Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress

Martina A. Friedl, Monika Schmoll, Christian P. Kubicek and Irina S. Druzhinina

Correspondence

Irina S. Druzhinina
druzhini@mail.zserv.tuwien.ac.at

Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9-1665, A-1060 Vienna, Austria

Light is a fundamental abiotic factor which stimulates growth and development of the majority of living organisms. In soil saprotrophic fungi, light is primarily known to influence morphogenesis, particularly sexual and asexual spore formation. Here we present a new function of light, the enhancement of mycelial growth. The photostimulated mycelial growth of the soil fungus *Hypocrea atroviridis* was detected on 17 (out of 95 tested carbon sources) carbohydrates and polyols, which are metabolically related to cellulose and hemicelluloses, and which are mainly available in the upper soil litter layer. This stimulation depends differently on the function of the two blue light receptor proteins BLR-1 and BLR-2, respectively, BLR-1 being responsible for carbon source selectivity and response to permanent light. Evocation of oxidative stress response in darkness imitates the photostimulation on nine of these carbon sources, and this effect was fully dependent on the function of BLR-1. We conclude that light in combination with the availability of litter-specific carbon sources serves as a signal for the fungus to be above ground, thereby stimulating fast growth in order to produce a maximum of propagules in the shortest time. We further deduce that this process involves oxidative stress response and the two blue light receptor proteins BLR-1 and BLR-2, the former playing the major role.

Received 20 October 2007
Revised 16 December 2007
Accepted 3 January 2008

INTRODUCTION

Light is a fundamental abiotic factor that influences the majority of living organisms. In fungi, light is primarily known to stimulate morphogenetic functions such as phototropism, hyphal branching, spore discharge, reproductive morphogenesis and conidiation (Tan, 1978; Cerdá-Olmedo & Corrochano, 2001; Berrocal-Tito *et al.*, 1999; Nagahashi & Douds, 2004). It also enhances the formation of pigments in order to protect the organisms against the deleterious effects of UV light (Li & Schmidhauser, 1995; Arrach *et al.*, 2001). In *Neurospora crassa*, all light-induced phenotypes are dependent on at least one of the two regulators white-collar-1 (WC-1; Linden, 2002) and white-collar-2 (WC-2; Linden *et al.*, 1997). These two proteins contain a zinc finger domain and a PAS domain through which they interact physically to form the heteromultimeric ‘white-collar complex’, which can then recruit further protein components (Corrochano, 2007). The WC-1 protein also functions as a blue light receptor via its LOV domain

and by its binding of an FAD chromophore (Liu *et al.*, 2003). Idnurm & Heitman (2005) recently demonstrated that the WC-1/WC-2 proteins of *N. crassa* are present in all asco- and basidiomycetous taxa, and represent an evolutionarily ancient and conserved system to control light-dependent processes.

Apart from the aforementioned studies, knowledge on other physiological properties of fungi that are influenced by light is scarce. Some authors have described effects of light on hyphal growth or branching, thereby mostly referring to inhibitory effects (Lauter *et al.*, 1998; Chen & Dickman, 2002; Ambra *et al.*, 2004; Casas-Flores *et al.*, 2004; Flaherty & Dunkle, 2005; Miyake *et al.*, 2005; Idnurm & Heitman, 2005; Brasch & Kay, 2006). For arbuscular mycorrhizal fungi, low-intensity light is also known to induce hyphal branching and the consequent increase of mycelial biomass (Nagahashi & Douds, 2003). This physiological response is explained by the improved probability of colonizing roots of young seedlings and establishing new arbuscular mycorrhizal relations. The same authors later showed the essential increase of hyphal branching of *Gigaspora gigantea* due to synergetic effect of light and root exudate compounds (Nagahashi & Douds, 2004).

Abbreviation: CSUP, carbon source utilization profile.

A supplementary table is available with the online version of this paper.

The photostimulation of the sporulation of soil fungus *Trichoderma* (mitosporic *Hypocrea*, Hypocreales, Ascomycota) has been known for many years. Gressel *et al.* (1971) showed that in *Hypocrea atroviridis* (*Trichoderma viride* in the original publication) the animal neurotransmitter acetylcholine stimulated asexual sporulation as efficiently as light. This fungus (incorrectly named *T. viride* or *Trichoderma harzianum* in many earlier papers) has been used as a simple experimental model to study photoinduced conidiation (for review see Betina & Farkas, 1998). As in other fungi, in *Hypocrea/Trichoderma* this process also depends on the function of WC-1/WC-2 orthologues (*blr-1* and *blr-2*; Casas-Flores *et al.*, 2004). Gresik *et al.* (1989, 1991) demonstrated that the stimulation of conidiation by light in *H. atroviridis* involves the cAMP/protein kinase signalling pathway. A cross-talk between a fungal blue light perception system and the cAMP signalling pathway has indeed recently been demonstrated in *H. atroviridis* (Casas-Flores *et al.*, 2006).

The influence of light on fungal development may not be restricted to sporulation: we have recently observed that in another *Hypocrea/Trichoderma* species – *Hypocrea jecorina* (= *Trichoderma reesei*) – light stimulates growth of the fungus on cellulose (Schmoll *et al.*, 2005). Also, Chovanec *et al.* (2001) reported that light stimulated hyphal growth of *H. atroviridis* on several hexoses as carbon sources. The objective of the present paper was (i) to test these findings with a broad range of carbon sources to arrive at a general picture; (ii) to investigate whether the two blue light receptors BLR-1 and BLR-2 are involved in this phenomenon; and (iii) to obtain insights into the physiological relevance of this finding.

METHODS

Strains. *H. atroviridis* strain IMI 206040 (wild-type; initially deposited as *T. harzianum* IMI 206040, but reidentified by ITS1 and 2 sequence analysis; GenBank accession AF278795) and the two $\Delta blr-1$ and $\Delta blr-2$ mutants derived from it (Casas-Flores *et al.*, 2004) were used throughout this study. All cultures were maintained on plates containing malt extract agar (MEA) and were subcultured monthly.

