



Cross-talk between light and glucose regulation controls toxin production and morphogenesis in *Aspergillus nidulans*

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ABSTRACT

Light is a major environmental stimulus that has a broad effect on organisms, triggering a cellular response that results in an optimal adaptation enhancing fitness and survival. In fungi, light affects growth, and causes diverse morphological changes such as those leading to reproduction. Light can also affect fungal metabolism, including the biosynthesis of natural products. In this study we show that in *Aspergillus nidulans* the effect of light on the production of the sterigmatocystin (ST) toxin depends on the glucose concentration. In cultures grown with 1% glucose and exposed to light, ST production was lower than when grown in the dark. This lower ST production coincided with an elevated rate of cellular damage with partial loss of nuclear integrity and vacuolated cytoplasm. However, in cultures grown with 2% glucose these effects were reversed and light enhanced ST production. Glucose abundance also affected the light-dependent subcellular localization of the VeA (velvet) protein, a key regulator necessary for normal light-dependent morphogenesis and secondary metabolism in *Aspergilli* and other fungal genera. The role of other VeA-associated proteins, particularly the blue-light-sensing proteins LreA and LreB (WC-1 and WC-2 orthologs), on conidiation could also be modified by the abundance of glucose. We also show that LreA and LreB, as well as the phytochrome FphA, modulate not only the synthesis of sterigmatocystin, but also the production of the antibiotic penicillin.

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1. Introduction

Light has a broad effect on living organisms from humans to fungi, triggering chemical and morphological responses as environmental adaptations. In fungi, light can cause diverse changes in fungal metabolism, growth, and the balance between sexual and asexual differentiation (Gyula et al., 2003; Purschwitz et al., 2006, 2008; Calvo, 2008; Corrochano, 2007; Blumenstein et al., 2005). Fungi are responsive to a broad spectral range of light, where blue and red light display prominent effects on fungal growth and morphogenesis (Mooney and Yager, 1990; Mooney et al., 1990; Kues, 2000; Cerda-Olmedo, 2001; Liu et al., 2003; Purschwitz et al., 2006, 2008).

Chemical and morphological development in the model fungus *Aspergillus nidulans* is regulated through interplay of two light-sensing systems, the red and the blue light-responsive systems, that interact with the VeA protein in the nucleus. Red light is sensed through a phytochrome-like receptor, FphA. This protein interacts directly with the VeA protein (Purschwitz et al., 2008). In addition,

FphA interacts with the blue-light-sensing protein apparatus that includes LreA and LreB, orthologs to the respective *Neurospora crassa* blue light-responsive WC-1 and WC-2 (He et al., 2002). FphA interacts with LreB, which was found to interact with LreA (Purschwitz et al., 2008). VeA also interacts with LaeA, a putative methyl transferase that regulates the expression of secondary metabolism gene clusters, and other proteins involved in the regulation of development (Bok and Keller, 2004; Bayram et al., 2008; Calvo, 2008).

In our previous studies we have demonstrated that VeA is necessary for the production of numerous secondary metabolites, including mycotoxins, not only in the genus *Aspergillus* (Kato et al., 2003; Calvo et al., 2004; Duran et al., 2007, 2009) but also in other fungal genera (Myung et al., 2009). In addition to VeA, other VeA-interacting proteins are also influential in regulating the biosynthesis of natural products, such as the production of the sterigmatocystin (ST) toxin in *A. nidulans*. This includes LaeA, VelB (Bayram et al., 2008), as well as FphA, LreA and LreB (Purschwitz et al., 2008). We showed that while LreA and LreB promote the formation of ST, FphA acts as a repressor of the synthesis of this mycotoxin (Purschwitz et al., 2008). The possible effect of these light receptors on the biosynthesis of other secondary metabolites has not yet been determined.

It is likely that the light response is also integrated in a cross-talk with those responses derived from additional external stimuli.

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Several environmental factors that influence secondary metabolism have been identified. For example, in *Aspergillus flavus* simple sugars such as glucose, fructose, sucrose and sorbitol when used as sole carbon sources strongly support fungal growth, increasing sporulation and aflatoxin production (Abdollahi and Buchanan, 1981; Buchanan and Stahl, 1984; Bu'Lock, 1961; Kacholz and Demain, 1983; Liu and Chu, 1998; Luchese and Harrigan, 1993). In contrast, peptone (Abdollahi and Buchanan, 1981; Bu'Lock, 1961; Feng and Leonard, 1998; Liu and Chu, 1998), galactose, xylose, mannitol and lactose (Kacholz and Demain, 1983) do not support aflatoxin production well. Our previous studies indicated that the effect of light on the production of ST by *A. nidulans* is medium-dependent (Kato et al., 2003). While production of ST in glucose minimal medium (GMM) was higher in the absence of light, light enhanced accumulation of ST in rich medium such as YGT (Kato et al., 2003). In *A. nidulans* extensive research has been done in deciphering the mechanisms of carbon catabolite repression, where the transcription factor CreA plays a key role as negative-acting regulator (Arst and MacDonald, 1975; Bailey and Arst, 1975; Dowzer and Kelly, 1991; Shroff et al., 1997). Our previous work on penicillin biosynthesis in *A. nidulans* has shown this pathway to be under carbon regulation, but CreA-independent, (Espeso and Peñalva, 1992; Espeso et al., 1993). The nature of this carbon regulatory system remains undetermined, however in the case of penicillin production, carbon regulation depends on the PacC-mediated pH regulatory system (Espeso et al., 1993); an example of a link between two regulatory signaling pathways responsive to different external cues.

In addition to the role of VeA in regulating secondary metabolism, it also regulates fungal morphogenesis. *A. nidulans* strains carrying a *veA* wild-type allele develop mainly asexually producing conidiophores in the light, while in the dark fruiting bodies called cleistothecia are formed predominantly (Yager, 1992). FphA is involved in the repression of sexual fruiting body formation and induction of conidiation (Blumenstein et al., 2005), while LreA and LreB have antagonistic roles. Similar to the WC-orthologs, VeA represses conidiation and promotes sexual development.

Previous studies have shown that glucose concentration also affects the morphogenesis balance between sexual and asexual development. For example, in *A. nidulans* high concentrations of glucose promote sexual development and simultaneously inhibit asexual development, while low glucose concentrations have the opposite effect (Han et al., 1990, 2001; Jeong et al., 2000).

In the present study we show a relationship between the effect of light and glucose as carbon source in the production of the mycotoxin ST and morphogenesis in the fungus *A. nidulans*. We also investigated the effect of blue and red-light-sensing proteins interacting with VeA on the biosynthesis of other secondary metabolites. Our results indicate that the perception of external stimuli and the morphological and metabolic responses are complex and integrated in order to achieve optimal adaptation to a plurality of environmental cues. This study also indicates that part of this integration of sensing mechanisms and cellular responses is mediated by VeA.

