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BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*

Sergio Casas-Flores,[1,2]† Mauricio Rios-Momberg,[2]† Martha Bibbins,[2] Patricia Ponce-Noyola[1] and Alfredo Herrera-Estrella[2]

[1]Instituto de Investigación en Biología Experimental, Facultad de Química, Universidad de Guanajuato, Apartado postal 187, Guanajuato 36050, Mexico

[2]Departamento de Ingeniería Genética de Plantas, CINVESTAV Unidad Irapuato, Apartado postal 629, Irapuato 36500, Mexico

Correspondence
Alfredo Herrera-Estrella
aherrera@ira.cinvestav.mx

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In fungi, phototropism, the induction of carotenogenesis and reproductive structures, and resetting of the circadian rhythm are controlled by blue light. *Trichoderma atroviride*, a fungus used in biological control, sporulates in a synchronized manner following a brief pulse of blue light. Due to its apparent simplicity, this response was chosen for pursuing photoreceptor isolation. Two genes were cloned, blue-light regulators 1 and 2 (*blr-1* and *blr-2*), similar to the *Neurospora crassa* white-collar 1 and 2, respectively. The BLR-1 protein has all the characteristics of a blue-light photoreceptor, whereas the structure of the deduced BLR-2 protein suggests that it interacts with BLR-1 through PAS domains to form a complex. Disruption of the corresponding genes demonstrated that they are essential for blue-light-induced conidiation. *blr-1* and *blr-2* were also shown to be essential for the light-induced expression of the photolyase-encoding gene (*phr-1*). Mechanical injury of mycelia was found to trigger conidiation of *T. atroviride*, a response not described previously. This response was not altered in the mutants. A novel effect of both red and blue light on mycelial growth was found involving another light receptor, which is compensated by the BLR proteins.

INTRODUCTION

Species of the common soil fungus *Trichoderma* are used for biocontrol of a variety of phytopathogenic fungi (Papavizas, 1985). *Trichoderma atroviride* is used as a photomorphogenetic model due to its ability to conidiate upon exposure to light. In total darkness, *T. atroviride* grows indefinitely as a mycelium provided that nutrients are not limiting. However, nutrient deprivation and light trigger the development of specialized asexual reproductive structures (conidia). A brief pulse of blue light (400–480 nm) given to a radially growing colony in a Petri dish induces synchronous sporulation. A ring of conidiophores bearing green conidia is produced at what had been the colony perimeter at the time of the light pulse (Gressel & Galun, 1967). The first event induced by light is a fast, first-order, photochemical reaction that does not require the presence of molecular oxygen and is independent of temperature. The photoinduction is 'remembered' while the culture is maintained in conditions that do not allow cellular growth (cold or absence of oxygen). When growth is resumed, under optimal conditions, the colony conidiates (Gressel *et al.*, 1975; Horwitz *et al.*, 1984a). According to the Bunsen–Roscoe law of reciprocity, a given quantity of photons could be delivered in pulses of different duration but the final response should be the same. A deviation from this rule indicates the participation of more than one photoreceptor or points to complexities of a single photoreceptor system such as photoreceptor recycling. For *T. atroviride* photoconidiation, reciprocity holds for pulses of blue light lasting from nanoseconds to minutes, indicating that photoconidiation is triggered by a single receptor system that is neither recycled to the photoreceptive form nor counted by enzymic processes during or immediately following irradiation (Horwitz *et al.*, 1990). Upon exposure to blue light, changes in membrane potential and in ATP levels, and a transient biphasic oscillation in intracellular cAMP levels, are observed (Gresik *et al.*, 1988). Exogenous cAMP promotes sporulation in the dark (Berrocal-Tito *et al.*, 2000; Nemcovic & Farkas, 1998), and a pulse of blue light results in the activation of adenylyl cyclase (Kolarova *et al.*, 1992).

A second light response in *T. atroviride* is the regulation of the expression of the photolyase gene *phr-1*. Blue light and

†These authors contributed equally to this work.

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development regulate expression of *phr-1*. No *phr-1* mRNA is detected in the dark, but it becomes detectable immediately after the light pulse (Berrocal-Tito *et al.*, 1999). cAMP bypasses the requirement for light for sporulation, whereas atropine, a compound known to reduce cAMP levels in fungal cells, prevents sporulation even after photoinduction. Light regulation of *phr1*, however, is indifferent to both these effectors (Berrocal-Tito *et al.*, 2000). Induction of photolyase expression behaves as a direct, rapid response to light, independent of the induction of sporulation (Berrocal-Tito *et al.*, 2000). These data suggest either that photoconidiation and light-induced expression of *phr-1* follow divergent signal transduction cascades or that conidiation is triggered indirectly by light, as a secondary response to the exposure to this stimulus.

