Enhanced responsiveness and sensitivity to blue light by blr-2 overexpression in *Trichoderma atroviride*

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Light is an environmental factor that regulates pivotal processes in living organisms, and appropriate perception is key to adaptation to the environment. Blue light activates asexual reproduction in *Trichoderma atroviride* through transcription factors BLR-1 and BLR-2 which regulate light-responsive genes. Here, we show that blr-2 expression is a limiting factor for photo-perception and photo-transduction. Overexpression of blr-2 resulted in increased photoconidiation and stronger expression of light-induced genes. In contrast, overexpression of blr-1 resulted in reduced photoconidiation and weaker expression of light-induced genes. blr-2 overexpression caused a marked reduction of growth when the fungus was grown under defined photoperiods, including a period of strong sensitivity to light, followed by a period of insensitivity. Long periods of incubation under this condition permitted recovery of a rhythmic growth similar to that of the wild-type. In addition, blr-2 expression is apparently regulated at the post-transcriptional level through the BLR proteins and its expression level is BLR-1-dependent even in the dark. Finally, we demonstrated that blr-2 overexpression caused higher sensitivity to blue light and we therefore propose that the preformation of BLR-1/BLR-2 complexes is key to adequate light perception in *T. atroviride*.

INTRODUCTION

Sunlight is a key environmental factor that determines the behaviour of living organisms. Thus proper perception of ambient light is essential for the adaptation of an organism to its environment. Among the diverse responses regulated by light, those commonly observed are associated with pivotal processes, such as growth, development, reproduction and metabolism (Linden et al., 1997; Franklin et al., 2005). In particular, the processes of blue-light perception, signal transduction and related responses have been extensively studied in bacteria, algae, plants and fungi.

In fungi, the best-studied responses to light are carotenogenesis, development, morphogenesis, tropism and resetting of the circadian clock (Idnurm et al., 2006; Linden et al., 1997; Liu et al., 2003; Lu et al., 2005; Silva et al., 2006). In *Neurospora crassa*, the proteins white collar 1 and 2 (WC-1 and WC-2) are essential for all its described light responses (Ballario et al., 1996; Linden & Macino, 1997). Analysis of the deduced amino acid sequences of these proteins revealed that they are PAS (Per-Arnt-Sim) domain-containing transcriptional factors with GATA type, zinc finger, DNA-binding domains. The WC-2 protein has only one PAS domain, whereas WC-1 contains three PAS domains (A–C). WC-1 and WC-2 form complexes through the interaction of the PAS-C domain of WC-1 and the PAS domain of WC-2 (Cheng et al., 2002, 2003). These complexes bind to the light-response element (LRE) found in the promoters of light-regulated genes even in the dark (Froehlich et al., 2002; He & Liu, 2005; Káldi et al., 2006). In the WC-1 protein, a specialized PAS domain essential for blue/UVA light perception, called LOV (light, oxygen, voltage), that binds FAD, similar to the first LOV domains described in plant phototropins, has been identified (Froehlich et al., 2002; He et al., 2002). WC-1 acts as a photoreceptor that, in combination with WC-2, tunes light input directly by activating the expression of genes required for the different responses described in *N. crassa* (Froehlich et al., 2002; He et al., 2002). A group of early light-induced genes showing transient expression patterns, typical of adaptive responses, have been identified (Lewis et al., 2002). Among these genes, one encodes a second blue-light photoreceptor, VIVID, necessary for adaptation to constant illumination and to detect changes in light intensity (Schwerdtfeger &
Light perception and subsequent transduction of the signal in fungi, suggesting that they are sufficient for their role in regulating genes that are BLR-dependent strictly require activation and repression of early gene expression, a dual role previously not described in any other fungus (Rosales-Saavedra et al., 2006). Interestingly, the BLR proteins participate in the light-dependent transcriptional activation and repression of early gene expression, a dual role previously not described in any other fungus (Rosales-Saavedra et al., 2006). Additionally, a number of biochemical and molecular data have provided support for the existence of yet another blue-light perception pathway. It appears that activation of both blue-light perception pathways is necessary for the regulation of photoconidiation (Berrocal-Tito et al., 2000; Rocha-Ramirez et al., 2002; Casas-Flores et al., 2006). Furthermore, the identification of light-regulated genes allowed us to identify red-light-regulated genes (Rosales-Saavedra et al., 2006). These data clearly indicate that multiple light perception pathways are functional in T. atroviride, in agreement with the different photoreceptors recently identified in fungal genomes.

To better understand the role of blr-2 in the light perception and transduction pathways of T. atroviride, we analysed its transcriptional regulation and the effect of its overexpression on the light responses of this organism. We found that expression of blr-2 is a limiting factor for light signal transduction and its overexpression leads to exacerbated responses to this stimulus. Furthermore, our results indicate that the expression of blr-2 is induced by light and autoregulated at the post-transcriptional level. Finally, blr-2-overexpression strains showed higher sensitivity to light. In contrast, strains overexpressing blr-1 were less responsive to light than the wild-type (WT). Our results indicate that BLR-2 plays key roles both in the perception and in the transduction of blue light in this fungus.

**METHODS**

**Growth conditions, media and strains.** Trichoderma atroviride WT strain IMI206040 and the corresponding Δblr-1 and Δblr-2 mutants were grown on PDA medium (Difco) at 27 °C. Conidiation of the Δblr-1 and Δblr-2 mutants was induced by mechanical injury to obtain inocula. Escherichia coli strain TOP10F' (Invitrogen) was used for plasmid DNA transformation.

To analyse growth in the dark, in photoperiods of 12 h dark/12 h light and in constant illumination with white light, the strains were grown on PDA in a controlled environment growth chamber operating at 27 °C (constant temperature) with a fluence of 230 μmol m⁻² s⁻¹, provided by cool-white fluorescent tubes. Petri plates (15 cm diameter) containing PDA were inoculated in the centre with plugs of mycelia (0.5 cm diam.) and incubated for 72 h at 27 °C (± 1 °C). The radial growth rate of the strains was determined by measuring colony diameter every 12 h. The experiment was carried out with six replicates for each strain.