2. Material and methods

2.1. Fungal strains and growth conditions

A. nidulans strains used in this study are listed in Table 1. These strains were cultured on glucose minimum medium (GMM, in this study the percentage of glucose is indicated in each case) plus the appropriate supplements for the corresponding auxotrophic markers (Kafer, 1977). Agar (15 g/l) was added in the case of solid medium. Strains were stored as 30% glycerol stocks at -80°C .

2.2. Mycotoxin analysis

Solid GMM containing different glucose concentrations (indicated in each case) was inoculated with FGSCA4 wild type (10^6 spores/plate) and cultured in white light (28 microEinstein/m²/s) or dark conditions. Experimental time points are indicated in each case. Four cores (16 mm diameter) from each replicate plate were collected in a 50 ml Falcon tube, and ST was extracted by adding 5 ml of CHCl_3 three consecutive times. The extracts were allowed to dry and then resuspended in 500 μl of CHCl_3 before 15 μl of each extract was fractionated on a silica gel thin-layer chromatography (TLC) plate using a toluene-ethyl acetate-acetic acid [80:10:10 (v/v/v)] solvent system. Alternatively, a solvent system consisting of benzene-acetic acid [95:5 (v/v)] was used. The TLC plates were sprayed with aluminum chloride (15% in ethanol) and baked for 10 min at 80°C to intensify ST fluorescence upon exposure to long wave (365 nm) UV light. ST purchased from Sigma was used as a standard.

2.3. Effect of cAMP on ST production

Solid GMM containing 1% or 2% glucose was supplemented with 400 μM of dibutyryl cyclic adenosine monophosphate (db-cAMP) or 8-bromo-cAMP. The plates were inoculated with FGSCA4 wild type (10^6 spores/plate). After 3 days of cultivation in light or dark conditions ST was extracted and analyzed according to the method described in Section 2.2.

Also as a separate experiment, internal cAMP levels were measured in the FGSCA4 strain when growing in liquid GMM cultures with different glucose concentrations that included a range from 0.3% to 6%. Spores (10^6 spores/ml) were inoculated in 150 ml of GMM with different glucose concentrations. After 24 h, 36 h and 40 h of incubation the medium was refreshed, maintaining the glucose concentrations as constant as feasible. Mycelia were harvested, lyophilized and ground. Fifty milligrams were extracted with 1.5 ml 5% TCA and glass beads in a bead mill for 7.5 min. The extracts were analyzed using the Cyclic AMP EIA Kit from Biomedical Technologies Inc. following the manufacturer instructions.

2.4. mRNA analysis

Total RNA was isolated from mycelia at the specified time points by using Trizol (Invitrogen) as described by the supplier. Four micrograms of total RNA were treated with DNaseI RQI (Promega) and reverse transcribed with MMLV reverse transcriptase (Promega). qRT-PCR was performed in a Mx3000P thermocycler (Stratagene) using SYBR Green JumpStartTM Tag Ready Mix (Sigma). Primers pairs used for qRT-PCR are listed in Table 2.

2.5. Microscopy analysis

Strains TRMD3.4.17 (Stinnett et al., 2007) and MAD3140 (Table 1) were used for microscopy studies to follow the localization of VeA wild-type protein in the cell as VeA::GFP fusion. MAD3140 was obtained by meiotic recombination between TRMD3.4.17 and MAD2446, a strain carrying *hhoA::mCherry* chimera (Ettxeste et al., 2009). Conidia were inoculated in liquid minimum medium with different glucose concentrations (as indicated) and cultured essentially as described in (Stinnett et al., 2007). Briefly, conidia were germinated onto the surface of a cover slip immersed in minimum medium and further incubated for 16 h with illumination or in the dark. Alternatively, conidia were point inoculated onto GMM containing 1%, 1.5% or 2% glucose. Cultures were incubated in light and dark conditions. Colonies grew under light, at 25 cm distance of the light source. Dark conditions were obtained by wrapping the

Table 1*A. nidulans* strains used in this study.

Strain	Genotype	Source
FGSCA4	Wild-type	Fungal Genetics Stock Center, Kansas City, Missouri
LAV+	<i>biA1</i> ; Δ <i>lreA::argB</i> ; <i>veA</i> +	Purschwitz et al. (2008)
LBV+	<i>biA1</i> ; Δ <i>lreB::argB</i> ; <i>pyroA4</i> ; <i>veA</i> +	Purschwitz et al. (2008)
SJP1	<i>pyrG89</i> ; Δ <i>argB::trpC</i> Δ B; <i>pyroA4</i> ; Δ <i>fphA::argB</i> ; <i>veA</i> +	Purschwitz et al. (2008)
SJP13.3	Cross between LBV + and SJP1— <i>biA1</i> ; Δ <i>lreB::argB</i> ; Δ <i>argB::trpC</i> Δ B; <i>pyroA4</i> ; Δ <i>fphA::argB</i> ; <i>veA</i> +	Purschwitz et al. (2008)
SJP15G	Cross between LAV + and SJP1— <i>pyrG89</i> ; Δ <i>argB::trpC</i> Δ B; <i>pyroA4</i> ; Δ <i>lreA::argB</i> ; Δ <i>fphA::argB</i> ; <i>veA</i> +	Purschwitz et al. (2008)
SJP21.3	Cross between SJP15G and SJP13.3— <i>pyrG89</i> ; Δ <i>lreB::argB</i> ; Δ <i>argB::trpC</i> Δ B; <i>pyroA4</i> ; Δ <i>lreA::argB</i> ; Δ <i>fphA::argB</i> ; <i>veA</i> +	Purschwitz et al. (2008)
TRMD3.4.17	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>veA::gfp::pyrG^{A.fumigatus}</i>	Stinnett et al. (2007)
MAD3140	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>veA::gfp::pyrG^{A.fumigatus}</i> , <i>hhoA::mCh::pyroA^{A.fumigatus}</i> , <i>inoB2</i>	This study

Table 2

Primers used in this study.

Primer name	Sequence
ActinF	5'-ATGGAAGAGGAAGTTGCTGCTCTCGTTATCGACAATGGTTC-3'
ActinR	5'-CAATGGAGGGGAAGACGGCAGCGG-3'
<i>stcU</i> -F	5'-CATGTCAAGGACGTTACGCCAGATGAATTCGACCGAGTATTTCCGGTC-3'
<i>stcU</i> -R	5'-GCGGCACACTCATCCACTGCTCATC-3'
<i>afIR</i> -F	5'-ATGGAGCCCCAGCGATCAGCCAG-3'
<i>afIR</i> -R	5'-TTGGTGATGGTGCTGCTCTTGGCTGCTCAAC-3'

plates with foil. The active growth front edge was labeled on the plate every 24 h. Detailed microscopic examination was performed on mycelium after 72 h. The agar piece obtained was washed with water and then smashed onto the microscope slide with the help of the coverslide and a drop (20 μ l) of 50% glycerol acting as mounting liquid. Samples were observed with an upright Nikon Eclipse 80i microscope equipped with Nomarski optics, a 63x oil objective 1.4 N.A. and Semrok filters adequate for GFP and mRFP fluorescence detection. Images were recorded with an Orca-ER camera (Hamamatsu Photonics). DIC images were taken with an exposure of 30 ms, mRFP with 150 ms and GFP with 750 ms. Images were processed minimally using Wasabi (Hamamatsu Photonics) and ImageJ 1.37v software package (<http://rsb.info.nih.gov/ij/>).