The shape of the action spectrum of photoconidiation (Gressel & Hartmann, 1968; Kumagai & Oda, 1969), which depicts the relative effectiveness of different wavelengths of light in eliciting the physiological response, is consistent with the absorption spectra of some flavoproteins. Experiments with the riboflavin structural analogue roseoflavin (Horwitz *et al.*, 1984b) also indicate the participation of a flavin as the photoreceptive pigment. Phototropism in higher plants (Thimann & Curry, 1960), photocarotenogenesis in *Neurospora crassa* (De Fabo *et al.*, 1976), and many other biological responses to blue light, have similar action spectra (Senger, 1980). Phototropism in higher plants is mediated by phototropins, serine-threonine protein kinases that undergo light-dependent autophosphorylation. The N-terminal region of these proteins contains two LOV (Light, Oxygen and Voltage) domains that bind FMN to function as light sensors (Christie *et al.*, 1999). The fluorescence excitation spectrum of the recombinant protein, and of isolated LOV domains, corresponds well with the action spectrum for phototropism (Christie *et al.*, 1998). All known responses to blue light in *N. crassa* are initiated by a pair of zinc finger transcription factors encoded by the white-collar genes (*wc-1* and *wc-2*). Additionally, WC-1 and WC-2 are the positive components in a negative feedback loop central to the occurrence of circadian rhythms in *Neurospora* (Crosthwaite *et al.*, 1997). These rhythms are entrained by blue light and WC-1 is the photoreceptor controlling this response. WC-1 possesses a FAD-binding LOV domain that is very similar to those of phototropins and that also functions as a light sensor (Froehlich *et al.*, 2002; He *et al.*, 2002). WC-1 and WC-2 interact through PAS domains to form the functional white collar complex (WCC) that binds to the promoters of light-regulated genes to rapidly activate transcription in response to light (Talora *et al.*, 1999). Similarly, in *T. atroviride phr-1* undergoes fast transcriptional activation in response to light (Berrocal-Tito *et al.*, 1999). The presence of putative WCC-binding boxes in the promoter region of *phr-1* suggested that blue light responses in *T. atroviride* could be under the control of white collar homologues.

Here we demonstrate that disruption of the *T. atroviride* *blr-1* and *blr-2* genes, orthologues of *wc-1* and *wc-2*, completely blocks photoconidiation and prevents the rapid blue-light-induced expression of *phr-1*. We also show for the first time the induction of a developmental process in an asexual fungus by injury, which is not altered in the *blr* mutants. Finally, we describe mycelial growth inhibition caused by both red and blue light, which is compensated by the presence of the BLR proteins.

METHODS

Strains, media and culture conditions. *Trichoderma atroviride* IMI 206040 (formerly *Trichoderma harzianum*) was used as the wild-type strain throughout this study. *Escherichia coli* strains DH5α and TOP10F′ (both from Invitrogen) were used for plasmid DNA transformation. The plasmids used were pBluescript (Stratagene), pCR2.1 (Invitrogen), pCB1004 (Carroll *et al.*, 1994), pBHY70 and pB (carrying a fragment of human 28S rDNA). Plasmids pCB1004 and pBHY70 contain the *E. coli* hygromycin phosphotransferase gene under the control of the *Aspergillus nidulans trpC* promoter. *T. atroviride* cultures were routinely grown at 25 °C on PDA plates (potato dextrose agar, Difco). For isolation of protoplasts, photoconidiation assays and light-induction experiments, cultures were grown in PDYC medium (24 g potato dextrose broth l^{-1}, 2 g yeast extract l^{-1} and 1·2 g casein hydrolysate medium l^{-1}, all from Difco). For protoplast transformation, PDA containing 200 μg hygromycin ml^{-1} was used as selection medium and 1 % agarose, 200 μg hygromycin ml^{-1} soft selection medium was used as overlay.

Southern and Northern blot analysis. Total RNA was extracted from mycelia as previously described (Berrocal-Tito *et al.*, 2000) and used for Northern analysis following standard techniques (Sambrook & Russell, 2001). For Northern blot analysis a 1·35 kb *Eco*RV fragment of *phr-1* was labelled with ^{32}P and used as a probe. A human 28S rDNA probe was used as loading control. DNA from each sample was isolated as described previously (Berrocal-Tito *et al.*, 2000). Southern blot analysis was performed following standard techniques (Sambrook & Russell, 2001). A *Bam*HI/*Pst*I *blr-1* fragment containing the complete gene and a *Hin*dIII *blr-2* fragment containing the complete open reading frame were ^{32}P labelled and used as probes for hybridization.

Isolation of *blr-1* and *blr-2*. The primers used for the amplification of the *T. atroviride* white-collar homologues were as follows. For the *wc-1* homologue, the forward primer fwc1 (5′-GATTGTGCAAATTGCCATACGAGG-3′) and reverse primer rwc1 (5′-CAACCCACAACTGTTGCATAGATC-3′), both identical to segments of the *wc-1* gene, were used. For the *wc-2* homologue, a set of degenerate primers was synthesized from reverse-translated segments of the WC-2 protein sequence, corresponding to the peptides EEYVCTDC for the forward primer (fwc2) and TLCNACGL for the reverse primer (rwc2). The resulting DNA fragments were labelled with a random-priming DNA-labelling system and used as probes to screen a λEMBL3 genomic library of *T. atroviride*. For *blr-1* the gene structure and coding sequence was predicted with the program GENSCAN (Burge & Karlin, 1997). For *blr-2* the coding sequence was determined by cDNA cloning and sequencing. Domain composition analyses for sequences were performed with Pfam (Bateman *et al.*, 2002) with an *E* value of 0·54; detecting the PAC motif in BLR-2 required lowering the sensitivity to $E=8$ (we define a PAS domain as the sum of Pfam-PAS and Pfam-PAC). The LOV domain alignments were performed with CLUSTAL X 1.83 (Thompson *et al.*, 1997).

