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Review

Trichoderma: sensing the environment for survival and dispersal

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Species belonging to the genus *Trichoderma* are free-living fungi common in soil and root ecosystems, and have a broad range of uses in industry and agricultural biotechnology. Some species of the genus are widely used biocontrol agents, and their success is in part due to mycoparasitism, a lifestyle in which one fungus is parasitic on another. In addition *Trichoderma* species have been found to elicit plant defence responses and to stimulate plant growth. In order to survive and spread, *Trichoderma* switches from vegetative to reproductive development, and has evolved with several sophisticated molecular mechanisms to this end. Asexual development (conidiation) is induced by light and mechanical injury, although the effects of these inducers are influenced by environmental conditions, such as nutrient status and pH. A current appreciation of the links between the molecular participants is presented in this review. The photoreceptor complex BLR-1/BLR-2, ENVOY, VELVET, and NADPH oxidases have been suggested as key participants in this process. In concert with these elements, conserved signalling pathways, such as those involving heterotrimeric G proteins, mitogen-activated protein kinases (MAPKs) and cAMP-dependent protein kinase A (cAMP-PKA) are involved in this molecular orchestration. Finally, recent comparative and functional genomics analyses allow a comparison of the machinery involved in conidiophore development in model systems with that present in *Trichoderma* and a model to be proposed for the key factors involved in the development of these structures.

Introduction

As a group, fungi have a deep impact on human life and ecosystem functionality. Fungi are the principal decomposers in the ecosphere, and are essential for recycling nutrients in the environment. Some of them have symbiotic associations with plants and algae, while others are used as biocontrol agents against phytopathogenic organisms (Druzhinina & Kubicek, 2005). Further, some groups of fungi are infectious agents and can cause a wide variety of diseases in animals, plants and humans (Idnurm & Heitman, 2005). Consequently, it is of major importance to understand the mechanisms of fungal development and reproduction in order to increase the benefits and decrease the costs that they represent.

Asexual sporulation is a common reproductive process for many species of fungi of medical, industrial and agricultural importance. Asexual spores (conidia) have either dispersal or resting functions and are also used as inocula. In this regard, conidia are used in commercial preparations of beneficial fungi, such as those that function as biocontrol agents against phytopathogenic organisms, as well as those used in industrial processes. It is within this area that *Trichoderma* is a genus of particular economic interest.

As a ubiquitous and often predominant component of the mycoflora in numerous soils (native and agricultural) in all climatic zones, *Trichoderma* species play an important role in ecosystem health (Klein & Eveleigh, 1998). Since the main mechanism for survival and dispersal of *Trichoderma* is through the production of conidia, understanding the factors that control this morphogenetic switch from vegetative growth to asexual reproduction is of major importance.

Fungi sense and interact with the environment, and there is an important crosstalk among environmental cues that determines the response of a fungus to its environment (Bahn *et al.*, 2007). Particular combinations of environmental cues trigger entry into a variety of developmental processes in fungi. Accordingly, some of these cues trigger conidiation in *Trichoderma*. The aim of this review is to summarize the current advances in knowledge of the process of conidiation in the genus *Trichoderma*, and to place them in the context of the state of our knowledge that has arisen from work in widely studied fungal model systems.

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Conidiophore morphology in the genus *Trichoderma*

Conidiophores in the genus *Trichoderma* can appear as paired branches that assume a pyramidal aspect, ending in one or a few phialides. Phialides may be held in whorls or may be penicillate, and can be densely clustered on a wide main axis or solitary. Conidia of most species of *Trichoderma* are less than 5 μm long and wide, and they may be globose, subglobose, ellipsoidal or oblong. Conidial pigmentation ranges from deep green to nearly grey, and ornamentation can be smooth, warted or tuberculate, and is a species character (Samuels, 1996). Since this review focuses on three species, in which the molecular mechanism of conidiation has been studied in greater depth, we briefly describe the morphology of the conidiophore for each one of these species.

Fig. 1 shows the most contrasting conidiophore structures found in the genus. The *Trichoderma atroviride* conidiophore is simple, with unilateral branching or in pairs (Fig. 1a). *Trichoderma virens* has conidiophores arising in clusters from an aerial mycelium, branching toward the tip, each branch ending in a penicillus of closely appressed phialides, with a sterile stipe (Fig. 1b). In *Trichoderma reesei*, on the other hand, conidiophores are found in minute pustules and along aerial hyphae, forming a well-defined main axis from which phialides arise singly toward the tip; further from the tip, a single phialide surmounts a single cell in addition to branches consisting of few cells, and each branch ends in one or two phialides, and phialides arising singly from intercalary cells of the branch; paired branching systems are rare.