2.6. Morphological studies

Asexual and sexual developmental studies were performed using the *A. nidulans* strains FGSCA4, LAV+, LBV+, SJP1, SJP13.3, SJP15G, SJP21.3 (Table 1). Plates containing 25 ml of solid GMM (1% and 2% glucose) plus the appropriate supplements were spread-inoculated with 100 μ l of water containing 10^6 spores. The cultures were incubated at 37 °C in the dark or in the light. After 7 days, a 16-mm-diameter core was removed from each spread plate culture and homogenized in water to release the spores. Conidia were counted using a hemacytometer. Identical cores were taken to examine cleistothecial production under a dissecting microscope. To increase visualization of cleistothecia the cores were spread with 70% ethanol to remove conidiophores.

2.7. Penicillin production

The penicillin (PN) bioassay was performed essentially as described by (Brakhage et al., 1992) by using *Geobacillus stearothermophilus* as a test organism. *Bacillus cereus* penicillinase controls were included in each case (five units per sample, purchased from Sigma) to evaluate whether the antibacterial activity observed was due to the presence of PN. This allowed us to distinguish the antibiotic activity of PN from those of other compounds that could have been produced.

3. Results

3.1. Effect of glucose and light on ST production in *A. nidulans*

Our previous studies indicated that the effect of light on the production of ST is medium-dependent (Kato et al., 2003). While production of ST in GMM was greater in the dark, accumulation of ST was greater in light in rich medium such as YGT (yeast extract, glucose and trace elements). Other authors have also described a light stimulating effect in ST production in rich medium (Guzman-de-Pena et al., 1998). Our present study revealed a cross-talk between the response to a single nutritional component, namely glucose, and light response in controlling the production of the mycotoxin ST. We found greater ST accumulation in the dark than in the light when the fungus was growing on GMM with 1% glucose, and surprisingly, the opposite was true when GMM with 2% glucose was used (Fig. 1A).

3.2. Effects of cAMP on ST production in *A. nidulans*

High cAMP level is an indicator of starvation (Lafon et al., 2006) that could cause a negative dominant effect resulting in possible alterations of the light effect on ST production. We investigated whether the addition of cAMP on 1% and 2% GMM could alter the effect on the ST biosynthesis stimulation by glucose under light in the wild-type strain *A. nidulans* FGSCA4. When 400 μ M of the cAMP analog db-c-AMP or 8-bromo-cAMP were added to the medium before inoculation, the ST biosynthesis pattern did not change in the light or dark conditions (Fig. 1B).

In another experiment we examined the internal levels of cAMP in cultures containing different glucose concentrations. Our results revealed that endogenous cAMP levels remain fairly constant when a range of 0.7–6% glucose concentration was used in the medium (Fig. 1C). cAMP levels increased only at glucose levels below 0.7%. The experiment was repeated several times with similar results in light or dark conditions.

3.3. Glucose concentration affects light-dependent subcellular localization of the VeA protein

An increase in ST production occurs as the glucose concentration in the minimal medium is increased, obtaining the highest accumulation of ST in the range close to 1.5% in the presence of light (Fig. 2A). The glucose concentrations at which ST production is maximized are different in the dark, where high accumulation of ST occurs at lower concentrations of glucose. Because VeA is a central component in light sensing, the effect of the glucose concentration on VeA localization was analyzed. We investigated the nuclear accumulation of a VeA::GFP fusion expressed by strain TRMD3.4.17 and MAD3140 (Table 1). Both strains display a *veA* wild-type phenotype (Stinnett et al., 2007, and data not shown).

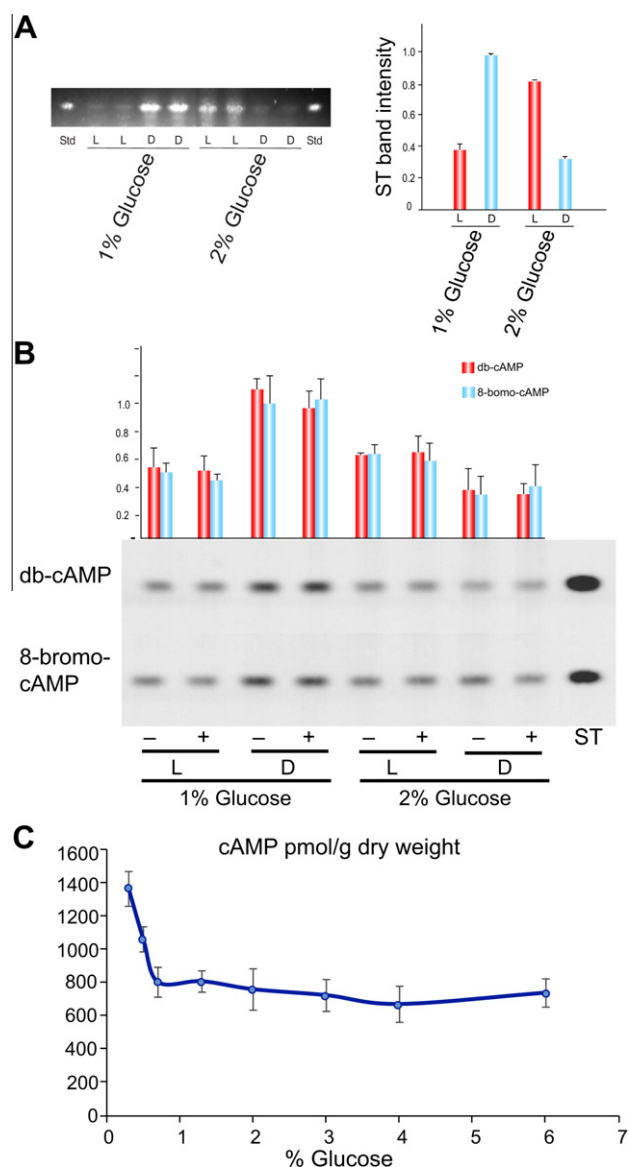


Fig. 1. Effect of light and glucose on ST production in *A. nidulans* and study of possible connections with cAMP levels. (A) Thin-layer chromatography analysis of ST produced by the wild-type *A. nidulans* FGSCA4 strain on GMM with 1% or 2% glucose cultured in light and dark conditions after 5 days of incubation. ST, standard. The densitometry was carried out with Scion Image Beta 4.03 software. The ST band intensity values were normalized with respect to the highest intensity considered as 1. (B) Effect of db-cAMP and 8-bromo-cAMP supplementation on ST production. Thin-layer chromatography analysis of ST produced by the wild-type *A. nidulans* FGSCA4 strain. Four-hundred μ M of db-cAMP or 8-bromo-cAMP was added to the medium before inoculation. ST was extracted after 3 days of cultivation. (C) Internal cAMP levels measured in strain FGSCA4 when growing in GMM cultures with different glucose concentrations. The error bars in graphs from panel A indicate standard error.