Generation of *blr* mutants by gene replacement. For the *blr-1* subclone, a 1425 bp *Eco*47III fragment containing the three PAS

domains was replaced by a 1·4 kbp *Hpa*I fragment of the pCB1004 plasmid (Carroll *et al.*, 1994). For the *blr-2* subclone the entire coding sequence was removed by inserting an upstream *Bam*HI/*Pst*I fragment and a downstream *Xho*I fragment into the corresponding restriction sites of plasmid pBHY70, which contains the hygromycin B resistance cassette derived from plasmid pCB1004 in the *Eco*RV site. The constructs were used for transformation of *T. atroviride*. *blr-1* mutants were obtained by biolistic transformation (Lorito *et al.*, 1993) and *blr-2* mutants by PEG-mediated protoplast transformation as previously described (Baek & Kenerley, 1998), except that mycelium was digested using 6 mg Novozyme ml^{-1} (Novo Biolabs). For screening of the gene-replacement events, the DNA of hygromycin-resistant colonies was subjected to PCR using primers with sequences taken from genomic DNA next to *blr-1* and *blr-2* but not present in the constructs used to transform, together with a primer hybridizing to the *hph* gene. Positive colonies were made to sporulate by injury, as described below, and serial dilutions of spore suspensions were plated to obtain monosporic cultures for three cycles.

RT-PCR. Total RNA of wild-type, Δ*blr-1* and Δ*blr-2* strains was obtained and incubated with DNase (Invitrogen) at 37 °C for 1 h. Two 5 μg samples of RNA of each mutant strain and four 5 μg samples of the wild-type strain were taken for standard RT reactions, except for controls without reverse transcriptase. For PCR amplification of the *blr-1* transcript cDNA, the forward primer BLR606 (5′-GGGATGACAGCCGAAC-3′) and the reverse primer BLR569 (5′-TCAGCTCCCGCGTGAC-3′) were used, spanning 1277 bp. Primer BLR569 corresponds to a sequence found in the second PAS domain of the protein that was deleted in the mutant and BLR606 to a sequence found in the 5′ translated portion of the gene that remained intact. For PCR amplification of the *blr-2* transcript cDNA, the forward primer '2316' (5′-CATTGCGGCTGCTAGG-3′) and the reverse primer '2180' (5′-GTCCCTTTCGCCATTC-3′) were used, spanning 629 bp. In this case both primers were designed within the deleted region of the gene. For the amplification of the *slt-2* transcript cDNA, the forward primer 'AHE-G300' (5′-AGACGCATCGTGCCA-3′) and the reverse primer '1738' (5′-TCGGCCTTGCTCGTGG-3′) were used, spanning 450 bp.

Light-effect experiments. Colonies were induced to conidiate as previously described (Berrocal-Tito *et al.*, 1999), by exposure to a standard blue light source consisting of light from cool-white fluorescent tubes filtered through a blue acrylic filter (LEE no. 183; fluence rate 3 μmol m^{-2} s^{-1}). For RNA extractions the colonies were inoculated over a washed cellophane sheet overlaying the solid media. At various times, the mycelia were scraped from the surface of the cellophane under red safelight (0·1 μmol m^{-2} s^{-1}). Samples of mycelia exposed to light or kept in the dark were immediately frozen in liquid nitrogen and used for RNA extraction. For colony growth inhibition experiments the same cool-white fluorescent tubes were used, without filter for white light (fluence rate 13 μmol m^{-2} s^{-1}), with the LEE no. 183 filter for blue light (fluence rate 4·3 μmol m^{-2} s^{-1}) and with a LEE no. 182 filter for red light (fluence rate 3·1 μmol m^{-2} s^{-1}); all incubations were done at 25 °C.

Stress-induced conidiation assays. For injury-induced conidiation, fungal colonies were grown in total darkness on PDA at 25 °C for 72 h, cut in stripes with a scalpel and incubated for an additional 24 h in the dark at 25 °C.