**Overexpression of blr genes in T. atroviride.** Plasmid pCB1004, carrying the hygromycin B resistance marker (FSGC), was used as the backbone for the construction of a constitutive expression vector. A 550 bp fragment of the Aspergillus nidulans trpC terminator and a 750 bp fragment containing the promoter of the Trichoderma reesei pki1 gene were cloned by PCR using the primers: TrpC-f (5'-GGTGACCTAGTGATTTAATAGCTCC-3') and TrpC-r (5'-GGTGACCTAGTGATTTAATAGCTCC-3') and Pki1-f (5'-CCGGGGGTCTGAGATCCGGCT-3') for the terminator, and Pki1-f (5'-CCGGGGGTCTGAGATCCGGCT-3') and Pki1-r (5'-CCGGGGGTCTGAGATCCGGCT-3') for the promoter. KpnI (terminator) and SstI (promoter) sites in the primers (underlined) were added to facilitate cloning into plasmid pCB1004. Plasmids pHatA and 68 ends (Casas-Flores et al., 2004) and pZEGA (Mach et al., 1994) were used as templates for the amplification of the trpC terminator (TrpCt) and pki1 promoter (Pki1t), respectively, under the following PCR conditions: one initial cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 45 s, and one final cycle of 68 °C for 7 min. The trpC terminator and pki1 promoter were sequenced and cloned into the KpnI and SstI restriction sites of pCB1004, respectively, obtaining the expression vector pUE08. The correct orientation of the regulatory regions in pUE08 was confirmed by PCR using universal and reverse primers, in combination with the primers described above.

To overexpress the blr genes, the corresponding complete blr-2 cDNA (1.6 kb) containing the UTR-5' and 3' ends (Casas-Flores et al., 2004) was cloned into the EcoRI site of pUE08, and the blr-1 ORF was cloned into the BamHI and HindIII sites of the same plasmid. The correct orientation of the blr-2 cDNA was corroborated by restriction
with different endonucleases. The plasmids were purified using the	he Qiagen plasmid Midi Kit and used to transform \( T. \) \textit{atroviride}
protoplasts using the PEG-CaCl\(_2\) method described by Baek &
Kenerley (1998). All transformants were subjected to three rounds of
monosporic culture. The identification and selection of the
transformants was performed by PCR using primers \( pki1\)-f and
\( trpC\)-r.

**Northern and Southern analysis.** Genomic DNA was isolated
following the procedure described by Raeder & Broda (1985). Total
RNA was isolated according to the protocol described by Jones \textit{et al.}
(1985). Southern and Northern blotting was performed using
Hybond-N+ membranes (Amersham), according to the manufac-
turer’s recommendations. Filters were hybridized with probes labelled
by random priming with [\( ^{32}\text{P} \)]dCTP and processed using standard
procedures (Sambrook \textit{et al.}, 1989).

**Analysis of light responses.** \( T. \) \textit{atroviride} cultures were grown in
the dark for 48 h at 27 °C on PDA plates and used as pre-inoculum.
Mycelial plugs (0.5 cm diam.) were taken from the colony growth
front and placed on the centre of PDA plates with (for RNA
extraction) or without (for photoinhibition analysis) a cellophane
overlay. Cultures were allowed to grow for further 36 h under these
conditions, and then photoinduced as described by Berrocal-Tito \textit{et al.}
(1999).

For analysis of the expression of light-responsive genes after a light
pulse, colonies were exposed to 1200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (unless otherwise
indicated) using a light source consisting of two cool-white
fluorescent tubes filtered with LEE filter #183 (fluence rate
5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and placed back in the dark at 27 °C. Mycelial
samples were subsequently collected at different times after exposure
to light. At the indicated times, mycelia were scraped from the surface
of the cellophane overlaying the PDA medium under low red safe-
light [LEE filter #106 (fluence rate 0.1 \( \mu \text{mol m}^{-2} \text{s}^{-1} \))] and
immediately frozen in liquid nitrogen for RNA extraction.

For photoadaptation analysis, we followed the procedure described
above, except that mycelia were kept under constant illumination.

Sensitivity to blue light was investigated following the procedure
described above, except that colonies were exposed to a different
fluence of blue light as indicated. Colonies were then placed back in
the dark at 27 °C for 15 min, and mycelia were collected and frozen
in liquid nitrogen for RNA extraction.

**Analysis of photoconidiation.** Colonies were incubated for 48 h in
the dark after photoinduction and conidia were collected in 8 ml
distilled water. Quantification of conidia was performed using an
Axiostar Plus microscope (Zeiss) in a Newbauer chamber.