Tests for growth of *H. atroviridis* on 95 different carbon sources. Druzhinina *et al.* (2006) developed a method using Biolog Phenotype MicroArrays to characterize carbon source utilization profiles (CSUPs) of wild-type and mutant strains of *H. jecorina*. Here we applied this technique to investigate the effect of constant and rhythmic light on CSUPs of *H. atroviridis*. For inoculum preparation, conidia of the parental strain IMI 206040 were obtained by cultivation on 3% MEA plates for 5 days. In the mutant strains $\Delta blr-1$ and $\Delta blr-2$, conidiation was stimulated by the injury of mycelia with a cold sterile scalpel (Casas-Flores *et al.*, 2004). Conidial inocula were prepared by rolling a sterile, wetted cotton swab over sporulating areas of the plates. The conidia were then suspended in sterile Biolog FF inoculating fluid (0.25% Phytagel, 0.03% Tween 40), gently mixed and adjusted to a transmission of 70% at 590 nm (using a Biolog standard turbidimeter calibrated to the Biolog standard for filamentous fungi). Ninety microlitres of the conidial suspension was dispensed into each of the wells of the Biolog FF microplates, which

were incubated at 28 °C under either constant white light (1800 lx), constant darkness or 12 h white light/darkness rhythm. The optical density (OD) at 750 nm (mycelial growth) and 490 nm (mitochondrial activity) was measured after 12, 18, 24, 36, 42, 48, 60, 66, 72, 96 and 168 h using a Biolog microplate reader. Each assay was repeated three to six times in series of independent experiments. Due to a generally slower growth rate of the two *blr* mutants, OD₇₅₀ at 66 h (not 48 h as used by Druzhinina *et al.*, 2006) was chosen as a reference for all three strains. At this time, all strains were in the linear phase of growth on the majority of assimilated carbon sources and the respective value was therefore proportional to the growth rate.

To study the effects of addition of dibutyryl cAMP and menadione, stock solutions in sterile distilled water were prepared separately and added to the corresponding inoculation fluids at a concentration of 1 mM for both.

Data analysis. Data from all experiments were combined in a single matrix, and analysed with the STATISTICA 6.1 (StatSoft) software package. All data were first subjected to descriptive statistical evaluations (mean, minimum, maximum and standard deviation values) and checked for outliers. Secondly, we used cluster analysis to detect possible groupings in our results. The significance of correlations was identified using product-moment or partial correlation algorithms. The significance of a hypothesis was tested by analysis of variance (ANOVA); post-hoc comparisons were done using Tukey's honest significant difference.

RESULTS

Carbon-source-specific photostimulation of mycelial growth

As a basal reference for this study, we characterized the CSUP of *H. atroviridis* IMI 206040 during growth in complete darkness using the algorithm of Biolog Phenotype MicroArrays (PM) as developed by Druzhinina *et al.* (2006). The resultant profile and the respective growth rates essentially resembled those obtained with *H. atroviridis* strain ATCC 74058 ('P1') (Seidl *et al.*, 2006) (ANOVA main effect, $P=0.49$), indicating that the data are representative for this species. The carbon sources were divided by joining cluster analysis into four principal groups that coincided with the shape of the corresponding growth curves. Thus clusters I, II and III contain carbon sources that allow fast, moderate and slow growth, respectively (see supplementary Table S1, available with the online version of this paper), while cluster IV contains carbon sources on which *H. atroviridis* grows very poorly or does not grow at all.

To identify a possible effect of light on growth, we used both constant illumination by intense white light (1800 lx) and alternating cycles of light and darkness (12 h each). We also verified that short exposure to light (<1 min), which was unavoidable during the reading of the microplates, did not reveal any significant effect (data not shown). Temperature was kept constant at 28 °C and was unaffected by these treatments.

The results presented in Fig. 1 show the comparisons of growth rates in complete darkness, under alternating cycles of light and darkness (12 h each) and under constant

illumination. Values shown reflect growth rates and were calculated from the difference in OD₇₅₀ between 24 and 66 h of incubation, where a linear increase in OD₇₅₀ took place on the majority of carbon sources (absolute OD₇₅₀ values for 66 h of growth on individual carbon sources are given in supplementary Table S1). The average growth rate of *H. atroviridis* IMI 206040 was enhanced in the presence of light (ANOVA, $P=0.005$ for rhythmic light and $P=0.002$ for constant illumination). The Tukey HSD post-hoc analysis showed that this was due to a significant stimulation of growth on 17 of the 95 carbon sources, i.e. the hexoses D-fructose, D-mannose (Fig. 1b), D-galactose and α -D-glucose; the pentoses D-xylose (Fig. 1c) and D-ribose; the polyols D-arabinitol and D-mannitol; the α -linked homoglucosides/glucans maltotriose, glycogen and D-trehalose; the α -1,3-glucosyl fructoside turanose; and the β -linked disaccharides D-cellobiose, sucrose, lactulose, gentiobiose and α -D-lactose (ANOVA main effect, $P<0.05$). Comparing these carbon sources with their position in the four clusters (I–IV) shows that most of them belonged to those which are well utilized by *H. atroviridis*; however, the fungus did not show a positive response towards light on all carbon sources of clusters I and II, indicating that fast growth is not a determinant of this effect. For example, growth on glycerol, the best carbon source for this fungus, was independent of light (Fig. 1d, ANOVA, $P>0.05$). Similarly, growth on N-acetyl-D-glucosamine, γ -aminobutyric acid (Fig. 1e), erythritol, and β -methyl-D-glucoside (all from cluster I), or on maltose, quinic acid and L-arabinose (all from cluster II) was not influenced by light. Growth on D-sorbitol was even significantly reduced by illumination (ANOVA main effect, $P=0.003$).

Exposure to constant white light basically yielded the same results as rhythmic illumination (Fig. 1a). However, we noted that the constant presence of light also stimulated growth on a small set of carbon sources (glycogen, maltotriose, turanose, dextrin) (factorial ANOVA, $P<0.05$), which did not show this effect under rhythmic illumination.