RESULTS

Cloning and characterization of *blr-1* and *blr-2*

The light responses of *T. atroviride* are similar in many ways to those of *N. crassa*, and putative WC-1 and WC-2 binding sequences were identified in the promoter of the *T. atroviride* light-responsive gene, *phr-1*. We therefore hypothesized that proteins homologous to WC-1 and WC-2 of *N. crassa* were involved in the regulation of blue-light responses in *T. atroviride*. Thus, we designed a PCR-based strategy to clone the corresponding genes. Briefly, two sets of primers for the amplification of the zinc finger domains of homologues of the *N. crassa wc-1* (fwc1 and rwc1) and *wc-2* (fwc2 and rwc2) genes were synthesized. The selected regions correspond to stretches highly conserved at the amino acid level among several zinc finger proteins. PCR amplification using *T. atroviride* DNA as template produced a 180 bp fragment with the *wc-1* primers and a 196 bp fragment with the *wc-2* primers. Sequencing and analysis of the PCR products indicated a high degree of similarity to the *N. crassa* white-collar genes. Screening of a *T. atroviride* genomic library with the fragments resulted in the identification of three identical clones for the *wc-1* homologue and one for the *wc-2* homologue. A 5·0 kb *Bam*HI/*Pst*I phage DNA fragment hybridizing with the probe for the *wc-1* homologue was subcloned into pUC18 and completely sequenced. A 6·0 kb *Sal*I phage DNA fragment hybridizing with the probe for the *wc-2* homologue was subcloned in pBluescript, and 3·1 kb from the central region of the subclone was sequenced. Analysis of the sequence of the subclones showed a significant degree of similarity to the *N. crassa wc-1* and *wc-2* genes, and indicated that they contained the complete *T. atroviride* genes, which were named blue-light-regulator 1 and 2 (*blr-1* and *blr-2*). In order to determine the copy number of *blr-1* and *blr-2*, Southern blot analysis of *T. atroviride* DNA was performed. For all the enzymes selected the number of bands indicated that *blr-1* and *blr-2* are present in a single copy in the *T. atroviride* genome (data not shown). The predicted proteins encoded by these genes are similar to their *N. crassa* counterparts. BLR-1 has 1020 aa and shows 53 % identity to WC-1, whereas BLR-2 has 484 aa and is 52 % identical to WC-2. The *Trichoderma* proteins have almost the same domain composition as the corresponding *Neurospora* proteins. Some differences are the presence in BLR-1 of a glutamine-rich region at the N-terminus instead of the long polyglutamine tract present in WC-1, and the absence in BLR-1 of the polyglutamine tract present in the carboxy-terminal region of WC-1. Both BLR proteins possess GATA zinc finger DNA-binding domains and BLR-1 has a nuclear localization signal at the same position as WC-1, suggesting that they function as transcription factors. The most distinctive feature of these proteins is the presence of several PAS domains, three in BLR-1 and one in BLR-2. PAS domains have been described as signal transduction domains monitoring the internal energy state of cells (Taylor & Zhulin, 1998), as protein–protein interaction domains, and also as sensors of environmental signals (Taylor & Zhulin, 1999). The first (N-terminal) PAS domain of BLR-1 belongs to the subgroup called LOV domains. Specifically, it belongs to the family of LOV domains that bind a flavin chromophore and sense blue light (Cheng *et al.*, 2003; Crosson *et al.*, 2003). The BLR-1 LOV domain possesses all the

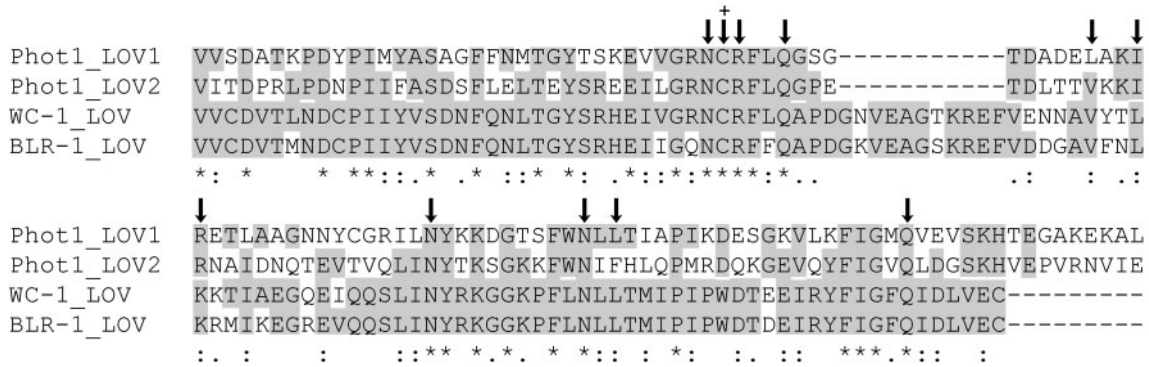

Fig. 1. Amino acid sequence alignment of LOV domains of BLR-1, WC-1 and Phot1 (LOV1 and LOV2). Identically conserved residues are shaded, asterisks mark completely conserved residues, and colons and dots indicate similar residues. The arrows mark residues that are predicted to interact with the flavin and the plus sign indicates the cysteine residue predicted to react with the flavin in response to light.

necessary residues to bind a flavin (Crosson & Moffat, 2001) and the conserved cysteine that participates in the light triggered formation of a flavin-cysteinyl adduct in plant LOV domains (Salomon *et al.*, 2000) (Fig. 1).

blr-1 and *blr-2* are required for light responses of *T. atroviride*

In order to assess the function of the *blr-1* and *blr-2* genes, we replaced them by modified incomplete versions interrupted by a hygromycin B-resistance cassette (Fig. 2a, b). DNA of hygromycin-resistant transformants was used in a PCR-based screening strategy with primers designed to detect the gene replacement events. Gene replacement in the positive candidates was confirmed by Southern blotting. The 3 kb band observed for the *blr-1* gene replacement candidates (Fig. 2c, left panel) and the absence of the two bands observed in the wild-type (1·95 and 1·05 kb, respectively), demonstrate the occurrence of the expected event. The bigger bands observed in the figure indicate that the construction is in the same genomic context as the original *blr-1* gene. The *blr-2* gene was also successfully replaced by the construct, as demonstrated by the presence of a single *Pst*I hybridizing band in the transformants, as compared to the two bands observed for the wild-type (Fig. 2c, right panel). The absence of any additional (unexpected) band proves that none of the selected transformants contains ectopic integrations. In order to further verify that there is no expression of these genes in the transformed strains, due to the possible presence of non-transformed nuclei, RT-PCR reactions were carried out using primers designed to detect the corresponding transcripts. Bands of the expected size were observed only when starting with total RNA of the wild-type strain but not of the transformed strains (Fig. 3a), whereas control RT-PCR reactions carried out using specific oligonucleotides for the constitutively expressed gene *slt-2* from *T. atroviride* showed the expected band for all strains (Fig. 3b).