**Analyses of \( blr \) gene expression by real-time PCR.** Primers for
the analysis of \( blr-1 \) (\( blr\)-f 5’-\text{GAATGGCGAGGGGGGCGAGT-3’}
and \( blr\)-r 5’-\text{CGGCGAGGGGCGCGAGT-3’}) and
\( blr-2 \) (\( blr\)-f 5’-\text{GTAAAAGGAGCAGCCCTACCCCTATC-3’}
and \( blr\)-r 5’-\text{GCCACCACCGACCGCAACACC-3’}) gene expression by real-time
PCR were designed. The primers gpd-f (5’-\text{GGCTGCAGGCTGGGCAACGCC-3’}) and
gpd-r (5’-\text{GGCTGCAGGCTGGGCAACGCC-3’}) were designed to use the gpd gene
(encoding glyceralde-
hyde-3-phosphate dehydrogenase) as an internal loading and
normalization control (Puyesky \textit{et al.}, 1997). Twenty micrograms of
total RNA was obtained and, as described above, treated with
amplification-grade RNase-free DNase (Invitrogen) and cleaned
using RNeasy mini kit columns (Qiagen). cDNA synthesis was
performed using 10 \( \mu \text{g} \) RNA, a reverse primer mixture (gpd-r, \( blr\)-r and
\( blr\)-2-r, 20 pmol each) and SuperScript RT III (Invitrogen),
following the manufacturer’s recommendations. After synthesis of
the cDNA, the mixture was treated with 0.2 M NaOH for 15 min at
37 °C to eliminate RNA and then neutralized with 0.7 M HEPES
before purification of the cDNA by using QIAquick Spin columns
(Qiagen). The optimal conditions and specificity for amplification of
the three genes were determined by PCR using a Corbett Research
thermocycler (version 2.2). Standard and relative efficiency curves
were performed using the cDNA sample obtained from the WT strain
grown in the dark to determine the dynamic range and to validate our
reaction conditions for these genes. To this end, five serial dilutions
(1:3) were made starting from 50 ng to 0.61 ng cDNA. The detection
of \( blr-2 \) transcript in amounts below 0.1 ng DNA was inconsistent.
PCR amplifications were performed under the following conditions: 1
cycle of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 64 °C for 30 s
and 72 °C for 35 s, using SYBR Green PCR Master Mix (Applied
Biosystems). Five replicates were made for each experimental
condition and all PCR reactions were performed in a 25 \( \mu \text{l} \) final
volume using a 7500 Real-Time PCR system (Applied Biosystems).
The specificity of amplification was monitored and evaluated by the
 corresponding dissociation curves. The relative quantification of \( blr \)
expression levels was performed with 6 ng cDNA per reaction and
their \( \Delta AC_T \) was determined as a function of the calibrator condition
(growth in the dark) to obtain comparative levels of relative
expression for both \( blr \) genes after treatment with light, using the
\( 2^{-\Delta AC_T} \) method (Livak & Schmittgen, 2001). To compare the
expression levels between \( blr-1 \) and \( blr-2 \), the \( \Delta AC_T \) values were
determined as a function of the \( AC_T \) obtained in the dark for the \( blr-2 \)
gen.

**RESULTS**

**\( blr-2 \) expression is induced by light**

The possible regulation of the \( blr \) genes by light was
analysed by real-time PCR when \textit{Trichoderma} was grown in
the dark and exposed to a saturating pulse of blue light. We
found increased levels of \( blr-2 \) transcripts upon exposure of
\textit{Trichoderma} to the pulse of blue light, reaching its
maximum level (27 % increase) compared to the dark
control 30 min after the pulse. Then, the \( blr-2 \) transcript
levels started to decrease, returning to the levels observed in
the dark by 60 min (Fig. 1). In contrast, the expression of
\( blr-1 \) decreased by 20 % at 60 min after exposure to the
light, coincident with the reduction of \( blr-2 \) expression.
Upon comparison of the expression levels of \( blr-1 \) and \( blr-2 \), it was
evident that the level of \( blr-1 \) transcripts was threefold higher than that
detected for \( blr-2 \) (Fig. 1), suggesting that BLR-2 protein levels could be a limiting
factor in blue light perception in \textit{T. atroviride}. Thus, we
decided to explore the effect of overexpression of \( blr-2 \) on the
blue light response.

**Exacerbated photoconidiation by \( blr-2 \) overexpression**

To test if \( blr-2 \) is a limiting factor for light input, we
constructed the constitutive expression vector pUE08. The
complete cDNA of \( blr-2 \) was cloned into plasmid pUE08.
The resulting construct (pUE08-\textit{OEblr2}) was used for
transformation of \textit{Trichoderma} (Qiagen). The optimal conditions and specificity for amplification of
the three genes were determined by PCR using a Corbett Research
thermocycler (version 2.2). Standard and relative efficiency curves
were performed using the cDNA sample obtained from the WT strain
grown in the dark to determine the dynamic range and to validate our
reaction conditions for these genes. To this end, five serial dilutions
(1:3) were made starting from 50 ng to 0.61 ng cDNA. The detection
of \( blr-2 \) transcript in amounts below 0.1 ng DNA was inconsistent.
PCR amplifications were performed under the following conditions: 1
cycle of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 64 °C for 30 s
and 72 °C for 35 s, using SYBR Green PCR Master Mix (Applied
Biosystems). Five replicates were made for each experimental
condition and all PCR reactions were performed in a 25 \( \mu \text{l} \) final
volume using a 7500 Real-Time PCR system (Applied Biosystems).
The specificity of amplification was monitored and evaluated by the
 corresponding dissociation curves. The relative quantification of \( blr \)
expression levels was performed with 6 ng cDNA per reaction and
their \( \Delta AC_T \) was determined as a function of the calibrator condition
(growth in the dark) to obtain comparative levels of relative
expression for both \( blr \) genes after treatment with light, using the
\( 2^{-\Delta AC_T} \) method (Livak & Schmittgen, 2001). To compare the
expression levels between \( blr-1 \) and \( blr-2 \), the \( \Delta AC_T \) values were
determined as a function of the \( AC_T \) obtained in the dark for the \( blr-2 \)
gen.
named OEblr2 (over-expression of blr-2), and T-9, one of the PCR-negative putative transformants, by Southern blotting. The analysis, carried out using EcoRV-digested DNA, showed the expected banding pattern for the endogenous gene (1.1 and 5.6 kb) in all strains, and the expected 0.6 kb band corresponding to the introduced endogenous gene (1.1 and 5.6 kb) in all strains, and the DNA, showed the expected banding pattern for the same place. Indeed, homologous integration appears to be a highly frequent event in transformants, except T-9 (Fig. 2a, b). The 7.1 kb band can only be explained if all integrations occurred at the same place. Indeed, homologous integration appears to be a highly frequent event in T. atroviride (unpublished data). Densitometric analysis of the hybridizing bands obtained indicated tandem integration of two copies of the vector in transformants OEblr2-1, -2, -3, -4, -6, -8, -12, and -14, and three copies in OEblr2-13. To demonstrate the overexpression of blr-2 in the transformants, we carried out Northern blot analysis. All transformants carrying the construct, as confirmed previously by the Southern blot assays, showed high levels of blr-2 transcripts, whereas in the WT strain and in strain T-9, used as an additional control, no detectable levels of blr-2 transcripts were observed (Fig. 2c).