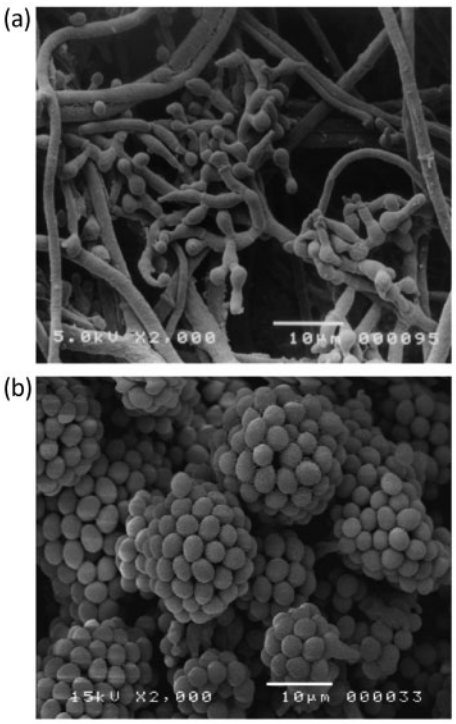

Fig. 1. Morphological differences between the conidiophores of *T. atroviride* and *T. virens*. Scanning electron microcopy images of (a) *T. atroviride* conidiophores, (b) *T. virens* conidiophores; bars, 10 μm.

Environmental stimuli that induce conidiation in *Trichoderma*

The environment represents a set of stimuli that do not arrive singly, and organisms have to sense and respond to each of them in an integrated way to survive and proliferate. For a better understanding of this process, researchers separate each stimulus to study, in more detail, the molecular mechanisms that allow fungi to sense, adapt to and respond to a specific environmental cue.

In particular, in *Trichoderma*, it has been shown that conidiation is regulated by light and mycelial injury (Horwitz *et al.*, 1985; Casas-Flores *et al.*, 2004; Steyaert *et al.*, 2010a). Other factors that influence conidiation include C : N status, low pH, extracellular calcium, and fungal-derived volatile organic compounds (VOCs) (Nemcovic *et al.*, 2008; Šimkovič *et al.*, 2008; Steyaert *et al.*, 2010a) (Fig. 2).

Light perception and gene expression at early stages of the developmental programme

Trichoderma species are, in most cases, common soil inhabitants that associate with plant roots. Thus, entry into the conidiation programme induced by light may reflect the behaviour of *Trichoderma* when it reaches the soil surface. Under such conditions it must prepare to deal with the potentially harmful effects of sunlight, and for dispersal into a different niche. In this sense, conidia are perhaps the best-suited structures.

The first description of the effect of light on *Trichoderma* was made in 1957 by Gutter, who reported that on nutrient-rich medium in the dark, *Trichoderma viride* grew indefinitely as mycelium, but that a brief pulse of light applied to the actively growing zone of the mycelium resulted in the formation of dark-green mature conidia, forming a ring at the periphery of the colony (Gutter, 1957). The fungus appears to be responsive to light (competent) only after 10–16 h of growth (Gressel & Galun, 1967). However, in constant light, conidiation occurs continuously across the fungal colony, whereas when exposed to cycles of light, concentric rings of conidia are observed. The amount of light to which a *Trichoderma* colony is exposed determines the amount of conidia produced. Since for *T. atroviride* photoconidiation can be induced with pulses of light lasting from nanoseconds to minutes, it would appear that in *Trichoderma*, photoconidiation is triggered by a single receptor system, according to the Bunsen–Roscoe law of reciprocity (Horwitz *et al.*, 1990).