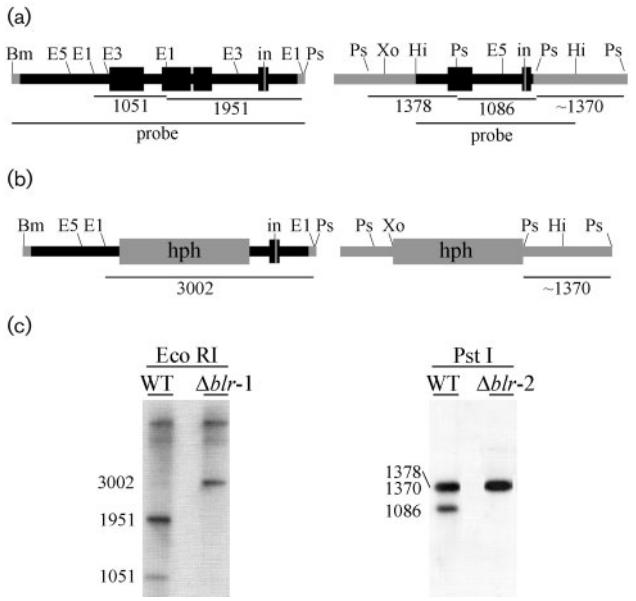

Fig. 2. (a) Schematic representation of the *blr-1* (left) and *blr-2* (right) genes with restriction sites and introns indicated. Bm, *Bam*HI; E1, *Eco*RI; E3, *Eco*47III; E5, *Eco*RV; Ps, *Pst*I; S1, *Sal*I; Xo, *Xho*I; Hi, *Hin*dIII; in, intron. The large boxes indicate PAS domains and the small boxes zinc finger domains. Bars span some restriction fragments of the indicated sizes (bp) and the probes (*blr-1*: *Bam*HI/*Pst*I; *blr-2*: *Hin*dIII) used in the Southern blot analysis shown in (c). The carboxy-terminal *Eco*RI site indicated in *blr-1* corresponds to two close sites giving a small fragment (not detectable) and a large barely detectable fragment (second from top). (b) Schematic representation of the constructs used to replace *blr-1* (left) and *blr-2* (right). The *blr-1* construct is the same size as the original gene. The *blr-2* construct is smaller. Bars span relevant fragments of the indicated sizes (bp). (c) Southern blot analysis of DNA of the wild-type and gene replacement candidate strains digested with the indicated enzymes and hybridized with the probes indicated in (a).

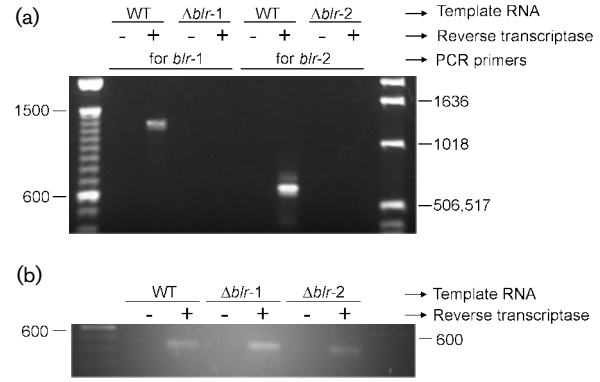

Fig. 3. (a) RT-PCR analysis of RNA from the wild-type and mutant strains, with primers designed to detect the *blr-1* and *blr-2* transcripts indicated in the corresponding lanes. Molecular size markers are 100 bp DNA ladder (left) and 1 kb DNA ladder (right), both from Invitrogen. (b) Control RT-PCR using the same RNA as in (a) and primers for the *T. atroviride slt-2* gene. Molecular size markers are 100 bp DNA ladder.

Several mutants from independent transformation events were used in this work; the figures show two representative strains (Δ*blr-1* and Δ*blr-2*) displaying the typical results obtained. Fig. 4(a) shows the results obtained in photoconidiation assays: neither of the mutants was able to conidiate in response to light, in contrast to the typical response obtained in the wild-type strain. The appearance of light-grown colonies of the mutants was identical to that of the dark-grown wild-type strain. Because the promoter of the *T. atroviride* light-induced photolyase gene, *phr-1*, contains putative regulatory elements similar to those implicated in the regulation of the *al-3* gene by the white-collar complex in *N. crassa* (Berrocal-Tito *et al.*, 1999), we analysed by Northern blotting the expression of *phr-1* after induction with a pulse of blue light. As expected, no detectable levels of *phr-1* mRNA were observed in either the *blr-1* or the *blr-2* deficient strains, whereas a clear *phr-1* induction, reaching its maximum level 30 min after exposure to the light pulse, was observed for the wild-type strain (Fig. 4b).