Light stimulation of mycelial growth requires functional *blr-1* and *blr-2* genes

Having demonstrated a photostimulation of *H. atroviridis* growth on selected carbohydrates and related carbon sources, we tested whether the blue light receptors BLR-1 and BLR-2 are involved in this response. We first studied the CSUPs of the two knockout mutants $\Delta blr-1$ and $\Delta blr-2$ of *H. atroviridis* IMI 206040. With the exception that both mutants grew slightly slower than IMI 206040 on all carbon sources (see supplementary Table S1), the CSUPs in darkness were essentially the same for both mutants ($r=0.9917$, $P<0.05$, product-moment correlation coefficient), and also very similar to those of the parent strain ($r=0.9140$ for $\Delta blr-1$ and $r=0.9058$ for $\Delta blr-2$). In order to investigate whether the *blr* mutants lack the photostimulation of growth, we plotted the growth rates of the parent strain and the two mutants in darkness and alternating illumination, respectively, against each other (Fig. 2a). In

this plot, photostimulation is evident if the respective data points are clearly above the 45° straight line. If the mean value of the observed ratio between rhythmic illumination and darkness is calculated for the two mutant strains, they are not sensitive to light, but in contrast are inhibited by it, and this is even stronger in $\Delta blr-2$ (Fig. 2a; ANOVA, main effect, $P=0.76$). However, a detailed inspection of the data revealed that the lack of stimulation by light is not complete, and different exceptions are noted for the two mutants. Rhythmic light clearly enhanced the growth rate of $\Delta blr-1$ on some carbon sources while the effect was not observed under constant illumination (Fig. 2b). Interestingly, photostimulation in $\Delta blr-1$ was also detected on N-acetyl-D-glucosamine, maltose, i-erythritol, L-proline and L-ornithine, on which the parent strain showed no photostimulation, indicating a different response of this mutant to light. A different pattern was observed for the $\Delta blr-2$ mutant, which did not differentiate between rhythmic light and darkness but was sensitive to the effect of constant illumination (Fig. 2c). Moreover, the effect was detected mainly on those carbon sources on which the parental strain had the strongest response to light: D-mannose, sucrose, D-ribose, D-trehalose, α -D-glucose, D-cellobiose and lactulose. Exceptions to this rule were i-erythritol and D-raffinose, on which growth of the mutant strain $\Delta blr-2$ but not of the parent strain was slightly stimulated by constant light. This finding suggests that BLR-1 is responsible for carbon source selectivity, whereas the intensity of this response requires both BLR-1 and BLR-2.

Addition of cAMP cannot rescue the light response in the $\Delta blr-1$ and $\Delta blr-2$ mutants

Farkas & Betina (1997) found that illumination results in a rise in intracellular cAMP levels in *H. atroviridis*, and Casas-Flores *et al.* (2006) have recently identified a cross-talk between light response and cAMP. We therefore investigated whether the reduced photosensitivity of $\Delta blr-1$ and $\Delta blr-2$ mutants could be rescued by an artificial increase in the intracellular pool of cAMP. Unfortunately, mutants overproducing adenylate cyclase or with a constitutively activated protein kinase display pleiotropic phenotypes (Saudohar *et al.*, 2002; Zhao *et al.*, 2006), and were thus not considered useful for investigation by Phenotype MicroArrays. We therefore chose to manipulate the internal cAMP pool of the wild-type strain and *blr* mutants by addition of the analogue dibutyl-cAMP which is able to penetrate the hyphae (Terenzi *et al.*, 1976) and which is also taken up by *H. atroviridis* (Reithner *et al.*, 2005; Casas-Flores *et al.*, 2006). The results (Fig. 3) show that the addition of cAMP to cultures growing in darkness does not influence the growth rate of the parent strain in general (ANOVA main effect, $P=0.09$). However, on four of the carbon sources – which all belonged to the group for which we previously detected an increased growth in the presence of light (i.e. α -D-glucose, gentiobiose, D-cellobiose and D-xylose, ANOVA main effect, $P=0.0004$, $P=0.002$,

$P=0.0000$, $P=0.0035$, respectively) – stimulation was indeed observed (Fig. 3b, c, d). This effect did not occur in the $\Delta blr-1$ and $\Delta blr-2$ mutants (ANOVA, $P>0.05$; data not plotted, but shown in supplementary Table S1), indicating that the involvement of cAMP in light stimulation of growth on these carbon sources is dependent on the function of BLR-1 and BLR-2.

Conditions of oxidative stress mimic photostimulation of growth in *H. atroviridis* IMI 206040 but not in the $\Delta blr-1$ and $\Delta blr-2$ mutants

Light is known to cause an oxidative stress response in fungi (see e.g. Iigusa *et al.*, 2005). In order to test whether the observed photostimulation of *H. atroviridis* may be mimicked by exposure to conditions of oxidative stress, we decided to expose the cultures to menadione, a superoxide generator (Pocsi *et al.*, 2005). Fig. 4(a) shows a comparison of growth rates under rhythmic light and in complete darkness with menadione added. In general, *H. atroviridis* IMI 206040 was able to grow in the presence of menadione on only 16 of the 95 carbon sources, and growth was completely inhibited on the others.