Analyses of conidiation showed an exacerbated response to blue light in all blr-2-overexpressing strains, showing no signs of conidiation in the dark, indicating that this phenotype is totally dependent on light (Fig. 2d). In contrast, strain T-9 showed a phenotype identical to that of the non-transformed strain, indicating that the transformation process did not affect its capacity to respond to light. The production of conidia increased significantly in the blr-2 overexpressers, with strain OEblr2-13 yielding the highest levels of conidia, producing 11.9-fold more conidia than the WT strain (Fig. 2e). Consistently, this strain was shown to carry three copies of the vector and to express the highest levels of the blr-2 transcript. These results are in agreement with the expression levels found for the blr genes and support our proposal that BLR-2 is a limiting factor in the phototransduction pathway of T. atroviride. Based on the results shown above, we selected the OEblr2-4 and OEblr2-13 strains, which represent all blr-2 overexpressers, for further analyses of light-regulated responses in T. atroviride.

The BLR proteins modulate mycelial growth in the presence of light

Previously, Casas-Flores et al. (2004) described a negative effect of light on mycelial growth of T. atroviride. Thus, we decided to investigate the effect of light on the growth of the blr-2-overexpressing strains compared to the WT strain, the transformation control strain T-9, and the gene replacement mutants Δblr-1 and Δblr-2. Under all conditions of growth analysed, we determined the radial growth every 12 h (radial growth rate). In the dark, the growth rate was similar among all strains, reaching a maximum after 24 h. Moreover, a constant radial growth rate was maintained after 24 h of growth, without further apparent changes. Furthermore, the total radial growth was similar among all the strains (Fig. 3a, d). However, under constant illumination, the pattern of radial growth rate was modified in all the strains when compared to the corresponding dark controls. The Δblr strains reached a maximum growth rate by 24 h, whereas the WT and T-9 strains reached their maximum between 36 and 48 h, and OEblr2-4 and -13 reached it only at 48 h (Fig. 3b). The growth rate of all strains decreased gradually to a minimum by 72 h. The growth pattern of the Δblr and OEblr2 strains showed an early maximum for the gene disruptants and a delayed maximum for the overexpressers, in contrast to the pattern observed for the WT strain. Under precise photoperiods, the growth rate was cyclic for the WT, T-9 and Δblr strains with faster growth rates in the dark and lower rates when exposed to light (Fig. 3c). Interestingly, OEblr2-4 and -13 showed an arrhythmic growth in the first four dark–light periods, developing an altered colony morphology, which recovered to a growth comparable to that of the rest of the strains by 60 h (Fig. 3c, e). Noticeably, the first light period provoked the strongest decrease in radial growth rate and the recovery was also slower in the next dark period. Interestingly, the blr-2-overexpressing strains were apparently insensitive to a second light period as suggested by the constant radial growth rate observed during this period (Fig. 3c). This behaviour can be observed in Fig. 3(e), where the formation of two nearly continuous rings of conidia, corresponding to the first two light periods (central part with dense conidiation), was observed. In subsequent periods the spacing between the conidiation rings became
similar among the different strains. The decrease in total radial growth could have been directly related to the arrhythmic growth provoked by the first two periods of exposure to light. This behaviour could not be associated with the higher photoconidiation of the \textit{blr-2} overexpressers, because the \textit{Δblr} strains, which did not conidiate, had a rhythmic growth similar to that of the WT and T-9 strains (Fig. 3c, e).

**Fig. 2.** Molecular and phenotypic analysis of \textit{blr-2} overexpressers. (a) Schematic representation of vector pUE08-OEblr2 and the \textit{blr-2} genomic locus. The number and position of the \textit{EcoRV} (EV) sites are indicated. (b) Southern analysis. Twenty micrograms of total DNA of the indicated strains was digested with \textit{EcoRV} and hybridized against \textit{\textsuperscript{32}P}-labelled \textit{blr-2} cDNA as probe. Molecular masses were estimated using a 1 kb ladder as marker. (c) Northern analysis. Twenty micrograms of total RNA isolated from mycelia grown in the dark were hybridized with \textit{\textsuperscript{32}P}-labelled \textit{blr-2} cDNA as probe. Hybridization with a 28S rRNA probe was used as a loading control. (d) Photoconidiation. Colonies grown in the dark were exposed to a fluence of 1200 \textit{μmol m}^{-2}, placed back in the dark for 48 h and photographed. (e) Quantification of conidia. The bars indicate the mean conidial yield per plate from three experiments; lines on bars indicate the mean value ± SD and the numbers on the bars denote the increase in conidial yield relative to the WT strain.
Expression of blue-light-regulated genes takes place faster and reaches higher levels in blr-2 overexpressers

We have identified a set of novel light-responsive genes, named blue-light-upregulated (blu) and -downregulated (bld), including subsets of genes whose expression is BLR-dependent and -independent, respectively (Rosales-Saavedra et al., 2006). We analysed the expression of some of these genes in the blr-2-overexpressing and WT strains. The expression of all the light-inducible genes (blu-1, -4, -7, -17 and phr-1) was clearly higher in the OEblr2 strains (Fig. 4a). These genes showed different patterns of expression and could be divided into three well defined groups. In group 1, blu-7 showed rapid expression with a maximum at 15 min, decreasing rapidly by 30 min after the light pulse, thus indicating a strong transient upregulation. In group 2, phr-1 and blu-17 showed an intermediate pattern of expression, reaching a maximum by 15–30 min and returning to basal levels of expression by 60 min. In group 3, blu-1 and blu-4 showed a peak between 30 and 60 min after the light pulse, which decreased by 2 h after the stimulus (Fig. 4a–c). In all cases, the expression was faster and sustained for longer periods of time in the OEblr2-4 and OEblr2-13 strains, compared to the WT (Fig. 4a–c). Interestingly, the expression of blu-4, which was previously considered independent of or partially dependent on the BLR proteins (Rosales-Saavedra et al., 2006), was clearly affected by the overexpression of blr-2 (Fig. 4a).