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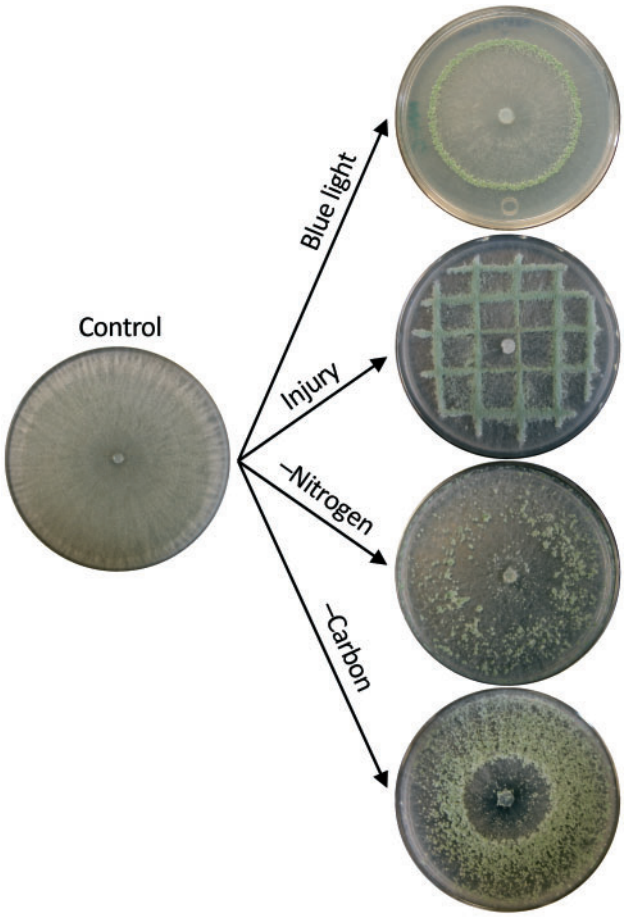

Fig. 2. Conidiation phenotype induced by different cues in *T. atroviride*. Wild-type *T. atroviride* strains were grown in the dark for 36 h and conidiation was induced by exposure to a flux of blue light (1200 μmol m^{-2}) or mechanical injury with a scalpel; nitrogen starvation (–Nitrogen) and carbon deprivation (–Carbon) conidiation was induced using Vogel's medium without nitrogen or carbon. Photographs were taken 36 h after treatment.

By means of scanning electron microscopy it has been determined that 3–7 h after photo-induction abundant branching of aerial hyphae with an increased number of septa can be observed, as well as the formation of new aerial hyphae (Galun, 1971). Branches form conidiophores, and the new aerial hyphae elongate, branch and also form conidiophores. This developmental programme can be divided into a determination state and a morphogenetic stage that includes reprogramming of gene expression, and the appearance of the corresponding physiological and morphological changes. Accordingly, the operation of this programme can be suppressed using RNA synthesis inhibitors, once it has been triggered by light, but only in a time window of approximately 7 h after illumination (Galun & Gressel, 1966; Gressel & Galun, 1967; Betina & Zajacová, 1978) (Fig. 3). As might be expected, protein synthesis inhibitors also block conidiation (Betina & Zajacová, 1978). In early studies it was shown that light-induced conidiation is inhibited in hyphal cells which develop in the absence of oxygen (Gutter, 1957). Later, Gressel *et al.* (1975) demonstrated that if oxygen is briefly removed from *T. viride* cultures and a pulse of light given, conidiation will begin when cultures are transferred back to the air. Thus, it was proposed that photoinduction is 'remembered' while a culture is maintained in conditions that do not allow cellular growth; as soon as growth is resumed, under optimal conditions, the colony conidiates (Gressel *et al.*, 1975; Horwitz *et al.*, 1990). Recently, Steyaert *et al.* (2010a) interpreted these observations as showing that oxidative processes are required for completion of the developmental programme, since there appears to be a clear differentiation between the initial photoreactions and the development of conidiation.

Determination of the different wavelengths of light that elicit the physiological response has shown a sharp peak in the near-UV at 350–380 nm, and a wider peak in the blue spectrum with a maximum at 440–450 nm, consistent with the participation of flavoproteins (Gressel & Galun, 1967; Kumagai & Oda, 1969). Casas-Flores *et al.* (2004) reported the identification of two genes (*blr-1* and *blr-2*); the corresponding proteins constitute the blue light regulator complex, one of them being a flavoprotein.

For a better understanding of light responses, gene expression analyses have been carried out in *T. atroviride* in search of light-responsive genes that could play key roles in photoconidiation. An initial study included the use of cDNA microarrays representing 1438 genes (Rosales-Saavedra *et al.*, 2006). This study led to the discovery of 30 genes (*blu*) upregulated by white light, and 10 down-regulated genes (*bld*). However, silencing of a set of *blu* genes (individually) did not block photoconidiation (Esquivel-Naranjo, 2007). More recently, the availability of the genome sequence has permitted genome-wide analysis of gene expression by high-throughput sequencing. Quantitative analyses have allowed the identification of 331 white light-regulated genes and 204 specifically responsive to blue light. The functional categories of the light-responsive genes fall into metabolism, stress, cellular transport, cofactor-binding proteins, cell cycle and DNA processing, transcription, and cell differentiation. It is noteworthy that of the stress-induced genes, most are related to oxidative stress. Additionally, genome-wide transcriptome analysis has shown that 10 transcription factors are regulated by light, suggesting, as expected, that the whole process involves a cascade of transcriptional events (Fig. 3; E. U. Esquivel-Naranjo and others, unpublished results). The global regulatory network for the blue light response has been also addressed in a proteomic approach. It has been reported that several polypeptides vary in abundance before and after structural changes are visible in *T. atroviride* (formerly *Trichoderma harzianum*), upon exposure to blue light (Baum & Horwitz, 1991).