VeA::GFP notably accumulated in nuclei in the dark at all glucose concentrations tested (Fig. 2B). Nuclear fluorescence accumulation, as described before (Stinnett et al., 2007; Araújo-Bazán et al., 2009), was visible in light although at a lower level (Fig. 2B). In the dark, a slight tendency of increased nuclear accumulation with larger amounts of glucose was detected, but significant differences were not found. ST production was increased in a narrow range of glucose concentrations (intermediate concentrations between 1% and 2%), particularly in light culture conditions (Fig. 2A). Coinciding with this increased production of mycotoxin, incubation with

1.5% glucose gave a notable accumulation of VeA fluorescent protein under light, with levels of fluorescence detected in nuclei similar to those found in cultures under dark growth conditions (Fig. 2B). When medium with glucose concentrations higher than 2% was used, the levels of ST production remained low, particularly in the dark (Fig. 2A).

In older cultures, 72 h after inoculation on solid medium, we observed that cellular integrity was partially compromised in those cultures exposed to light where medium with low levels of glucose concentration (1%) was used. In approximately 80% of vegetative cells the cytoplasm appeared vacuolated and the nuclear structures were not well defined (Fig. 3). However, in the medium containing higher glucose levels this detrimental effect of light was minimized and most of the cells presented defined nuclei in healthy cells. This effect on cell integrity in low glucose medium was not observed in cultures growing in the dark.

3.4. Effect of light and glucose on *A. nidulans* ST gene expression

We analyzed ST production and the expression levels of ST genes in *A. nidulans* FGSCA4 wild-type strain growing on solid GMM with 1%, 1.5% and 2% glucose in the light and in the dark (Fig. 4A and B). The ST TLC analysis results were consistent with other results shown in this work (Figs. 1, 2 and 4A). We examined *aflR* and *stcU* transcription levels. *aflR* encodes a ST-specific transcription factor necessary for the activation of the ST gene cluster (Yu et al., 1996; Fernandes et al., 1998). The expression of the structural gene *stcU* is often used as an indicator of ST cluster activation (i.e. Kato et al., 2003).

Our qRT-PCR analysis shows that at 48 h *aflR* expression was greater in 1% glucose cultures, particularly in those growing in the dark. At this same time point, *aflR* levels were very low in light cultures with 1.5% and 2% glucose. However, over time *aflR* expression was greater when those same glucose concentrations (1.5% and 2%) were used in cultures growing in the light (Fig. 4B), while the *aflR* transcript levels decrease in 1% glucose cultures.

In the case of *stcU*, at 48 h transcripts were detected in 1% and 1.5% glucose cultures (particularly in the latter) in the dark, while only low transcript levels were observed in 2% glucose cultures (Fig. 4B). Similarly to *aflR*, *stcU* transcripts increased at 72 h in 2% cultures exposed to light, while transcript levels notably decreased in cultures in which lower glucose concentrations were used.

3.5. Effect of light and glucose on wild-type *A. nidulans* development

In *A. nidulans* the presence or absence of illumination governs the developmental decision towards asexual (light) or sexual (dark) differentiation (Yager, 1992). In addition to the effect of light, other factors, such as nutrient presence or abundance, also influence fungal morphogenesis. For example, it has been previously shown that high levels of glucose promote sexual development, while repressing conidiation (Han et al., 1990, 2001; Jeong et al., 2000). We investigated the possible integrative effect of illumination and glucose concentration in the model fungus *A. nidulans*.

As expected conidiation was higher at 48 h in the light than in the dark, and increased over time (72 h) at all concentrations tested, particularly when low glucose concentrations were used (Fig. 4C and D). At those same time points Hülle cells were also counted as an indicator of the initiation of sexual development (Yager, 1992). Hülle cell numbers increased in dark cultures with higher glucose concentration and this increase continued over time. Few Hülle cells were observed in 1.5% and 2% glucose cultures exposed to light (Fig. 4C).

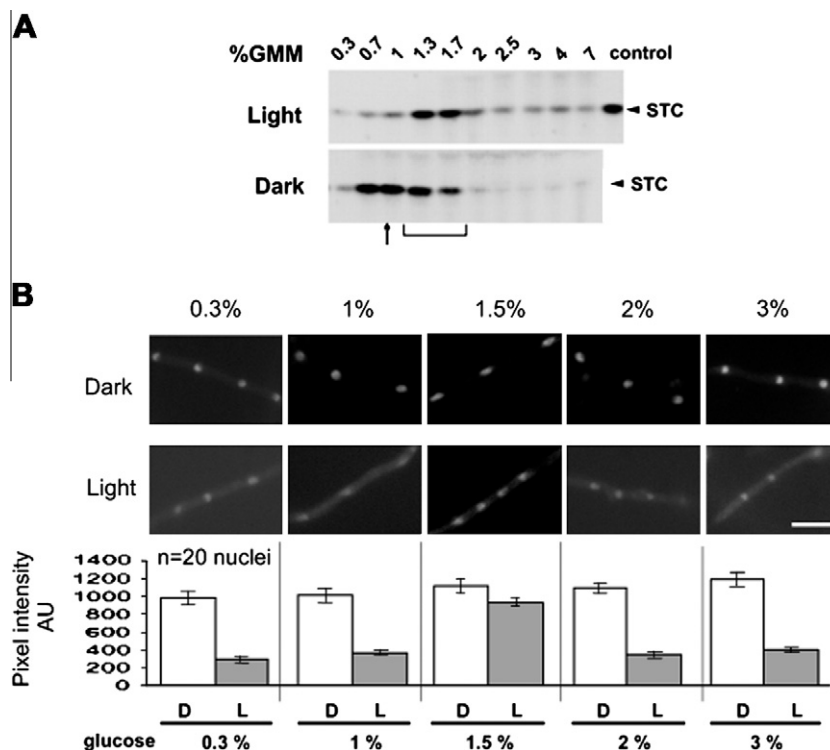


Fig. 2. Dependence of VeA nuclear localization on glucose concentration and its relationship with ST biosynthesis. (A) Thin-layer chromatography analysis of ST produced by the wild-type *A. nidulans* FGSC4 strain on GMM for 72 h with a range of different glucose concentrations from 0.3% to 7%. (B) Images of TRMD3.4.17 grown in minimum medium with glucose concentrations as indicated on the top, and with or without illumination. A quantification of the nuclear fluorescence, in arbitrary units (for 20 nuclei of different hyphae) is shown at the bottom of each condition. For each condition 20 nuclei were measured. The error bars in graphs from panel A indicate standard error. Each experiment was repeated at least three times.