Repression of bld-2 was apparently similar in both the WT and blr-2-overexpressing strains. However, the dark-related recovery of expression was slower in the OEblr2-4 and OEblr2-13 strains, compared to the WT (Fig. 4a, d). These results provide further evidence of the role of the BLR proteins in the transcriptional activation and repression of blue-light-responsive genes.

The expression of blr-2 is regulated through the BLR complex in both the dark and the light

In N. crassa, the expression of the wc genes is mainly regulated at the transcriptional level by means of the WC
Interestingly, the transcript levels of both situation to the behaviour found in the WT strain (Fig. 5a). The expression of the blr genes (Fig. 4a), indicating that it regulates its own expression pattern found in the blue-light-upregulated genes after a light pulse. (a) Colonies of the WT, OEblr2-4 and -13 strains were grown in the dark for 36 h and samples were collected (D) or further exposed to a pulse of blue light (1200 μmol m⁻²) and collected at the times indicated after the pulse. Total RNA from the samples was isolated and subjected to Northern blot analysis. Blots were sequentially hybridized with ³²P-labelled probes of the indicated genes. Hybridization with gpd was used as loading control. The time-course expression of phr-1 (b), blu-17 (c) and bld-2 (d) was determined by densitometry analysis of scanned autoradiographs; values indicate expression levels relative to those of gpd, which was used as a loading control. WT; OEblr2-4; OEblr2-13.

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proteins. This WC protein dependence has been found both in the dark and after light induction (Ballario et al., 1996; Cheng et al., 2003; Káldi et al., 2006). Thus, we investigated, first, if blr-2 overexpression had any effect on the expression of the blr genes and, second, if they were involved in regulating their own expression. In this regard, we found that blr-2 expression levels in the dark were 12.4-fold higher in the OEblr2-13 strain than in the WT strain, and up to 21.6-fold higher upon light induction (Fig. 5a), thus demonstrating that blr-2-overexpressing strains express higher levels of blr-2 than of blr-1, an inverted situation to the behaviour found in the WT strain (Fig. 5a). Interestingly, the transcript levels of both blr genes increased similarly in response to blue light, showing a 75% increase for both blr-1 and blr-2 in the OEblr2-13 strain, and the transcript level of blr-1 fluctuated over time (Fig. 5a). Furthermore, light-induced accumulation of blr-2 transcripts in the OEblr2-13 strain was faster and stronger, reaching its maximum level 15 min after a light pulse. This change in blr-2 expression is similar to the expression pattern found in the blue-light-upregulated genes (Fig. 4a), indicating that it regulates its own expression (Fig. 5a). Noticeably, the 75% increase in the blr-2 transcript levels upon light induction in the blr-2 overexpresser represents a 9.2-fold increase compared to the level found in the WT strain. Northern blot analysis of a T. atroviride transformant carrying a transcriptional fusion of the gene encoding the green fluorescent protein (gfp) with the pki-1 promoter indicated that there were no significant changes in gfp expression in response to light (data not shown). Furthermore, conidiation and the expression of blu genes induced by light in that strain were similar to those of the WT. Given the fact that the overexpression of blr-2 was achieved using a constitutive promoter, these data suggest autoregulation at the post-transcriptional level. In the dark, the levels of blr-1 expression were not altered in the blr-2 overexpresser (Fig. 5a), directly linking the observed phenotypes to blr-2 overexpression. Additionally, we analysed the expression of blr-2 in the Δblr-1 strain to investigate the dependence of blr-2 expression on BLR-1. We found approximately 3.5-fold lower levels of blr-2 expression in the mutant compared to the expression levels in the WT strain, even in dark (Fig. 5b), indicating that BLR-1 is necessary to maintain the expression of blr-2 in the absence of light. Furthermore, the light induction of the steady-state levels of blr-2 transcripts in the Δblr-1 strain was not observed. Thus, our data suggest that the BLR complex regulates the induction of blr-2 transcripts occasioned by light at the post-transcriptional level.

Expression of blr genes is altered by blr-2 overexpression under constant illumination

Blue-light-regulated genes are subjected to photoadaptation in T. atroviride, as occurs in other organisms (Rosales-Saavedra et al., 2006). Therefore, we analysed the expression of the blr genes under constant illumination. The expression of blr-2 in the WT strain under constant blue light was upregulated by 40% and showed an expression pattern identical to the induction provoked by

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a light pulse, with transcript levels decreasing to those found in the dark (Fig. 6a) after 60 min. However, the expression pattern of blr-2 was modified in the OEblr2-13 strain, losing the typical transitory expression found in the WT strain (compare Fig. 6a and Fig. 6b). Light induction was only detected after 30–60 min of continuous exposure to light, showing a similar increase in transcript levels of 40% in both blr genes, but no decrease was observed in the time frame analysed (Fig. 6b, c). Furthermore, expression of blr-1 under constant illumination was different in both strains. In the WT strain, light had a modest negative effect on blr-1 expression (25% decrease) that apparently coincided with a decrease in blr-2 transcript levels (data not shown), whereas in the OEblr2-13 strain light stimulated blr-1 expression (Fig. 6c). These results indicate altered light regulation of the blr genes under constant illumination occasioned by the overexpression of blr-2.

We analysed the expression of light-regulated genes to determine if the changes in the expression patterns of the blr genes were associated with a general alteration of photoadaptation caused by blr-2 overexpression. Under the same conditions, we found that the expression pattern of light-regulated genes was similar between the WT and OEblr2-13 strains. Although it appeared that the messengers decreased more slowly in OEblr2-13 than in the WT strain, this may be only an effect of the higher levels of transcripts accumulated in this strain (Fig. 6d–f). In addition, repression of the bld-2 gene was also comparable in both strains, even though recovery of the transcript levels observed in the dark was not evident in the time frame analysed here, considering that its expression starts increasing only after 6 h of growth under these conditions (data not shown). These results suggest that blr-2 overexpression does not alter the photoadaptative response of the light-regulated genes of Trichoderma and that a different mechanism regulates the temporary expression of the blr genes under constant illumination.