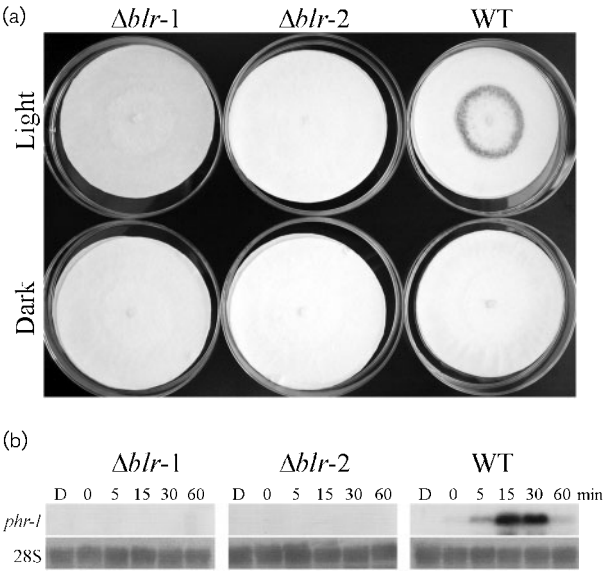

Fig. 4. (a) Photoconidiation assay for the *blr* mutants and wild-type strains. The photographs show dark-grown colonies 24 h after a 5 min blue-light pulse (Light) and colonies treated identically except for the light pulse (Dark). The hyaline mycelium is not visible on the white filter paper. (b) Northern blot analysis of total RNA from *blr* mutants and the wild-type, hybridized with a fragment of the *phr-1* gene. RNA was extracted at the indicated times (min) after a 5 min light pulse. D, dark control.

blr-1 and *blr-2* are not necessary for conidiation triggered by injury

T. atroviride cultures grown in the dark conidiate several days after reaching the border of a Petri dish, presumably in response to nutritional stress. The Δ*blr-1* and Δ*blr-2* strains were also affected in this behaviour, remaining as a mycelium for an indefinite period after filling a dish, suggesting that the BLR proteins could have a general role in conidiation. We tested several other types of stress to determine if they induced conidiation in *T. atroviride*. Among these stresses, injury of the mycelia caused a very particular response, triggering conidiation in the damaged area (Fig. 5). In order to verify if the *blr* mutants are impeded in conidiation, we injured mycelia of these strains. As shown in Fig. 5, injury readily triggered conidiation along the damaged area, as in the wild-type strain, demonstrating that the conidiation pathway is intact in the manipulated strains and that the BLR proteins are not necessary for this response.

blr-1 and *blr-2* control growth in *T. atroviride*

Until now, photoconidiation and the induction of *phr-1* mRNA were the only light responses described in *T. atroviride*. We observed that *T. atroviride* has a slower growth rate under constant illumination relative to dark-grown colonies. Initially, this effect was attributed to the diversion of energetic resources to conidiation, but a proportionally (~20 %) similar light-dependent reduction of the growth rate in the *blr* mutant strains, without conidiation, was observed (Fig. 6a). In addition, as shown in Fig. 6(a), the *blr* mutants have higher growth rates than the wild-type, both under illumination and in the dark, suggesting a light-independent function for the BLR proteins influencing growth rate. To determine the wavelength component of light responsible of the mycelial growth reduction effect, cultures of the wild-type and mutant strains were grown under constant light using blue and red filters. Surprisingly, as there are no known responses to red light in *Trichoderma*, red light caused a marked reduction of the colony diameter of the wild-type strain (Fig. 6b). Interestingly, both types of light significantly

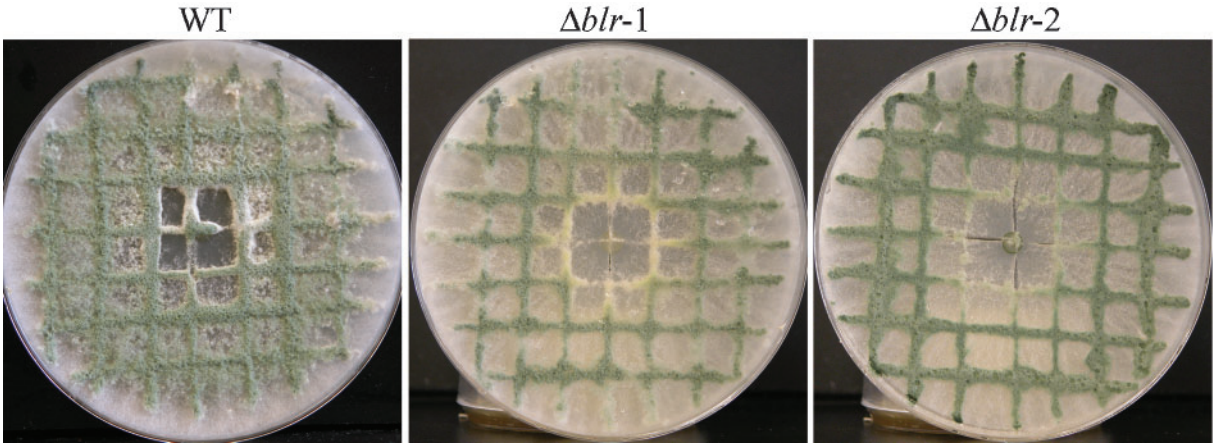

Fig. 5. Conidiation triggered by mechanical injury. The mycelium of the indicated strains was cut in stripes with a scalpel and photographed 24 h later. Normal conidia developed along the cut lines.

reduced mycelial growth in the mutant strains, in a far more pronounced manner than white light (Fig. 6b).