To reveal possible cross-talk(s) between photostimulation and the stimulation by menadione, we applied joining cluster analysis to the growth rates obtained after incubation of IMI 206040 (i) in darkness, (ii) under rhythmic illumination, (iii) in darkness with addition of menadione, and (iv) in constant light with menadione. Nine distinct groups of carbon sources were detected by this analysis (Fig. 4b). The most pronounced menadione stimulation (group I) was observed on dextrin (232 % compared to growth rate in darkness), where photostimulation by constant light was significantly stronger than the effect of rhythmic light (208 and 132 %, respectively; see also Fig. 1a). The second group (II) contained five sugars (D-cellobiose, gentiobiose, D-xylose, D-mannose and α -D-glucose) on which photostimulation by light was fully mimicked by addition of menadione (Fig. 4a, b). Interestingly, four of these carbon sources (i.e. all except D-mannose) were identical to those on which growth in darkness was also stimulated by addition of cAMP (cf. Fig. 3). This may hint at a carbon-source-dependent cross-talk between photostimulation, intracellular level of cAMP and response to oxidative stress. The third group (III) contained 'cluster I' carbon sources, which showed strong photostimulation, but on which menadione inhibited growth rather than stimulating it (i.e. γ -aminobutyric acid, D-trehalose, D-fructose, D-ribose, sucrose, D-galactose, D-mannitol and D-arabitol; Fig. 4b). Groups IV, V and VII are characterized by weak photostimulation and nearly

complete inhibition of growth by menadione. The statistical difference between these groups was only due to different growth rates in darkness. Interestingly, menadione completely blocked growth on glycerol – the best carbon source for *H. atroviridis*. The effect of menadione was the same under illumination and in darkness for all carbon sources (ANOVA, $P=0.55$), indicating that the operation of oxidative stress is not affected by light.

Stimulation of growth by menadione was not detected in the $\Delta blr-1$ and $\Delta blr-2$ mutants. In contrast, growth of both was inhibited by menadione on the same carbon sources as the parental strain, and was unaffected on those on which growth of the parent strain was stimulated by menadione (data not shown). However, addition of menadione completely blocked growth of both mutants on sucrose on which IMI 206040 grew in all conditions tested. When the two mutants were compared with each other, clear differences were noted on D-trehalose, D-fructose, D-arabitol, maltotriose and glycogen, where mutant $\Delta blr-1$ was more strongly inhibited by menadione than mutant $\Delta blr-2$ (Fig. 5). However, on D-cellobiose, gentiobiose and D-galactose this difference did not occur when menadione-added plates were incubated under constant illumination (Fig. 5). Thus, the resistance of both *blr* mutants to conditions of oxidative stress is carbon-source dependent and is notably weaker compared to the wild-type strain. Moreover, the $\Delta blr-2$ mutant exhibited higher menadione resistance than the $\Delta blr-1$ mutant

DISCUSSION

In this paper, we report that photostimulation of growth of the soil fungus *H. atroviridis* is not a universal effect but is restricted to certain available carbon sources. It can be observed on many but not on all carbohydrates and carbohydrate-related compounds tested, thus raising the question as to the benefit for the fungus from this trait.

Ecological aspects of photostimulation

Vegetative growth of fungi has been reported to be influenced by light, both negatively (Casas-Flores *et al.*, 2006; Ambra *et al.*, 2004; Flaherty & Dunkle, 2005; Schmoll *et al.*, 2005) and positively (Lauter *et al.*, 1998; Chen & Dickman, 2002; Chovanec *et al.*, 2001). On a biochemical level, exposure of *Aspergillus ornatus* to a pulse of light led to protein phosphorylation and decreased glucose uptake (Hill, 1976), and in the slime mould *Physarum polycephalum* it stopped glucose consumption, and decreased the

Fig. 1. Effect of light on growth of *H. atroviridis* IMI 206040. (a) Growth rate on 95 carbon sources and water in darkness (squares), constant light (open circles) and an alternating 12 h light/darkness cycle (red circles) calculated as difference between OD₇₅₀ values at 24 and 66 h incubation. Error bars correspond to SD at 66 h, and were calculated from three parallel experiments. The grey shaded area indicates the level of negative control (growth rate on water). (b–d) Growth curves of *H. atroviridis* on some individual carbon sources used in these experiments. Symbols are as in (a).

Fig. 2. Effect of light on growth of *H. atroviridis* $\Delta b l r - 1$ and $\Delta b l r - 2$ mutants. (a) Plot of growth rates (OD₇₅₀ at 66 h; see Methods) of *H. atroviridis* IMI 206040 (circles), mutant strains $\Delta b l r - 1$ (diamonds) and $\Delta b l r - 2$ (triangles) in darkness vs growth rates under alternating cycles of light. Each symbol indicates the value for one individual carbon source. The red, dotted and black lines are trend lines for the three respective strains. The grey area shows the 95% confidence interval in the case of no light stimulation, i.e. values lying over the trend line indicate stimulation by light. (b) and (c) Plots of the growth rate of mutant strain $\Delta b l r - 1$ and mutant strain $\Delta b l r - 2$, respectively, in darkness vs constant illumination (open circles) and vs 12 h light/darkness cycles (red circles). Red and black lines correspond to the fitting function with 95% confidence interval (corresponding dashed lines) for 12 h light/darkness cycles and constant illumination conditions, respectively. Selected carbon sources are named.

Fig. 3. Effect of elevated level of exogenous cAMP on growth of *H. atroviridis* IMI 206040 in comparison to effect of light. Plots of growth rates (OD₇₅₀ at 66 h; see Methods) on 95 carbon sources under alternating cycles (12 h rhythm) of light (full red circles) against growth rates in darkness plus 1 mM dibutyl cAMP (green crosses). The red and green lines indicate the mean correlation between the respective values (darkness vs alternating light, and darkness vs darkness plus cAMP, respectively), and the dashed lines specify the 95 % confidence intervals for these straight lines. (b–d) Examples of full growth curves in darkness (black), under alternating light and darkness (red) and in darkness plus cAMP (green). SD was estimated as explained for Fig. 1.

accumulation of glucans and D-trehalose (Schreckenbach *et al.*, 1981). On the other hand, photoinduction or photostimulation of conidiation has been known for a long time and is well studied in fungi (Sulová & Farkás, 1991; Lauter *et al.*, 1997). Chovanec *et al.* (2001) further noted that photostimulation or photoinhibition of growth and conidiation of *H. atroviridis* is dependent on the carbon sources.