Transcriptomic analyses have revealed that components of the oxidative stress pathway are responsive to light. Thus, a

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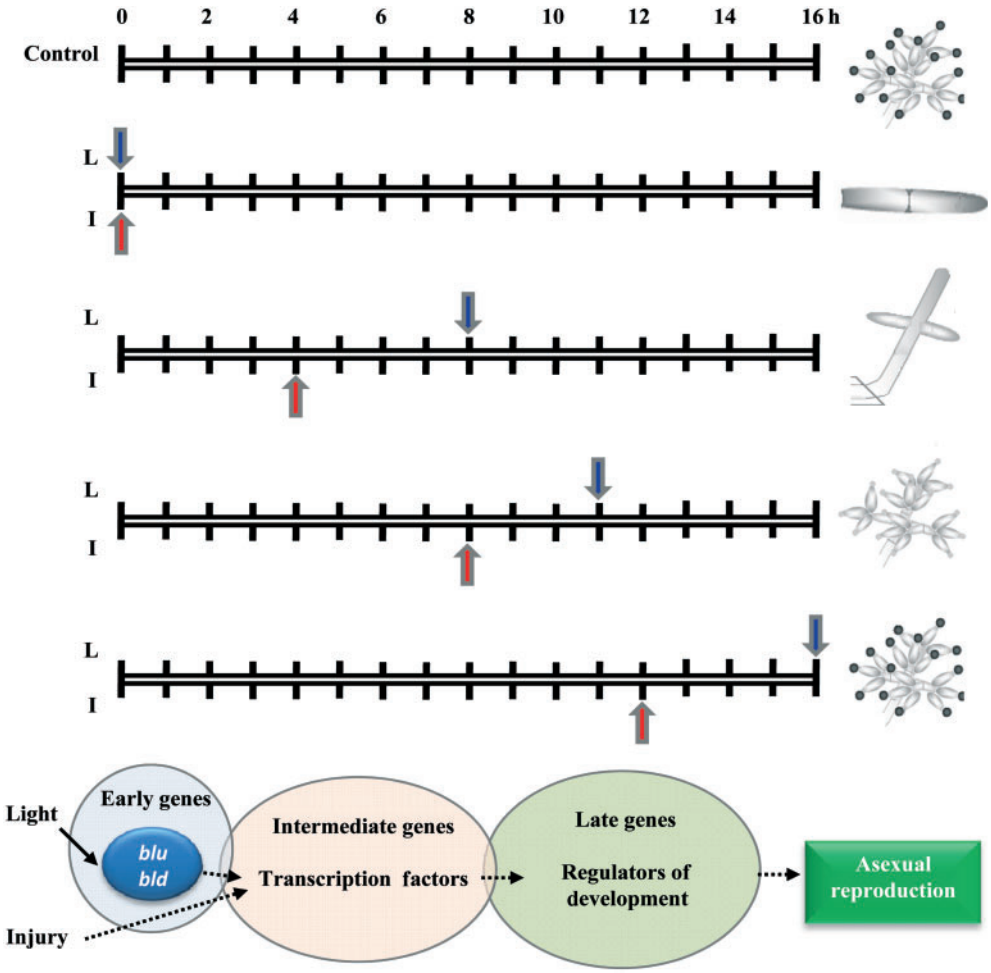

Fig. 3. A cascade of transcriptional events drives the conidiation process. Colonies were exposed to a pulse of 5-fluorouracil (5-FU) given at the indicated times after a light pulse (blue arrows) or mechanical damage (red arrows). Drawings at the right depict the structures formed 30–36 h after induction of conidiation, upon exposure to 5-FU at the indicated times after induction (blue light, L; injury, I). Control: *T. atroviride* without inhibitor. The lower panel represents a proposed model of the transcriptional cascade, in which early genes, *blu* and *bld* (including transcription factors), activate a second pool of genes that include other transcription factors that in turn activate intermediate genes, and then late genes, allowing completion of the process.

role for reactive oxygen species (ROS) in conidiation induced by light is proposed, in agreement with early studies that suggested the involvement of oxidation steps in conidiation (Gutter, 1957; Gressel *et al.*, 1975). Nevertheless, it is evident that several processes are turned on in response to light, and further analyses are required to determine which components of the different response pathways affected by light have a direct role in conidiation.