**A high level of blr-2 expression enhances sensitivity to blue light in T. atroviride**

Based on the possibility that the BLR proteins act as a complex, we rationalized that the formation of such complexes in the dark should be important for appropriate light perception. Therefore, blr-2 overexpression should result in changes in the sensitivity to blue light. In this context, we decided to investigate if the blr-2 overexpressers were altered in their photosensitivity. The WT strain, and the OEblr2-4 and -13 strains were exposed to different fluences of blue light. As shown in Fig. 7(a), both transformants showed a conidiation ring when exposed to 225 μmol blue light m⁻², comparable to the ring observed in the WT strain when exposed to 1200 μmol m⁻² using the same light source. Statistical analysis of the fluence response allowed us to estimate a saturation dose of 2400 and 1200 μmol m⁻² for the WT and OEblr2 strains, respectively (Fig. 7b). The half-saturating doses for photoconidiation were 1859 μmol m⁻² for the WT strain, and 612 and 560 μmol m⁻² for OEblr2-4 and -13, respectively, indicating a greater than threefold higher sensitivity of both transformants than that detected in the WT strain (Fig. 7b).

The difference between the two transformants was statistically not significant, indicating that the BLR-2 levels were saturating, even in the OEblr2-4 strain, which had lower blr-2 expression. Additionally, the yield of conidia was evidently higher in the blr-2-overexpressing strains than that in the WT strain (Fig. 7a, b).
Similar results were obtained for the expression of blue-light-regulated genes. The overexpression of blr-2 caused a higher accumulation of mRNA for the light-responsive genes, at the same light fluence, than that observed in the WT strain (compare OEblr2 and WT in Fig. 7c). Furthermore, blu-1 and phr-1 reached their maximum expression, in the OEblr2 strains, with a fluence of 600 μmol m⁻², whereas in the WT strain their expression was still increasing when 1200 μmol m⁻² was applied. In the case of blu-7 and blu-17, the response was saturated at 300 μmol m⁻² in the OEblr2 strains, whereas saturation in the WT strain was reached using 600 μmol m⁻². The expression of blu-4 did not appear to reach saturation even when exposed to the maximum fluence used in this analysis (Fig. 7c). Taken together, these results show that the higher photosensitivity and early saturation with light are directly associated with the overexpression of blr-2.

**Effect of blr-1 overexpression in photoconidiation and gene expression**

To provide further support to our hypothesis of blr-2 expression being a limiting factor in light signal transduction, we cloned the complete cDNA of blr-1 into plasmid pUE08. The resulting construct (pUE08-OEblr1) was used for transformation of *T. atroviride*. Out of 40 putative transformants analysed by PCR, only eight carried the transformation vector and were confirmed by Southern analysis (data not shown). Northern analysis of the transformants carrying the construct showed, in all cases, high levels of blr-1 transcript even in the dark, whereas in the WT and OEblr2-13 strains no detectable levels of blr-1 transcript could be observed (Fig. 8a). Unexpectedly, overexpression of blr-1 caused a strong decrease in light-induced conidiation compared to the WT and OEblr2-13 strains (Fig. 8b). Additionally, photoconidiation of the blr-1-overexpressing strains induced by different doses of light was always lower than for the WT and OEblr2-13 strains. However, conidiation induced by mycelial injury was similar among all strains (data not shown), indicating a light-specific alteration.

Because of the negative impact of blr-1 overexpression on photoconidiation, we analysed the effect of blr-1 overexpression on light-induced gene expression. Accordingly, analysis of the expression of blue-light-regulated genes after a light pulse in a selected strain overexpressing blr-1...
showed lower levels of *blu-1* and *blu-17* transcripts compared to that observed for the WT and OEblr2-13 strains under the same conditions. The negative effect of *blr-1* overexpression on the regulation of *blu-1* and *blu-17* was clearly different, with *blu-1* showing a much lower level of induction. Downregulation of *bld-2* was less drastic (Fig. 8c). These results suggest that the regulation and levels of the BLR proteins are very important for appropriate induction of gene expression.

**DISCUSSION**

Homologues of the WC proteins have been found in other Ascomycetes, as well as in the Basidiomycetes and Zygomycetes (Ambra *et al.*, 2004; Casas-Flores *et al.*, 2004; Idnurm *et al.*, 2006; Lu *et al.*, 2005; Silva *et al.*, 2006). In *T. atroviride*, *blr-1* and *blr-2* encode essential proteins for photoconidiation and regulation of a set of blue-light-responsive genes (Casas-Flores *et al.*, 2004; Rosales-Saavedra *et al.*, 2006). The expression of these genes is apparently very low, similar to the level found for the *Cryptococcus neoformans* *cwc* genes (Lu *et al.*, 2005). The *blr-2* transcript levels were lower than those detected for *blr-1*. *T. atroviride* strains overexpressing *blr-2* had a higher yield of conidia in response to blue light. Our results are consistent with the hypothesis that *blr-2* expression is a limiting factor in the transduction of the light signal. In *N. crassa*, WC-1 is limiting, compared to WC-2, and its overexpression enhanced the expression levels of blue-light-regulated genes and the rhythmic expression of *frq* (Cheng *et al.*, 2001; Franchi *et al.*, 2005). Consistent with the higher levels of WC-2 compared to WC-1, overexpression of *wc-2* did not cause any obvious photo-sensorial alteration in *N. crassa*.