3.6. Role of *A. nidulans* FphA, LreA and LreB in morphogenesis in response to different glucose concentrations under light or dark conditions

In our present study we investigated the role of light-sensing proteins in morphogenesis under light/dark conditions at different glucose concentrations. Previously we showed that the blue-light-sensing proteins, LreA and LreB, have a repressing function on conidiospore production while the red-light sensing phytochrome-like protein, FphA, activates asexual development when growing on 2% glucose GMM (Purschwitz et al., 2008). A decrease in glucose (1%) did not alter the FphA/LreA/LreB-regulatory pattern of conidiation with respect to that previously described at a higher glucose concentration (2%) (Purschwitz et al., 2008, Fig. 5A and Suppl. Fig. 1). However, our current experiments show that de-repressed conidiation in *lreA* and *lreB* deletion mutants was enhanced at lower glucose concentration (1%) in both light and dark growing conditions. Conidiation of strains carrying *fphA* single null or in combination with either *lreA* or *lreB* null alleles or in the triple mutant combination was increased in the dark when 1% instead of 2% glucose was used in the medium (Fig. 5A, Suppl. Fig. 1).

Regarding cleistothelial formation, whether the fungus was growing on 1% or 2% glucose media, FphA repressed the formation of these structures, while LreA and LreB are activators of the sexual cycle (Fig. 5B, Suppl. Fig. 1). However, we observed a clear reduction of cleistothelial production in all strains tested in 1% glucose medium when compared with 2% glucose grown cultures (Fig. 5B). Production of cleistothecia in the *lreA* and *lreB* deletion mutants in the dark was particularly reduced when growing on 1% glucose. The increase in cleistothelial formation on 2% glucose cultures under light was limited in the *lreA* and *lreB* deletion mutants (Fig. 5B).

3.7. FphA, LreA and LreB regulate penicillin production

Our previous work revealed that *A. nidulans* VeA regulates the production of a variety of secondary metabolites, including mycotoxins, such as ST, and antibiotics, such as penicillin (PN) (Kato et al., 2003). Here we investigated whether other VeA-interacting and light-sensing components also participate in the regulation of PN biosynthesis.

Our results in PN-inducing medium (Fig. 6) show that the light sensors FphA, LreA and LreB, play different roles in PN production. While PN biosynthesis was reduced in the *fphA* deletion mutant, *lreA* and *lreB* mutations resulted in a slight increase of PN production. Interestingly, the pattern observed (Fig. 6) was opposite with respect to that observed for ST production (Purschwitz et al., 2008). Double and triple mutants also resulted in a regulatory role opposite to that described for ST production. The PN-inducing medium (as in Brakhage et al., 1992) is opaque; therefore the mycelium was grown in the absence of light. As shown in Fig. 6, the antibacterial activity detected was exclusively due to the presence of PN.

4. Discussion

Metabolic changes, as well as changes in growth and sexual and asexual development, are part of the adaptive response to illumination (Purschwitz et al., 2006; Herrera-Estrella and Horwitz, 2007). Light affects the biosynthesis of certain metabolites, for example the production of carotenoids in *N. crassa* (Rau and Mitzka-Schnabel, 1985) or *Fusarium oxysporum* (Ruiz-Roldan et al., 2008). Light also influences polysaccharide and carbohydrate metabolism, nucleotide and nucleoside metabolism, fatty acid metabolism (Schuster et al., 2007; Tisch and Schmoll, 2010) and

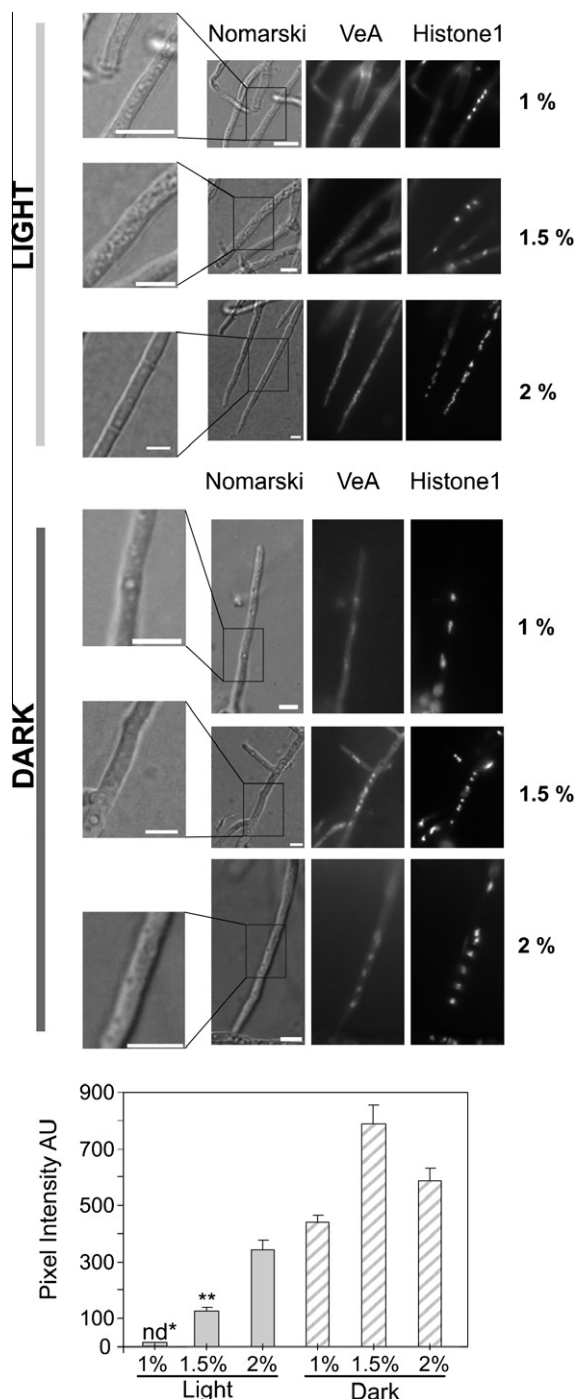


Fig. 3. Localization of VeA in mycelia grown on solid media. Images of hyphae from strain MAD3140 grown for 72 h on supplemented minimal media with the indicated percentage of glucose (on the right) as main carbon source. Localization of VeA::GFP was observed in the dark or in the light under the same conditions as in Fig. 2. Number, position and integrity of nuclei were in these experiments followed by using histone 1 tagged with mCherry as nuclear marker. On the left side, 2× magnifications of hyphae interferential images (Nomarski) show the presence or abundance of vesicles and vacuoles. Bars indicate 5 μ m. The graph bar shows the quantification of nuclear VeA::GFP fluorescence by measuring pixel intensities. Grey and striped bars show measurements in the light and in the dark, respectively. Error bars indicate S.D. nd* and ** indicate that those VeA::GFP fluorescent measurements were taken in cells displaying intense nuclear H1-mCherry fluorescence.