DISCUSSION

We have isolated two genes from *T. atroviride*, *blr-1* and *blr-2*, which are essential mediators of the previously characterized blue/UV-A light responses in this organism. BLR-1 has three PAS domains and one GATA zinc finger DNA-binding domain; BLR-2 has one PAS domain and one GATA zinc finger. In the N-terminal region of the *N. crassa* WC-1 there is a glutamine-rich region that includes a continuous tract of 28 glutamine residues; this region is necessary for the transcriptional activation of a subset of light- and clock-regulated genes (Lee *et al.*, 2003; Toyota *et al.*, 2002). There is also a glutamine-rich region at the C-terminus. In BLR-1 these motifs are not present as such, but rather 10 out of 40 residues at the N-terminus are glutamine, which in principle is enough to function as an activation domain (Mitchell & Tijian, 1989). An alternative is that in *T. atroviride* another protein containing activation domains could be necessary to form an active transcriptional factor complex. The first PAS domain of BLR-1 belongs to a family of LOV domains that bind flavin non-covalently and are specialized in blue/UV-A light reception. The presence of this subtype of LOV domain in a protein required for the light responses of *T. atroviride* is in accordance with the requirement of flavin for photoinduction. Moreover, the mechanism of light perception by LOV domains (Salomon *et al.*, 2001) and the fact that the light-driven formation of a flavin-cysteinyl adduct occurs at temperatures as low as 77 K in plant LOV domains (Iwata *et al.*, 2003) explain the observed independence from temperature of the photoinductive event, and, since the photoproduct is a stable covalent bond, this mechanism could explain the 'memory' of photoinduction reported in *Trichoderma* (Gressel *et al.*, 1975). This effect has also been reported for phototropism in higher plants (Pickard *et al.*, 1969) and for two light responses in *Phycomyces* (Ebrey & Clayton, 1969; Petzuch & Delbruck, 1970). The PAS domain present in the deduced BLR-2 protein is more likely to function in protein–protein interactions.

Northern blots carried out to detect the mRNA of both *blr-1* and *blr-2* showed that they are transcriptionally active and that the corresponding transcripts do not appear to be significantly affected by light (data not shown). As expected, *T. atroviride* mutants in either *blr-1* or *blr-2*, where the gene was replaced by a deleted version, were shown (by RT-PCR, Fig. 3) to lack the corresponding transcript. These mutants were unable to conidiate in response to blue light and no induction of the light-responsive gene *phr-1* could be observed. An interesting observation showing that the conidiation process is not affected in the BLR mutants is the response to injury. The induction by injury of a developmental process in fungi was described in *Schizophyllum commune* for haploid fruiting-body formation (Leonard & Dick, 1973). However, to the best of our knowledge, induction of asexual reproduction by injury has not been previously reported in filamentous fungi.

As mentioned before, PAS domains are implicated in signal transduction in a wide variety of organisms and also in the formation of protein–protein interactions, as between WC-1 and WC-2 (Cheng *et al.*, 2002). By analogy to what occurs in *Neurospora*, the BLR proteins may function as a complex to activate transcription of fast light-regulated genes such as *phr-1*. The DNA-binding domains and the nuclear localization signal in BLR-1, the presence in the *phr-1* promoter of elements similar to those necessary for the WC complex binding to the promoter of *Neurospora* genes, the similar time-course of fast light-regulated genes in both organisms, and the fact that the induction of *phr-1* mRNA is abolished in the *blr* mutants, argue in favour of this possibility. However, a third protein carrying typical activation domains could be necessary to form an active

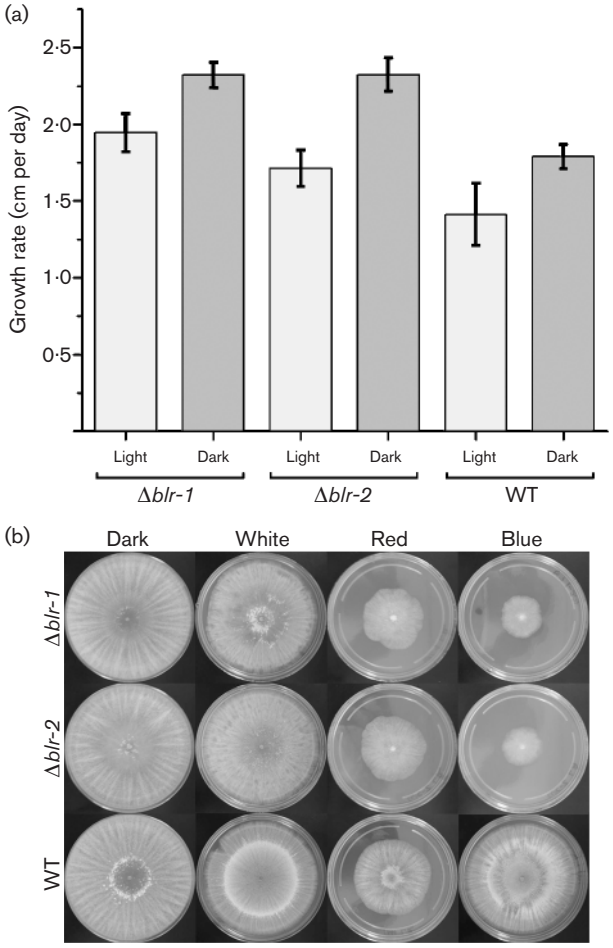

Fig. 6. Effect of light on mycelial growth of *T. atroviride*. (a) Growth rates of *blr* mutants and wild-type colonies grown on PDA either in the dark or under constant illumination with white light, as indicated. Each column represents the mean of radial growth rate; bars indicate the standard deviation for nine independent measurements. (b) The indicated *T. atroviride* strains were grown under constant light of the indicated quality at 25 °C for 5 days and photographed.

complex if the glutamine-rich region in the N-terminus of BLR-1 is not functional.