Exposure of *T. atroviride* to continuous white light results in a reduction in growth, even in *blr* deletion mutants. This effect was intensified when only the blue or red regions of the spectrum were applied (Casas-Flores *et al.*, 2004). Additionally, growth of a *T. reesei* strain with a mutation in *envoy*, encoding a putative secondary light receptor (ENVOY), was strongly affected by white light (Schmoll *et al.*, 2005). In *Tuber borchii* and *N. crassa*, light also affects radial growth during light–dark periods which is partially modulated through WC-1 (Ambra *et al.*, 2004). We did not detect significant differences among the different strains analysed when grown in the dark. However, notable changes were observed during growth under constant illumination and during photoperiods. In all cases, light had a negative effect on growth, being stronger in the *blr-2* overexpressors. Under constant illumination, we found different growth patterns with consistent radial growth rate profiles among the phenotypes analysed (WT, OEblr2 and Δblr), therefore suggesting that the BLR proteins modulate *T. atroviride* growth as proposed by Casas-Flores *et al.* (2004). Under defined photoperiods, we found a rhythmic growth rate in the WT strain and the Δblr mutants. Interestingly, the *blr-2* overexpressors showed a temporary arrhythmic growth in the first period of exposure to light. This growth alteration was slightly stronger in OEblr2-13 than in OEblr2-4, directly correlating the observed phenotype with the level of expression of *blr-2*. Furthermore, there was a clear state of insensitivity in the second light period, suggesting an alteration of
photoadaptation in the blr-2-overexpressing strains. However, we did not find significant changes in the expression pattern of light-regulated genes under continuous light exposure, indicating that various mechanisms may be operating in photoadaptation of *T. atroviride*. Although the *envoy* mutant is defective in growth under constant illumination, and the corresponding gene is clearly light-regulated (Schmoll et al., 2005) through the action of BLR proteins (unpublished data), we did not observe such strong inhibition in the Δblr mutants, indicating that, at least in part, the BLR proteins are negative modulators of mycelial growth and that ENVOY may participate in a negative feedback loop that desensitizes the light input through the BLR complex, as indicated in Fig. 9(a). Furthermore, the fact that this growth disturbance could be observed even in the Δblr strains strongly suggests the participation of an alternate light perception system, which at some point must establish cross-talk with the BLR-dependent system (Fig. 9a). Noticeably, this growth disturbance is temporary and the recovery after the fourth photoperiod suggests the existence of interrelated feedback loops among different light perception systems.

Fig. 8. Molecular and phenotypic analysis of blr-1 overexpressers. (a) Northern blot analysis. Total RNA (20 μg) isolated from mycelia grown in the dark was hybridized with 32P-labelled probe of the blr-1 cDNA. Hybridization with gpd probe was used as loading control. (b) Photoconidiation. Colonies grown in the dark were exposed to 1200 μmol m\(^{-2}\) placed back in the dark for 48 h and photographed. (c) Expression analysis of light-regulated genes for the WT, OEblr1-8 and OEblr2-13 strains after a light pulse as described in the legend to Fig. 4(a).

Fig. 9. Model for light regulation of gene expression through the BLR complex in *T. atroviride*. (a) Light is sensed through the LOV domain of BLR-1, activating the BLR complex, which in turn regulates the expression of light-responsive genes (*blu* and *bld*), through LREs present in their promoter regions and subsequent photoconidiation. The BLR complex regulates blr-2 expression at the transcriptional level in the dark (grey area), and at the post-transcriptional level after light induction. The expression of light-regulated genes is transient under constant illumination, a process that is putatively regulated through a negative feedback loop where ENVOY could be part of that circuit. White light reduces the growth of *T. atroviride* through a hypothetical photoreceptor (HP) that interacts with the light input through BLR proteins. (b) BLR-2 is a limiting factor in light perception in *T. atroviride* (left panel), and its overexpression saturates the blue light perception system through the formation of a BLR complex, leading to the generation of more photoreceptor complexes (right panel).
A set of blue-light-regulated genes whose expression was both dependent and independent of BLR proteins has been identified in T. atroviride (Rosales-Saavedra et al., 2006). All genes analysed showed an earlier and stronger response to a light pulse in OEblr2 strains, compared to the WT strain. These results were consistent with the higher level of photoconidiation observed, directly associated with blr-2 overexpression. Overall, our results clearly indicate that blr-2 expression is a limiting factor in light signal transduction as demonstrated by the lower transcript levels detected compared to those detected for blr-1, as well as the enhanced responsiveness to light. This conclusion was corroborated through blr-1 overexpression. The negative effect caused by blr-1 overexpression was reflected both in the activation and repression of light-regulated genes, and finally in photoconidiation, suggesting a global alteration in the perception and transduction of blue light by the alteration of the balance of the amounts of the BLR proteins. Although these results were unexpected and previously not described in fungi, they suggest that a delicate balance in BLR-1/BLR-2 levels is a major factor for the appropriate sensing and transduction of blue light. An alternative explanation is that overproduction of the blr-1 transcript might trigger a strong negative regulation mechanism, affecting the efficiency of translation of the transcript. The effect of blr-1 overexpression on light signal transduction is certainly interesting and deserves future in-depth analysis.

It is noteworthy that, in addition to its regulation by red light (Rosales-Saavedra et al., 2006), we found that the expression of blu-4 is clearly regulated by blue light through the BLR complex. In agreement with this observation, analysis of the blu-4 promoter region in T. reesei indicated the presence of an LRE (GATTC-N21-CGATT) 725 bp upstream of the putative ATG, as well as other GATA-type elements, as shown for other BLR-dependent light-regulated genes (Rosales-Saavedra et al., 2006). To date, blu-4 is the first gene that represents a point of convergence of the signal transduction cascades involved in the response to two different light inputs in fungi. Furthermore, the cross-talk between these two light perception systems might be, at least in part, responsible for the altered growth provoked by the overexpression of blr-2, probably due to an imbalance in the light regulation between the two (or more) inputs (Fig. 9a). In plants, cross-talk between cryptochromes and phytochromes that regulate plant growth and architecture has been well documented (Franklin et al., 2005).