secondary metabolism (Tisch and Schmoll, 2010; Calvo, 2008). We have previously shown that VeA is necessary for the synthesis of numerous natural products (Kato et al., 2003; Calvo et al., 2004;

Duran et al., 2007, 2009; Myung et al., 2009; Calvo, 2008), and that FphA, LreA and LreB also have a role in regulating the production of the mycotoxin ST in *A. nidulans* (Purschwitz et al., 2008). In our present study we demonstrated that the effect of light-sensing proteins in metabolism is broader, also affecting the formation of the antibiotic PN (Fig. 6). Interestingly, the role of FphA, LreA, LreB on PN production is opposite to that described for ST regulation (Purschwitz et al., 2008). In PN-inducing medium, the production of this antibiotic decreased in the *fphA* deletion mutant, indicating that FphA is an activator of PN biosynthesis. On the other hand, *lreA* and *lreB* mutation resulted in a slight increase of PN production. A differential expression of fungal secondary metabolites, specifically ST and PN in *A. nidulans*, was also described for FadA, an alpha-subunit of a heterotrimeric G-protein (Tag et al., 2000). Future research will include the study of light-sensing proteins on the synthesis of additional natural products as well as exploring whether a possible connection with the FadA signaling pathway exists.

Previous studies showed some conflicting findings regarding the effect of light on secondary metabolism. For example, production of aflatoxin in *A. flavus* has been described as being negatively regulated by light (Joffe and Lisker, 1969). While Aziz and Moussa report that aflatoxin production by *A. flavus* is enhanced upon cultivation in light (Aziz and Moussa, 1997). Ochratoxin production in *A. ochraceus* appears to increase in cultures growing in the light (Aziz and Moussa, 1997). However, the photoperiod did not show an effect on ochratoxin production under field conditions (Belli et al., 2006). These differences in the outcomes of light regulation may be explained by different cultivation conditions, including abundance of nutrients. Our previous work indicates that the effect of light on the production of ST toxin in *A. nidulans* is medium-dependent (Kato et al., 2003). While production of ST in GMM was greater in the dark, accumulation of this toxin was greater in light in a rich medium such as YGT. The stimulating effect of light on ST production in rich medium is also supported by other studies (Guzman-de-Pena et al., 1998). One of the most significant findings in our current study is the interaction between the responses to different external stimuli that results in an integrated regulation of secondary metabolism. Specifically we found a relationship between the effect of glucose abundance and light in the control of ST biosynthesis, in which glucose concentration changes the effects of light on ST production (Figs. 1, 2A and 4A). In the dark, it is clear that increasing glucose concentrations exerted a repressive action on ST production, while in the light increasing amounts of glucose induced mycotoxin production.

In order to better understand the effect of glucose on the light regulatory role in ST biosynthesis we investigated whether variations in cAMP play a role in the interaction between these two regulatory systems. cAMP levels vary depending on glucose availability, increasing under glucose starvation (see review by Lafon et al., 2006 and references therein, and Lafon et al., 2005). cAMP is known to activate cAMP-dependent protein kinase A (PKA) (Neves et al., 2002; Pawson and Scott, 2005; Pierce et al., 2002). In addition, light has been shown to cause alterations in the internal cAMP level (Gresik et al., 1988; Farkas et al., 1990). cAMP/PKA signaling is known to govern morphogenesis of a number of fungi (Lee et al., 2003), including *A. nidulans* (Shimizu and Keller, 2001). This signaling pathway also has inhibitory effects on the expression of the *afIR* gene, encoding the ST gene cluster-specific transcription factor, and consequently inhibits ST production by *A. nidulans* (Shimizu and Keller, 2001). Our results suggest that the observed effect of glucose on light-dependent ST biosynthesis regulation was not mediated by cAMP under the experimental conditions tested.

We previously showed that light regulates the subcellular localization of VeA, and therefore, the VeA nuclear protein interactions