T. atroviride was chosen as a photobiological model due to the apparent simplicity of its single known response to light. Here we describe another response that must depend on other blue and/or red light receptor(s). The growth rate of the wild-type and *blr* mutant strains is reduced in constant white light relative to dark-grown colonies. The effect of light on growth rate is exacerbated when the colonies are grown in constant blue or red light, suggesting that a balance is attained when both types of light are given in conjunction (white light). Mycelial growth inhibition by blue but not red light has recently been described in the hypogeous fungus *Tuber borchii*. The authors describe a similar effect of blue light in *N. crassa*, and suggest that this response in *N. crassa* depends on the presence of a functional WC-1 protein (Ambra *et al.*, 2004). In contrast, in *Trichoderma* the exacerbated mycelial growth inhibition by exposure to constant blue light can only be observed in the absence of either *blr-1* or *blr-2*. The presence of the BLR proteins in the wild-type strain seems to protect against the exacerbated effect of constant blue but not red light. This apparent contradiction can be explained by the fact that in *T. atroviride* all strains, mutant and wild-type, grow faster in the dark than when exposed to white light and that the *blr* mutants have a higher growth rate, both in the dark and when exposed to light, than the wild-type (see Fig. 6a). In fact, after careful examination of the data reported for *N. crassa* a similar behaviour can be observed; we believe that this phenomenon is what is actually described by Ambra *et al.* (2004), where if only growth under illumination is considered the phenomenon seems to be white-collar 1 dependent. Further support for our interpretation comes from the fact that the data presented by Ambra *et al.* (2004) also show a marked growth inhibition by white light in the *wc-1* mutant, similar to the effect observed in the *T. atroviride blr-1* mutant when using this type of light. Unfortunately, no data are available on the effect of different types of light on mycelial growth inhibition in *Neurospora* and *Tuber borchii* does not appear to respond to red light. Additionally, the conditions to which both fungi were exposed are not comparable and we know of no further photobiological work on the effect of different light intensities in *T. borchii*. Taken together, our data suggest that mycelial growth inhibition at least in *T. atroviride* is mediated by an as-yet-unidentified blue-light receptor. The effect of red light was surprising because these wavelengths were previously thought to have no effect in *Trichoderma*.

A possible explanation for these phenomena is that normal mycelial growth of the *T. atroviride blr* mutants when exposed to constant white light can only be achieved if there is an interaction between the two putative blue and red light receptors or their corresponding signalling pathways. Crosstalk between blue and red light signalling pathways is well documented in plants (Nagy & Schafer, 2002). This would imply that the separate effects of blue and red light are not additive when the fungus is exposed to both types of light but rather that the interaction blocks both detrimental effects as a result of signal integration. An alternative explanation is that yet another receptor perceiving light outside of the blue and red regions of the spectrum, perhaps green light, compensates for the negative effect of blue and red light. Recently, a green-light-absorbing rhodopsin was reported in *N. crassa* (Brown *et al.*, 2001). There is increasing evidence of green light antagonizing the effects of blue and red light in plants, such as stomatal opening and stem elongation (Folta, 2004; Talbott *et al.*, 2002). Normal growth under constant blue light would necessarily require the interaction between the BLR photoreceptor system and that of the novel blue light receptor proposed. This would imply that the BLR proteins are involved in sustaining mycelial growth when mycelium is exposed to constant blue light and it is not competent for sporulation. It is possible that

photoreceptor(s), similar to *cry-1*, *vvd*, *phy-1* or *phy-2*, recently found in the *N. crassa* genome (Borkovich *et al.*, 2004) play a role in the control of mycelial growth.

Blue light is a potent stimulus with the potential to induce harmful photosensitized reactions inside cells; most organisms compensate for this in ways that are not always obvious. Fungi are no exception: *N. crassa* responds by synthesizing screening pigments and the soil fungus *T. atroviride* sporulates. The effect of light on growth rate shown here resembles that of stressful conditions such as nutrient limitation and high temperature. It is thus possible that the BLR proteins have a more general role in dealing with stress. The absence of 'end of plate' conidiation and the faster growth of the mutants point to light-independent functions of these proteins. One such function could be participation in a probable circadian rhythm observed in *Trichoderma*; an approximately 24 h oscillation of light sensitivity for the photoinduced conidiation was reported (Deitzer *et al.*, 1988), and a light pulse results in a shift of the banding pattern of a mutant that sporulates in the dark (Deitzer *et al.*, 1984). BLAST searches against several newly sequenced fungal genomes revealed proteins similar to BLR-1 and BLR-2 in several of them, such as *Fusarium graminearum*, *Podospora anserina*, *Nectria haematococca*, *Magnaporthe grisea*, *Aspergillus nidulans* and *Ustilago maydis*. Thus it appears that BLR-like proteins are conserved fungal regulators possibly involved in coping with blue-light-induced stress and other stressing stimuli, although until now the function of proteins belonging to this family had only been shown for the *N. crassa* WC-1 and WC-2.

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