In N. crassa, the expression of wc genes is mainly regulated at the transcriptional level through WC proteins. This dependence on both WC proteins has been found both in the dark and after light induction (Ballario et al., 1996; Cheng et al., 2003; Káldi et al., 2006). In T. atroviride, the induction of blr-2 by light was faster and higher in the OEblr2-13 strain compared to the WT strain, similar to the expression pattern found for the blue-light-induced genes. We determined a 75% induction of blr-2 in OEblr2 that represents a 9.2-fold increase with respect to the transcript levels found in the WT strain, strongly suggesting a positive post-transcriptional autoregulation. Analysis of the expression of blr-2 in the Δblr-1 mutant showed that its expression in the dark and accumulation induced by light depends on BLR-1, providing further support to the notion that the BLR proteins act as a complex in light-activated post-transcriptional regulation (Fig. 9a). The fact that the transcript levels of blr-2 decreased 3.5-fold in the Δblr-1 strain may be explained by the presence of a putative LRE (GATGC-N23-GATGC-N7-CGATT) 1122 nt upstream of the ATG in the blr-2 promoter, similar to what is found in other light responsive genes, suggesting regulation at the transcriptional level mediated by the BLR complex in the dark (Fig. 9a). Consistently, the WC complex regulates the expression of wc-1 through two different promoters upstream of its ORF, named the distal and proximal promoters. The distal promoter is necessary for expression in the dark and the proximal promoter is essential for light-responsive expression (Káldi et al., 2006). Altogether, our data suggest that the BLR complex is a transcriptional regulator active even in the dark and that after a light pulse it can regulate expression of light-responsive genes at the transcriptional level, and that of blr-2 at the post-transcriptional level (Fig. 9a).

Recently, we determined that the expression of light-regulated genes in T. atroviride is subjected to photoadaptation (Rosales-Saavedra et al., 2006). Similarly, blr-2 expression under constant illumination showed a typical adaptive response, similar to the expression pattern found after a light pulse, indicating the existence of a turn-off mechanism operating in its post-transcriptional regulation. Conversely, the expression pattern of the blr genes was different in the blr-2-overexpressing strain. However, the expression pattern of light-regulated genes was the same in the WT and OEblr2-13 strains, indicating that different adaptation mechanisms may be operating. In N. crassa, a negative feedback loop involving the flavoprotein VIVID and phosphorylation by different kinases that destabilize WC-1, and block the transcriptional activity of the WC complex, are the main mechanisms that control adaptation under continuous exposure to light (Schwerdtfeger & Linden, 2003; He & Liu, 2005). T. reesei, a close relative of T. atroviride, has a homologue (ENVOY) of VIVID that might play a similar role in photoadaptation (Scholl et al., 2005). In our view, the mechanisms controlling the expression of blr-2 at the post-transcriptional level and, possibly, mycelial growth under continuous exposure to light differ from those reported for N. crassa. The expression of blr-1 was not altered by light in the WT strain and an increase in the transcript levels was only evident in the blr-2 overexpresser. The increase in the blr-1 transcript steady-state levels observed only in a blr-2-overexpressing strain was comparable to the change found in the blr-2 expression levels. The fluctuations observed in blr-1 transcript levels have been observed in autoregulated genes, and typically arise from interference in the half-life...
of mRNA and the resulting time gap between transcription and translation, as shown for the zebrafish somitogenesis oscillator (Lewis, 2003; Schmoll et al., 2005). This may be indicative of the existence of a post-transcriptional regulation mechanism for both transcripts. However, since we did not find light-induced changes in the blr-1 mRNA steady-state levels in the WT strain, these results must await further experimental support.

Formation of complexes between the WC proteins (WCC) has been demonstrated (Cheng et al., 2002; Froehlich et al., 2002). The WC proteins have been found in the dark as a heterodimer able to bind LREs and, after a light pulse, a large WCC is formed that transiently binds the LREs of light-regulated genes in vivo (Froehlich et al., 2002; He & Liu, 2005). Additionally, although WC-2 regulates WC-1 steady-state levels, WC-2 overexpression did not seem to alter the levels of WC-1 found in the WT strain (Cheng et al., 2001, 2002). Our data suggest that, similar to the WC proteins, the T. atroviride BLR proteins act as a complex. Additionally, blr-2 overexpression did not alter the transcript levels of blr-1 in the dark. Therefore, we consider that all phenotypes found may be associated directly with higher BLR-2 levels. Overexpression of blr-2 resulted in a threefold higher sensitivity to light of the blr-2-over-expressing strains than that of the WT strain. The fluence required for the light response was lower for the OEblr2 strains than for the WT strain. This parameter, directly associated with photoreceptors, was modified by blr-2 overexpression. The enhanced sensitivity to blue light of the OEblr2 strains can also explain the arrhythmic growth observed in the first light period, which possibly occurred as a result of an imbalance in the photosensitivity of different perception systems that regulate growth under constant illumination with white light. Our data strongly suggest that a BLR-1/BLR-2 complex is necessary for light perception. Although we cannot discard any effect associated with changes in the levels of BLR-1 caused by blr-2 overexpression, our data support the notion that a preformed photoreceptor complex between BLR proteins in the dark is key for the adequate perception and transduction of the light signal, as indicated in Fig. 9(b).

The BLR proteins regulate a set of specific genes, as demonstrated by the deletion of either of them (Rosales-Saavedra et al., 2006), and the overexpression of blr-1 and blr-2. Together, these data clearly demonstrate a role dual for the BLR complex in light-dependent induction and repression of transcription (Fig. 9a). Here, we show evidence of additional roles of the BLR complex in the positive post-transcriptional regulation of blr-2 triggered by light, as well as of transcriptional regulation in the dark (Fig. 9a). The expression analysis of blr and early light-responsive genes under constant illumination clearly indicates that a negative feedback loop is operating in the downregulation of the expression of these genes (Fig. 9a). Our results indicate that the adaptation mechanism to light is different between the blr and light-responsive genes, even though their regulation clearly occurs through the BLR complex. On the other hand, we found that the BLR proteins are dispensable for the regulation of growth of T. atroviride under defined photoperiods. However, there is strong evidence for cross-talk among the different light perception pathways. We demonstrated regulation of blu-4 expression by blue light through the BLR complex, in addition to its regulation by red light as described previously (Rosales-Saavedra et al., 2006), thus supporting cross-talk between the blue and red light transduction pathways. Finally, our results support a key role of BLR-2 in blue light sensing and transduction, and we propose that pre-formation of a BLR photoreceptor complex is key for appropriate light perception, and for subsequent robust regulation of all blue light responses (Fig. 9b).

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