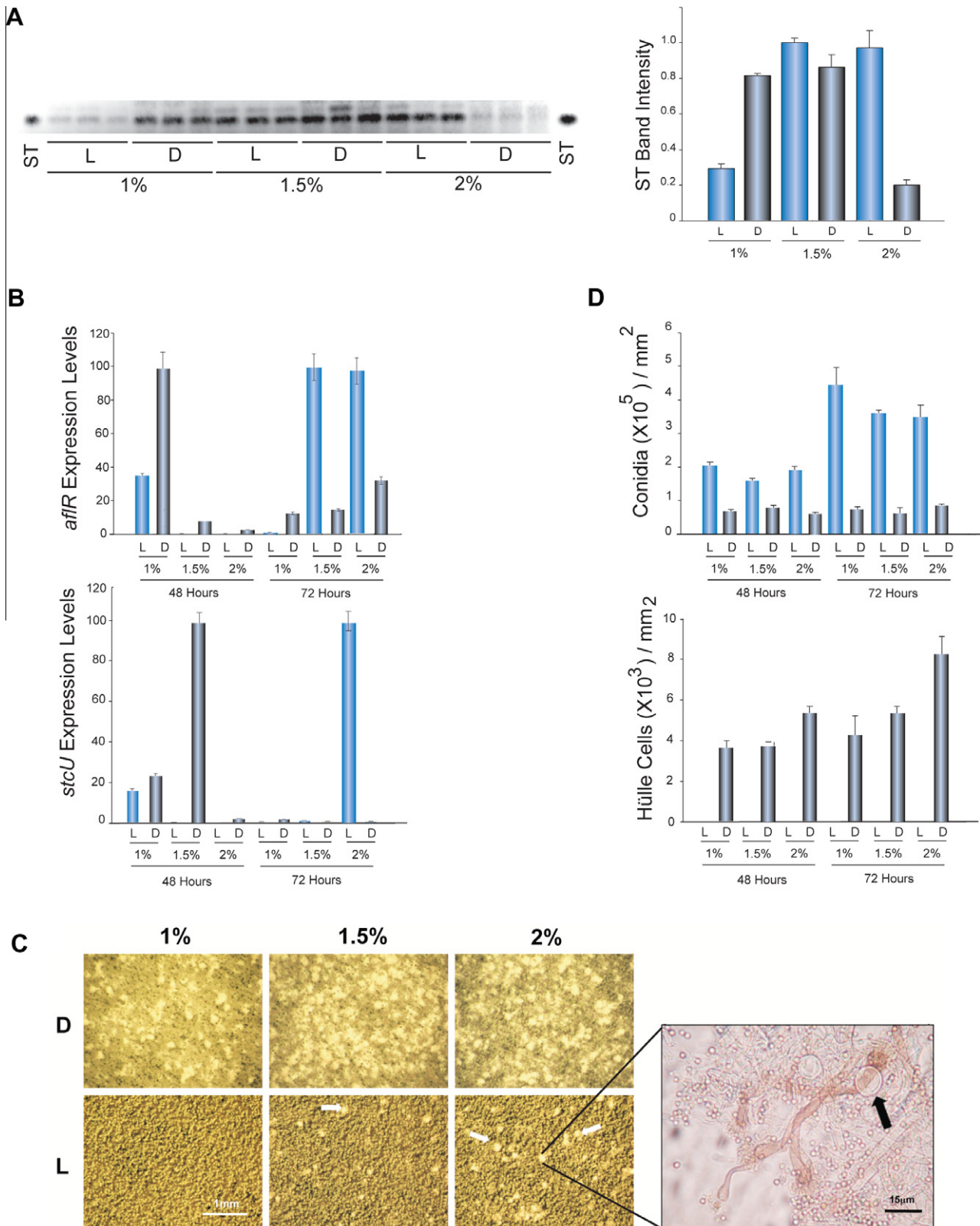


Fig. 4. Effect of light and glucose on wild-type ST gene expression, ST accumulation and morphogenesis. (A) TLC analysis of ST from strain FGSC44 grown for 96 h on supplemented minimal media with the indicated percentage of glucose as main carbon source. On the right, densitometry showing the intensity of the ST bands. The densitometry was carried out using the Scion Image 4.03 software. (B) qRT-PCR results showing expression levels of *aflR* and *stcU* genes. The relative expression levels were calculated using the method described by [Kenneth and Schmittgen \(2001\)](#), and all values were normalized to the expression of the *A. nidulans* tubulin gene. (C and D) Developmental studies performed under the same experimental conditions. In C, micrographs of conidiophores and cleistothelial primordia (white arrows) with a magnification showing Hülle cells (black arrow) surrounding the primordia. In D, quantification of conidia and Hülle cells. The error bars indicate the ranges for three replicates.

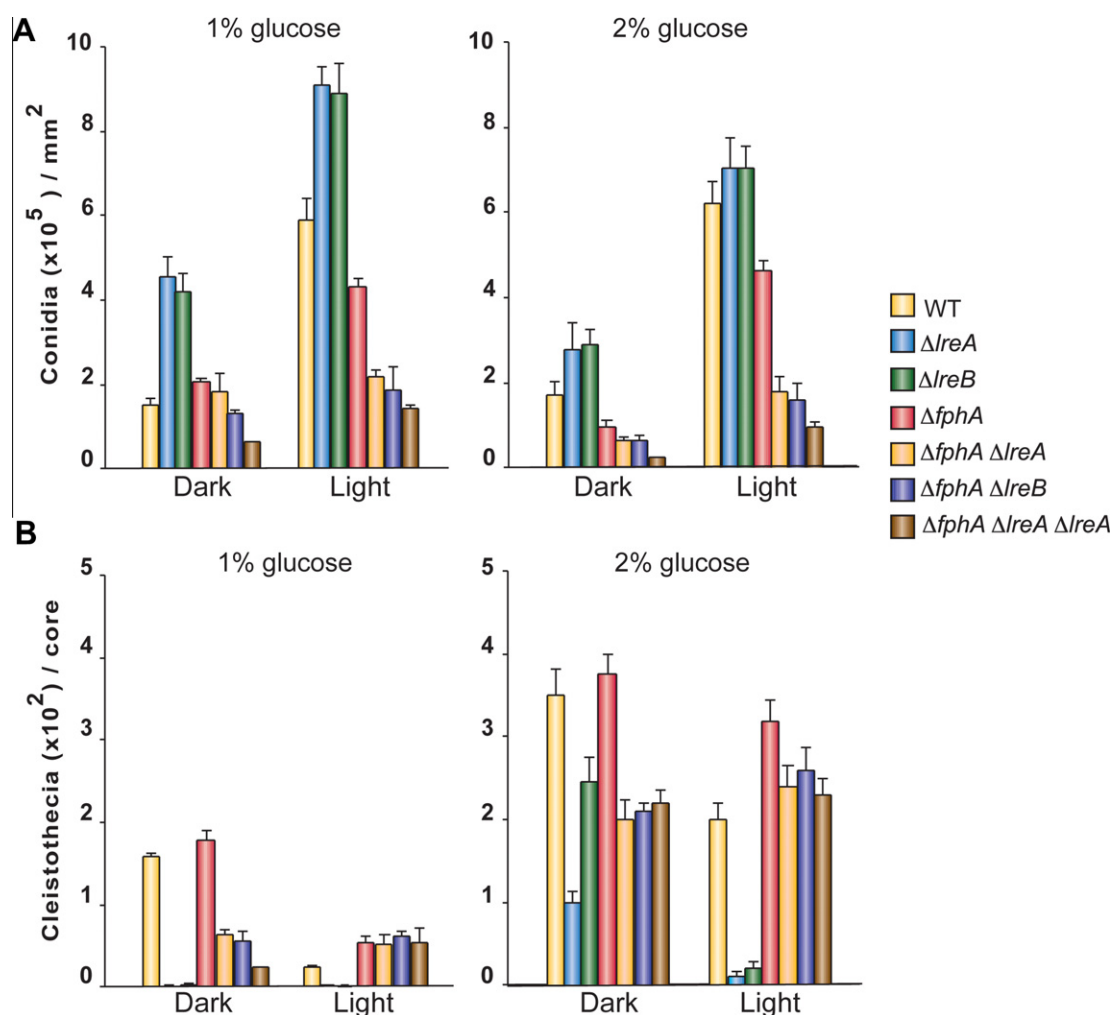


Fig. 5. Role of light-sensing components in the effect of light and glucose on *A. nidulans* morphogenesis. (A) Asexual development in *A. nidulans* FGSC4 (WT), $\Delta fphA$, $\Delta lreA$, $\Delta lreB$ single mutants, $\Delta lreA$, $\Delta fphA$ and $\Delta lreB$, $\Delta fphA$ double mutants and triple mutant growing on 2% or 1% GMM in the dark or under white light for 7 days. (B) Sexual development in the *A. nidulans* strains mentioned above under the same experimental conditions.

that include the light-sensing protein apparatus and other proteins including LaeA, a putative methyl transferase that regulates the expression of secondary metabolism gene clusters, including the ST gene cluster (Bok and Keller, 2004; Bayram et al., 2008; Calvo, 2008). Here we tested whether differences in glucose concentration could affect the VeA subcellular localization pattern determined by light. Essentially VeA accumulates in the nuclei in the dark under all conditions tested, where it has been shown to maintain interactions with photoreceptors and transcription factors (Calvo, 2008; Bayram et al., 2008). A key question here is whether VeA in different glucose conditions may alter its range of possible interactors/effectors. Interestingly, we have observed light independent nuclear accumulation when 1.5% glucose was added. Although on 1% and 1.5% solid medium at 72 h VeA visualization was prevented due to partial cellular damage caused by light, in the dark we also observed higher nuclear accumulation of VeA in cultures with 1.5% glucose. The narrow range of glucose effect on VeA nuclear accumulation might reflect a highly specific regulatory system depending on the carbon source. In this context it is possible that two ways of VeA cellular regulation could exist depending on either activating nuclear transport via the NLS recognized by the importin a KapA or altering VeA protein turn over, since there is no evidence of a nuclear export pathway involved (see Araújo-Bazán et al., 2009). Additional protein–protein interactions with carbon-regulators or effectors might account for the latter,

although experiments done by us and others gave no evidence for such interactions however the conditions used differ from those now analyzed in this work.