On the basis of the present results, we offer the following speculation as to why these two different physiological processes are both affected by light. Species of *Hypocrea*/*Trichoderma* are soil saprotrophs and/or facultative mycoparasites, which can also be found in the rhizosphere or

become plant symbionts (endophytes). They are frequently detected on the surface of decaying plant debris in a litter layer (Klein & Eveleigh, 1998). Thus, the open air environment of these fungi almost exclusively consists of pre-digested celluloses and hemicelluloses of decaying plant materials (Albersheim *et al.*, 1994). For many soil fungi, aerial conidiation appears to be the most successful strategy for reproduction, dispersal and general survival. Therefore these fungi require a reliable mechanism to sense the most favourable environmental conditions to initiate conidiation. The soil litter layer, the optimal niche for sporulation, is usually a very heterogeneous substratum composed mainly of dead plant materials of various degradation stages and usually essentially mixed by soil

Fig. 4 (continued on facing page). Growth of *H. atroviridis* IMI 206040 under conditions inducing oxidative stress (1 mM menadione). (a) Plot of growth rates (OD_{750} at 66 h; see Methods) in the presence of alternating light and darkness (red circles) vs growth rates in darkness plus addition of 1 mM menadione (blue triangles). The red and blue lines indicate the mean correlation between the respective values (darkness vs alternating light/darkness, and darkness vs darkness plus menadione, respectively), and the dotted lines specify the 95% confidence intervals for these straight lines. Shaded areas I–IX define groups of carbon sources which are characterized by a different pattern of cross-talk between photostimulation and response to oxidative stress, as determined by cluster analysis (see b). (b) Growth rates in darkness (grey bars), under alternating light and darkness (red bars) and in the presence of menadione (blue bars). Carbon sources were ordered in order to fit to the positions in the joining cluster analysis (cladogram on the left).

fauna. This implies that the availability of one or another single nutrient compound alone can not guarantee that the hyphae will reach the most successful area for spore dispersion. Thus, light may serve as a second necessary criterion by which the fungus senses the optimal conditions for sporulation. Therefore, we reason that when at least two abiotic factors (availability of certain carbon sources and illumination) signal the presence of an aerial environment to the fungus, it will increase its rate of growth and branching in order to reach the state of conidiophore production and sporulation in the shortest possible time. This interpretation is also supported by the findings that conidiation and its photostimulation are also carbon-source dependent (Chovanec *et al.*, 2001; M. A. Friedl & I. S. Druzhinina, unpublished data).

A comparison of individual carbon sources for which photostimulation of growth was observed with the potential chemical composition of this 'aerial' niche of *Hypocrea/Trichoderma* shows that they coincide. Growth stimulation was observed on (i) monosaccharides, which

occur as major constituents of the plant polysaccharides (Albersheim *et al.*, 1994); (ii) polyols, which accumulate from the metabolism of the pentoses in hemicelluloses (Seiboth *et al.*, 2003); and (iii) oligosaccharides, which are degraded by enzymes secreted for complete degradation of the aforementioned polysaccharides (e.g. Aro *et al.*, 2005). The assumption that this correlation is not accidental is supported by the fact that the growth rate on other carbon sources, which are well assimilated but usually not easily available in the soil litter layer, is not stimulated by light: a notable example is *N*-acetyl-D-glucosamine, the monomer of chitin. It becomes available for *H. atroviridis* nutrition in the form of the cell wall of other soil fungi, which it can attack as a mycoparasite, or from the exoskeleton of dead arthropoda. Another example is glycerol, which arises from triacylglycerides from various materials but only rarely from dead plant material.

Hence, we conclude that the nature of the carbon sources for which photostimulation of growth was observed reflects the composition of the potential niche for rapid growth of

Fig. 5. Effect of menadione on the growth rates of the two *blr* mutants in darkness and alternating light and darkness. All experiments were done under alternating light and darkness. Grey and white bars correspond to growth rates (OD_{750} at 66 h; see Methods) of $\Delta blr-1$ and $\Delta blr-2$, respectively. Bars surrounded in red indicate treatments with 1 mM menadione. The horizontal bars over the bars for the $\Delta blr-1$ and $\Delta blr-2$ mutants indicate the growth rates of the respective mutant under constant darkness. They were assayed with the same carbon sources as indicated for the graphs. The light grey area indicates the level of growth detected on water (control). GABA, γ -aminobutyric acid.

H. atroviridis, with the aim of accelerating subsequent conidiation.

Role of BLR proteins in photostimulation of growth

The blue light photoreceptors BLR-1 and BLR-2 have previously been shown to regulate photostimulation of conidiation in *H. atroviridis* (Casas-Flores *et al.*, 2004), and it was therefore plausible to expect their involvement also in the photostimulation of hyphal growth. This expectation could indeed be confirmed. However, the responses in the two Δblr mutants were significantly different: the $\Delta blr-1$ mutant strain still showed photostimulation of growth on some carbon sources – even on ones where the parental strain exhibited no response – whereas growth of the $\Delta blr-2$ strain in the presence of rhythmic light was essentially the same as in darkness. These findings imply that the two BLR proteins must have different functions in the sensing of light: assuming that the residual sensing of light by the $\Delta blr-1$ mutant is due to the function of BLR-2, the latter must be involved in determining the intensity of this stimulation. In this context it is noteworthy that the stimulatory effects in the $\Delta blr-2$ strain were only observed in the presence of constant light, and were almost absent in the presence of alternating cycles of light and darkness,

thus suggesting that BLR-1 only senses high, probably deleterious doses of light in the absence of BLR-2. No such finding was obtained with the $\Delta blr-1$ mutant, suggesting that in the wild-type genotype, BLR-1 plays the major role in the photostimulation of growth. This is consistent with the recent demonstration that *blr-2* expression is a limiting factor for photo-perception and photo-transduction (Esquivel-Naranjo & Herrera-Estrella, 2007). We note that this conclusion is in contrast to findings in *N. crassa*, where the BLR-1 orthologue WC-1 has been reported to be the limiting factor for the formation of the WC-1/WC-2 photoreceptor complex (He *et al.*, 2005).

