the same regulatory mechanism mediates nitrogen catabolism and carotenoid and bikaverin biosynthesis.

The regulation of carotenoid and gibberellin production by nitrogen also applies to early genes in both pathways, i.e., *hmgR* and *fppS*, which encode enzymes used for the synthesis of FPP- and GGPP-derived terpenoids, and *ggs1* and *gibA*, which encode specific GGPP synthases. Both enzymes catalyze the same reaction, which yields the GGPP substrates for the next specific enzymes, CarRA and GibB, which produce phytoene and kaurene, respectively. GibA was formerly assigned as a specific enzyme of the gibberellin pathway because of the linkage and coregulation of the encoding gene with others in the GA cluster (44). Our results on the regulation of *gibA* are consistent with this functional role. In contrast, earlier results indicated that *ggs1* was not regulated by light or by nitrogen or carbon levels (31).

The overexpression of *ggs1* in the *carS* strains strongly suggests that the Ggs1 enzyme participates in the synthesis of carotenoids. However, *ggs1* also could be involved in the synthesis of other fungal GGPP-derived compounds, such as ubiquinones (26, 48) or dolichols (40). Lack of any additional GGPP synthase gene in the *Fusarium* genome suggests that the gene or genes mutated in the *carS* strains have a role not only in carotenoid biosynthesis but also in the regulation of these metabolites. In contrast, the *carS* mutations do not affect the expression of the former gene in the terpenoid pathway, *fppS*, also involved in the synthesis of sterols. Earlier experiments with transfer to a nitrogen-free solution showed opposite regulations for the syntheses of the gibberellins and sterol precursors, i.e., kaurene and squalene (18), suggesting a repression of the gene responsible for the conversion of FPP to squalene under nitrogen starvation.

Activation of carotenogenesis by nitrogen starvation was independent of that produced by light. This was indicated by the similar photoinduction of the pathway, either in carotenoid content or in *carRA* and *carB* mRNA levels, found in wild-type mycelia incubated in excess nitrogen or under nitrogen starvation. As earlier described (4), carotenoid photoinduction was not apparent in the *carS* mutants. However, the expression data are consistent with light induction of *carRA* and *carB* mRNA levels in the *carS* strains. Because of their high background levels, such induction is proportionally less pronounced in the *carS* strains than in the wild type. Furthermore, light induction of the carotenoid content in these strains may be compensated for by a loss produced by light-induced degradation.

The increased carotenoid content found in the *carS* mutants incubated for 16 h under nitrogen starvation (Fig. 6A) was not accompanied by the increase in *carRA* and *carB* mRNAs found in a former experiment (compare Fig. 3B and Fig. 6B). This could be explained by the air-exposed incubation step used in this experiment. Carotenoid biosynthesis is regulated by oxidative stress in other fungi (24, 30), and 1 h of exposure to air may result in changes of *carRA* and *carB* mRNA levels. Experiments to investigate the effect of this and other environmental stresses on carotenogenesis in *F. fujikuroi* are in progress.

Our data show that the putative CarS protein is not responsible for the regulation by nitrogen or by light but confirm a key role in the control of carotenoid biosynthesis. The deregulation of *carRA* and *carB* in the *carS* mutants and their easy isolation in mutagenesis screenings are consistent with the loss of a transcriptional repressor. Such putative regulatory protein(s) could interact with the regulatory network that controls gibberellin and bikaverin biosynthesis, as shown by the alterations in the production of these compounds and the differential expression of key structural genes in the mutants in comparison to the wild type. The pleiotropic phenotype of the *carS* mutants also could be consistent with the alteration of a regulatory protein involved in a more general control mechanism. Indeed, our data do not allow us to infer if only nitrogen-regulated gene expression is altered or if these and many other genes are misregulated independently of nitrogen availability.

The four *carS* mutants investigated exhibit more phenotypic similarities than discrepancies. The differences observed among them could be due to allelic-specific effects as well as to secondary mutations in other genes. Genetic mapping of the *carS* mutation and *carS* cloning by complementation are hindered by low sexual fertility and transformation frequency (17) of their parental wild-type strain. Complementation experiments between *carS* strains to confirm the mutation in the same gene and experimental approaches to pursue its identi-

fication are in progress. The characterization of such a gene will represent a landmark in the understanding of the underlying regulatory mechanisms for carotenoid biosynthesis and their eventual connections with the nitrogen regulation of other pathways in *Fusarium*, a paradigmatic model fungus for secondary metabolism.

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