Our microscopy studies showed that at low glucose concentration (1%), light had a particularly harmful effect on fungal cells over time. This was evidenced by the loss of nuclear integrity in most of the hyphal compartments and the vacuolated cytoplasm at 72 h after inoculation onto solid medium (Fig. 3). It is possible that light might have caused a more rapid consumption of glucose and that glucose starvation resulted in partial cellular damage. This damage was accompanied by, and might have contributed to, a decrease in the ability of *A. nidulans* to produce ST toxin over time under these conditions. At the same time, under illumination the fungus produces abundant air-born conidia, slightly more at low glucose concentrations, that allow dissemination to a more suitable environment. Higher glucose concentrations (1.5% and 2%) remediate the detrimental effects of light on the cells, allowing more ST accumulation. In addition, our results revealed that while ST gene expression levels were low in 48 h light cultures, these levels increased in older 2% glucose cultures, which could contribute to the accumulation of ST in the 2% glucose light cultures.

In the dark grown cultures, cellular integrity was not compromised at any glucose concentration studied over time, and ST production was abundant at 1%, where ST gene transcription was higher than in light cultures as observed after 48 h of incubation.

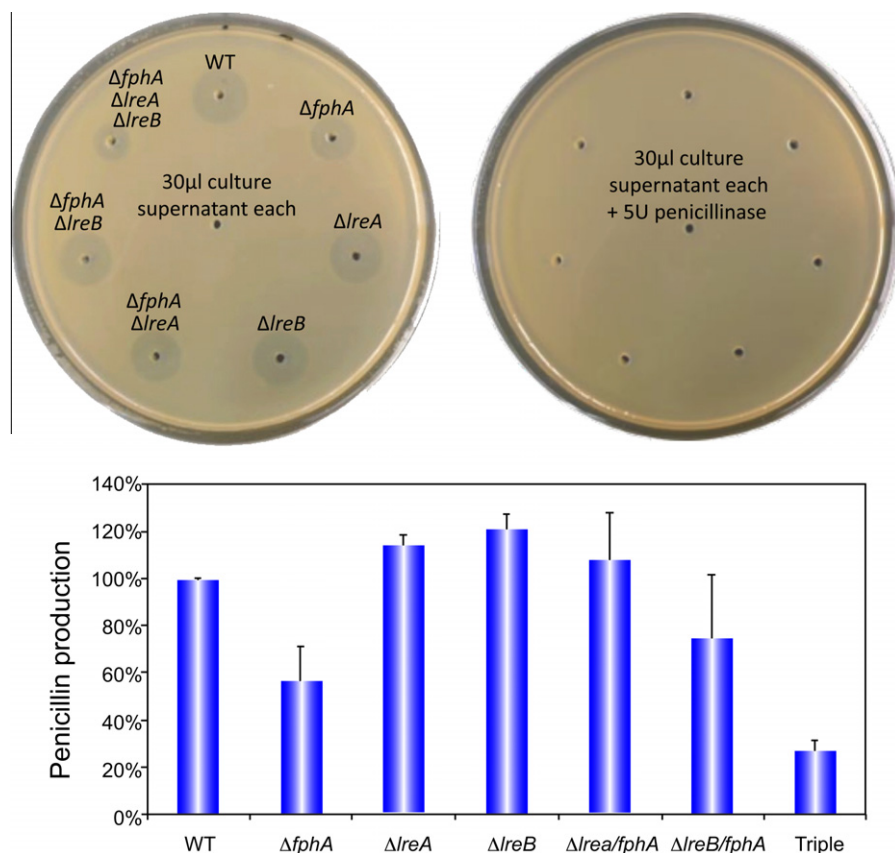


Fig. 6. Penicillin production is regulated by light-sensing proteins. *Bacillus stearothermophilus* was used as testing organism against fungal culture supernatants. Amounts of penicillin are indicated as a percentage with respect to the amount produced by the wild-type strain. WT, wild type. Error bars indicate standard error.

At glucose concentrations greater than 2%, ST levels remain low, particularly in the dark. It is possible that under those conditions ST production could be repressed by carbon-regulators perhaps interacting with VeA, and/or by a VeA independent mechanism. Overall, our results suggest that carbon metabolism conditions the light effect on toxin production.

As expected, in the absence of light the development of cleistothecial primordia was much more extensive than in the light. The increase in Hülle cells occurred more rapidly at higher glucose levels. Fungal morphogenesis is also regulated by VeA. Deletion of the *veA* gene results in a complete blockage in cleistothecial formation and increased conidiation (Kim et al., 2002; Kato et al., 2003; Calvo, 2008), indicating that VeA is indispensable for sexual development while repressing asexual development. FphA represses sexual development and promotes conidiation while LreA and LreB have antagonistic effects with respect to the role of FphA (Purschwitz et al., 2008). In the present study we show that induction of *A. nidulans* conidiation at low glucose concentration is particularly notable in the absence of LreA and LreB, in both light and dark conditions (Fig. 4 and Suppl. Fig. 1). This suggests an additional role for LreA and LreB in negatively modulating the response to a decrease in carbon source availability that leads to conidiation in this fungus. In *Trichoderma atroviridae*, a link between blue-light-sensing proteins BLR1 and BLR2 (also orthologs of *N. crassa* WC-1 and WC-2) and carbon starvation-induced conidiation has been shown (Casas-Flores et al., 2006). However, this was not the case in *N. crassa* (Arpaia et al., 1999; Sokolovsky et al., 1992), indicating that the relationship between the responses to light and carbon source abundance that conditions conidiation could vary in different fungi. With respect to sexual reproduction, while cleistothecial production was

drastically enhanced in most of the strains tested when growing on high glucose medium, that increase was modest in the absence of LreA and LreB in cultures exposed to light, suggesting a possible interaction among light stimulus, LreA/B presence and glucose abundance. Our studies, together with those in *T. atroviridae*, further support a multi-tasking role for WC-1 and WC-2 orthologs in cross-talks with other signaling pathways responsive to external stimuli regulating fungal morphogenesis.

In conclusion, our study revealed that changes in light and carbon source concentration are important environmental cues, and that the sensing of these simultaneous stimuli and the concomitant cellular response does not occur independently but in an interdependent manner and in part mediated by VeA and VeA-interacting proteins, resulting in metabolic and morphological changes that could differ from those individually induced by variations in light or glucose concentration. Indeed, in nature fungi sense and react to a complexity of stimuli occurring simultaneously. Thus, it is predictable that future studies will reveal additional interactions linking diverse and interactive sensing and signaling mechanisms that fungi utilize to reach an optimal adaptive response to a complex environment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fgb.2010.08.007](https://doi.org/10.1016/j.fgb.2010.08.007).

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