Photostimulation of mycelial growth due to oxidative stress

While the above considerations support a role for the BLR proteins in the photostimulation of hyphal growth, they do not explain the biochemical mechanism responsible for this effect. From our data, we propose that the observed photostimulation is – at least in part – due to oxidative stress. UV light is known to cause the accumulation of reactive oxygen species and lead to oxidative stress in mammals and fungi (Yoshida & Hasunuma, 2004; Iigusa *et al.*, 2005). Even in *N. crassa*, which is protected against UV light by formation of carotenoid pigments (Yoshida &

Hasunuma, 2004; Iigusa *et al.*, 2005), UV light has been demonstrated to exert oxidative stress. Hyaline hyphae of *H. atroviridis* lack such pigments, and we consequently conclude that this fungus must perceive light as a signal for a potentially hazardous environment. Support for such an assumption comes from recent findings with *N. crassa*, where rhythmic activation of the osmotically stress-activated p38-type MAPK by the *N. crassa* circadian clock allows anticipation and preparation for the hyperosmotic stress and desiccation that begin at sunrise (Vitalini *et al.*, 2007). Moreover, the idea is also supported by our recent observation that in another species of *Hypocrea* (*H. jecorina*), the gene encoding the heat-shock factor HSF1 is strongly upregulated by exposure to light (A. Schuster *et al.*, unpublished observations). The heat-shock response mediated by HSF1 is known to cooperate in the response to oxidative stress in fungi (Noventa-Jordao *et al.*, 1999; Hahn *et al.*, 2006; Yamamoto *et al.*, 2007). In further support of our assumption, oxidative stress has been shown to increase the formation of aerial hyphae in *N. crassa* (Michan *et al.*, 2003) and cause increased hyphal extension in arbuscular mycorrhizal fungi (Pawlowska & Charvat, 2004). In view of these and our own findings that the evocation of oxidative stress by addition of menadione in darkness mimics photostimulation of growth of *H. atroviridis* on several carbon sources, we hypothesize that the observed effect of light is at least partly due to an oxidative stress response. If this hypothesis is correct, it also identifies a new role of the BLR proteins: our data show clearly that the two BLR proteins are essential for the response to oxidative stress. The fact that the oxidative stress response is only observed on some carbohydrates and not on others, and also not on non-carbohydrate compounds, suggests that the oxidative stress response is dependent on carbon signalling and/or carbon metabolism. While there is no direct proof for this at the moment, Hong & Carlson (2007) showed that the Snf1 kinase (a central carbon-specific signalling component in fungi, which differentiates between carbohydrate and non-carbohydrate carbon sources; Schuller, 2003), is involved in the oxidative stress response. Also, Magherini *et al.* (2007) identified the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase as a potential signalling component in oxidative stress. While related findings are not yet available for *H. atroviridis*, these data are indicative that oxidative stress has a link to carbohydrate metabolism.

A cross-talk between effect of light, oxidative stress response and carbohydrate assimilation

Addition of cAMP to the cultivation medium in darkness stimulated the growth rate only on a narrow range of those carbon sources where photostimulation was also observed, i.e. α -D-glucose, gentiobiose, D-cellobiose, D-mannose and D-xylose. These carbon sources can all be considered as the main degradation products from cellulose and hemicellulose, and we conclude that the mechanism of photostimulation of their assimilation involves the cAMP/

protein kinase pathway. In agreement with Casas-Flores *et al.* (2006), the phenotype of $\Delta blr-1$ and $\Delta blr-2$ mutants was not rescued by the addition of cAMP. Interestingly, protein kinase A is known to be involved in the fungal response to oxidative stress (Zhao *et al.*, 2006). Casas-Flores *et al.* (2006) suggested that BLR-1/BLR-2 may activate proteins upstream of protein kinase A, which would be consistent with the lack of effect of cAMP addition to the two *blr* mutants. However, growth on most of the carbon sources on which stimulation by light was observed was insensitive to the addition of cAMP. It is possible that this is due to the existence of a cAMP-insensitive response to oxidative stress. Yamamoto *et al.* (2007) reported that while cAMP generally activates gene expression in response to oxidative stress, it also negatively regulates the expression elicited by superoxide radicals such as menadione. This would be consistent with our findings, because the addition of cAMP was done in the dark, and thus in the absence of oxidative stress. In any case, our data imply that the oxidative stress response caused by light may involve different mechanisms on different carbohydrates.

While some of the conclusions drawn above still require the functional characterization of the components of oxidative stress response in *H. atroviridis* for full confirmation, our data nevertheless demonstrate the existence of a cross-talk between light, the oxidative stress response and carbohydrate assimilation. They also document the existence of different roles for the two blue light receptor proteins in this process. Given the fact that a deletion in these two proteins is not lethal (Casas-Flores *et al.*, 2004), together with the observation of their role in hyphal growth in the presence of light and in stress response, and owing to their high conservation throughout the fungi (Idnurm & Heitman, 2005), BLR proteins present themselves as promising candidates for targeted control of plant-pathogenic fungi.

ACKNOWLEDGEMENTS

We thank Dr A. Herrera-Estrella, Irapuato, Mexico, for providing us with the $\Delta blr-1$ and $\Delta blr-2$ mutant strains. This work has been supported by grants from the Austrian Science Foundation (FWF-P17325) to C. P. K.

REFERENCES

- Albersheim, P., An, J., Freshour, G., Fuller, M. S., Guillen, R., Ham, K. S., Hahn, M. G., Huang, J., O'Neill, M. & other authors (1994). Structure and function studies of plant cell wall polysaccharides. *Biochem Soc Trans* **22**, 374–378.
- Ambra, R., Grimaldi, B., Zamboni, S., Filetici, P., Macino, G. & Ballario, P. (2004). Photomorphogenesis in the hypogeous fungus *Tuber borchii*: isolation and characterization of Tbw-1, the homologue of the blue-light photoreceptor of *Neurospora crassa*. *Fungal Genet Biol* **41**, 688–697.
- Aro, N., Pakula, T. & Penttilä, M. (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev* **29**, 719–739.
- Arrach, N., Fernandez-Martin, R., Cerda-Olmedo, E. & Avalos, J. (2001). A single gene for lycopene cyclase, phytoene synthase, and

- regulation of carotene biosynthesis in *Phycomyces*. *Proc Natl Acad Sci U S A* **98**, 1687–1692.
- Berrocal-Tito, G., Sametz-Baron, L., Eichenberg, K., Horwitz, B. A. & Herrera-Estrella, A. (1999).** Rapid blue light regulation of a *Trichoderma harzianum* photolyase gene. *J Biol Chem* **274**, 14288–14294.
- Betina, V. & Farkas, V. (1998).** Sporulation and light-induced development in *Trichoderma*. In *Trichoderma and Gliocladium*, vol. 1, *Basic Biology, Taxonomy and Genetics*, pp. 75–94. Edited by C. P. Kubicek & G. E. Harman. London: Taylor & Francis.
- Brasch, J. & Kay, C. (2006).** Effects of repeated low-dose UVB irradiation on the hyphal growth of *Candida albicans*. *Mycoses* **49**, 1–5.
- Casas-Flores, S., Rios-Momberg, M., Bibbins, M., Ponce-Noyola, P. & Herrera-Estrella, A. (2004).** BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology* **150**, 3561–3569.
- Casas-Flores, S., Rios-Momberg, M., Rosales-Saavedra, T., Martinez-Hernandez, P., Olmedo-Monfil, V. & Herrera-Estrella, A. (2006).** Cross talk between a fungal blue-light perception system and the cyclic AMP signaling pathway. *Eukaryot Cell* **5**, 499–506.
- Cerdá-Olmedo, E. & Corrochano, L. (2001).** Genetics of *Phycomyces* and its responses to light. In *Photomovement*, vol. 1, pp. 589–620. Edited by D. P. Hader & M. Lebert. Amsterdam: Elsevier.
- Chen, C. & Dickman, M. B. (2002).** *Colletotrichum trifolii* TB3 kinase, a COT1 homologue, is light inducible and becomes localized in the nucleus during hyphal elongation. *Eukaryot Cell* **1**, 626–633.
- Chovanec, P., Hudecova, D. & Varecka, L. (2001).** Vegetative growth, aging- and light-induced conidiation of *Trichoderma viride* cultivated on different carbon sources. *Folia Microbiol (Praha)* **46**, 417–422.
- Corrochano, L. M. (2007).** Fungal photoreceptors: sensory molecules for fungal development and behaviour. *Photochem Photobiol Sci* **6**, 725–736.
- Druzhinina, I. S., Schmoll, M., Seiboth, B. & Kubicek, C. P. (2006).** Global carbon utilization profiles of wild-type, mutants and transformant strains of *Hypocrea jecorina*. *Appl Environ Microbiol* **72**, 2126–2133.
- Esquivel-Naranjo, E. U. & Herrera-Estrella, A. (2007).** Enhanced responsiveness and sensitivity to blue light by *blr-2* overexpression in *Trichoderma atroviride*. *Microbiology* **153**, 3909–3922.
- Farkas, V. & Betina, V. (1997).** The intracellular level of ATP during the photoinduced sporulation of *Trichoderma viride*. *Folia Microbiol (Praha)* **22**, 438.
- Flaherty, J. E. & Dunkle, L. D. (2005).** Identification and expression analysis of regulatory genes induced during conidiation in *Exserohilum turcicum*. *Fungal Genet Biol* **42**, 471–481.
- Gresik, M., Kolarova, N. & Farkas, V. (1989).** Light-stimulated phosphorylation of proteins in cell-free extracts from *Trichoderma viride*. *FEBS Lett* **248**, 185–187.
- Gresik, M., Kolarova, N. & Farkas, V. (1991).** Hyperpolarization and intracellular acidification in *Trichoderma viride* as a response to illumination. *J Gen Microbiol* **137**, 2605–2609.
- Gressel, J., Strausbauch, L. & Galun, E. (1971).** Photomimetic effect of acetylcholine on morphogenesis in *Trichoderma*. *Nature* **232**, 648–649.
- Hahn, J. S., Neef, D. W. & Thiele, D. J. (2006).** A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. *Mol Microbiol* **60**, 240–251.
- He, Q., Shu, H., Cheng, P., Chen, S., Wang, L. & Liu, Y. (2005).** Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. *J Biol Chem* **280**, 17526–17532.
- Hill, E. P. (1976).** Effect of light on growth and sporulation of *Aspergillus ornatus*. *J Gen Microbiol* **95**, 39–44.
- Hong, S. P. & Carlson, M. (2007).** Regulation of Snf1 protein kinase in response to environmental stress. *J Biol Chem* **282**, 16838–16845.
- Idnurm, A. & Heitman, J. (2005).** Light controls growth and development via a conserved pathway in the fungal kingdom. *PLoS Biol* **3**, e95.
- Iigusa, H., Yoshida, Y. & Hasunuma, K. (2005).** Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*. *FEBS Lett* **579**, 4012–4016.
- Klein, D. & Eveleigh, D. E. (1998).** Ecology of *Trichoderma*. In *Trichoderma and Gliocladium*, vol. 1, *Basic Biology, Taxonomy and Genetics*, pp. 57–73. Edited by C. P. Kubicek & G. E. Harman. London: Taylor & Francis.
- Lauter, F., Yamashiro, C. T. & Yanofsky, C. (1997).** Light stimulation of conidiation in *Neurospora crassa*: studies with the wild-type strain and mutants *wc-1*, *wc-2* and *acon-2*. *J Photochem Photobiol* **37**, 203–211.
- Lauter, F. R., Marchfelder, U., Russo, V. E. A., Yamashiro, C. T., Yatzkan, E. & Yarden, O. (1998).** Photoregulation of *cot-1*, a kinase-encoding gene involved in hyphal growth in *Neurospora crassa*. *Fungal Genet Biol* **23**, 300–310.
- Li, C. & Schmidhauser, T. J. (1995).** Developmental and photo-regulation of *al-1* and *al-2*, structural genes for two enzymes essential for carotenoid biosynthesis in *Neurospora*. *Dev Biol* **169**, 90–95.
- Linden, H. (2002).** Circadian rhythms. A white-collar protein senses blue light. *Science* **297**, 777–778.
- Linden, H., Ballario, P. & Macino, G. (1997).** Blue light regulation in *Neurospora crassa*. *Fungal Genet Biol* **22**, 141–150.
- Liu, Y., He, Q. & Cheng, P. (2003).** Photoreception in *Neurospora*: a tale of two white collar proteins. *Cell Mol Life Sci* **60**, 2131–2138.
- Magherini, F., Tani, C., Gamberi, T., Caselli, A., Bianchi, L., Bini, L. & Modesti, A. (2007).** Protein expression profiles in *Saccharomyces cerevisiae* during apoptosis induced by H₂O₂. *Proteomics* **7**, 1434–1445.
- Michan, S., Lledias, F. & Hansberg, W. (2003).** Asexual development is increased in *Neurospora crassa cat-3*-null mutant strains. *Eukaryot Cell* **2**, 798–808.
- Miyake, T., Mori, A., Kii, T., Okuno, T., Usui, Y., Sato, F., Sammoto, H., Watanabe, A. & Kariyama, M. (2005).** Light effects on cell development and secondary metabolism in *Monascus*. *J Ind Microbiol Biotechnol* **32**, 103–108.
- Nagahashi, G. & Douds, D. D., Jr (2003).** Action spectrum for the induction of hyphal branches of an arbuscular mycorrhizal fungus: exposure sites versus branching sites. *Mycol Res* **107**, 1075–1082.
- Nagahashi, G. & Douds, D. D., Jr (2004).** Synergism between blue light and root exudate compounds and evidence for a second messenger in the hyphal branching response of *Gigaspora gigantea*. *Mycologia* **96**, 948–954.
- Noventa-Jordao, M. A., Couto, R. M., Goldman, M. H., Aguirre, J., Iyer, S., Caplan, A., Terenzi, H. F. & Goldman, G. H. (1999).** Catalase activity is necessary for heat-shock recovery in *Aspergillus nidulans* germlings. *Microbiology* **145**, 3229–3234.
- Pawlowska, T. E. & Charvat, I. (2004).** Heavy-metal stress and developmental patterns of arbuscular mycorrhizal fungi. *Appl Environ Microbiol* **70**, 6643–6649.
- Pocsi, I., Miskei, M., Karanyi, Z., Emri, T., Ayoubi, P., Pusztahelyi, T., Balla, G. & Prade, R. A. (2005).** Comparison of gene expression signatures of diamide, H₂O₂ and menadione exposed *Aspergillus nidulans* cultures – linking genome-wide transcriptional changes to cellular physiology. *BMC Genomics* **6**, 182.

- Reithner, B., Brunner, K., Schuhmacher, R., Peissl, I., Seidl, V., Krška, R. & Zeilinger, S. (2005). The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genet Biol* **42**, 749–760.
- Saudohar, M., Bencina, M., van de Vondervoort, P. J., Panneman, H., Legisa, M., Visser, J. & Ruijter, G. J. (2002). Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*. *Microbiology* **148**, 2635–2645.
- Schmoll, M., Franchi, L. & Kubicek, C. P. (2005). The *Hypocrea jecorina* PAS/LOV domain protein ENVOY renders cellulase gene expression light-dependent. *Eukaryot Cell* **4**, 1998–2007.
- Schreckenbach, T., Walckhoff, B. & Verfurth, C. (1981). Blue-light receptor in a white mutant of *Physarum polycephalum* mediates inhibition of spherulation and regulation of glucose metabolism. *Proc Natl Acad Sci U S A* **78**, 1009–1013.
- Schuller, H. J. (2003). Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. *Curr Genet* **43**, 139–160.
- Seiboth, B., Hartl, L., Pail, M. & Kubicek, C. P. (2003). D-Xylose metabolism in *Hypocrea jecorina*: loss of the xylitol dehydrogenase step can be partially compensated for by *lad1*-encoded L-arabinitol-4-dehydrogenase. *Eukaryot Cell* **2**, 867–875.
- Seidl, V., Druzhinina, I. S. & Kubicek, C. P. (2006). A global screening for carbon sources stimulating N-acetyl- β -D-glucosaminidase formation in *Trichoderma atroviride*. *Microbiology* **152**, 2003–2012.
- Sulová, Z. & Farkás, V. (1991). Photoinduced conidiation in *Trichoderma viride*: a study with inhibitors. *Folia Microbiol (Praha)* **36**, 267–270.
- Tan, K. K. (1978). Light-induced fungal development. In *The Filamentous Fungi*, vol. 3, pp. 334–357. Edited by J. E. Smith & D. R. Berry. London: Edward Arnold.
- Terenzi, H. F., Flawia, M. M., Tellez-Inon, M. T. & Torres, H. N. (1976). Control of *Neurospora crassa* morphology by cyclic adenosine 3',5'-monophosphate and dibutyryl cyclic adenosine 3',5'-monophosphate. *J Bacteriol* **126**, 91–99.
- Vitalini, M. W., de Paula, R. M., Goldsmith, C. S., Jones, C. A., Borkovich, K. A. & Bell-Pedersen, D. (2007). Circadian rhythmicity mediated by temporal regulation of the activity of p38 MAPK. *Proc Natl Acad Sci U S A* **104**, 18223–18228.
- Yamamoto, A., Ueda, J., Yamamoto, N., Hashikawa, N. & Sakurai, H. (2007). Role of heat shock transcription factor in *Saccharomyces cerevisiae* oxidative stress response. *Eukaryot Cell* **6**, 1373–1379.
- Yoshida, Y. & Hasunuma, K. (2004). Reactive oxygen species affect photomorphogenesis in *Neurospora crassa*. *J Biol Chem* **279**, 6986–6993.
- Zhao, W., Panepinto, J. C., Fortwendel, J. R., Fox, L., Oliver, B. G., Askew, D. S. & Rhodes, J. C. (2006). Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infect Immun* **74**, 4865–4874.

Edited by: N. L. Glass