Mycelial injury as a cue that triggers conidiation

During the analysis of mutants affected in the photoreceptor complex of *T. atroviride*, it was inadvertently discovered that injury to the mycelium induced conidiation (Casas-Flores *et al.*, 2004). Later it was reported that the phenomenon is common to multiple species of *Trichoderma* (Steyaert *et al.*, 2010b, c). Similar to the observed response to light, the completion of this developmental programme can be suppressed using RNA synthesis inhibitors, but in a different time window of approximately 12 h after injury (Fig. 3). In a recent transcriptome analysis using high-throughput sequencing, 415 and 518 genes were found to be transiently repressed and induced after injury, respectively, reinforcing the notion of the existence of a cascade of transcriptional events leading to conidiation (Fig. 3). Consistent with what has been observed in response to light, a significant number of injury-responsive genes encode proteins related to oxidative stress. During early stages of the response to injury, genes known to generate ROS are induced, whereas those known to scavenge ROS are repressed. In addition, the use of antioxidant agents prevents conidiation, strongly suggesting that an oxidative burst may trigger conidiation (M. Hernández-Oñate and others, unpublished results).

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Influence of nutritional status on conidiation

Nutrient deprivation or different sources of nutrients are universal signals for conidiation in fungi. Analysis of conidiation in *T. viride* and *T. atroviride* on various carbon sources has revealed that this process is strongly carbon source-dependent in both light and darkness, and that light plays a catalytic role, enhancing the extent of conidiation (Chovanec *et al.*, 2001; Friedl *et al.*, 2008). Chovanec *et al.* (2001) observed conidiation in *T. viride* cultures grown on 30 out of 32 carbon sources, including polysaccharides, amino acids and alcohols. Conidiation rates varied depending on the carbon source, and the level of variation in dark-grown cultures was comparable with that observed in response to a light pulse. In contrast, conidiation is not observed in *T. atroviride* when amino acids or alcohols are the sole carbon source. In addition, 48 out of 95 tested carbon sources induce conidiation in the dark, and light enhances the response to most of them. However, light appears to inhibit conidiation on D-arabinose and D-gluconic acid (Friedl *et al.*, 2008). In most of these 48 carbon sources, growth rates do not correlate with conidiation; in some cases they allow slow or very poor mycelial growth of the fungus; thus, conidiation could be a response to starvation. However, there are other carbon sources in which there is fast mycelial growth and clear conidiation; these conidiation patterns are suggested to be due to different redox potentials upon catabolism of the carbon sources used (Friedl *et al.*, 2008). Primary sources of nitrogen strongly promote photoconidiation in *Trichoderma asperellum*, *T. atroviride* and *Trichoderma pleuroticola*, but not *Trichoderma hamatum* or *T. virens*, suggesting the interactive effect to be species (isolate)-specific (Steyaert *et al.*, 2010c).

Acid environments are associated with the conidiation process

In several species of *Trichoderma*, low pH seems to be a determinant for conidiation. Conidiation induced by light and mechanical injury is strictly low pH-dependent, with maximum response values below pH 4.4 in *T. atroviride*, *T. hamatum* and *T. pleuroticola* (Steyaert *et al.*, 2010b), whereas in *T. harzianum*, conidiation is higher at pH 5.5 (Moreno-Mateos *et al.*, 2007); hence, the quality and quantity of the response seems to be species-specific. It is proposed that photoconidiation is dependent on a low intracellular pH, achieved by the low pH in the environment and the acidification that occurs when mycelia are exposed to light (Gresík *et al.*, 1991; Steyaert *et al.*, 2010b). Transcriptional regulation of gene expression by pH is mediated in filamentous fungi by the zinc finger transcription factor PacC (Peñalva & Arst, 2002, 2004). Accordingly, the use of strains silenced in or expressing modified versions of the *T. harzianum* orthologue (*pac1*) indicates that pH-dependent conidiation is regulated through Pac1 (Moreno-Mateos *et al.*, 2007).

Consequently, the influence of pH in conidiation induced by other environmental cues is clear, although pH by itself appears not to be sufficient, as denoted by the absence of conidiation in darkness at pHs at which photoconidiation is clearly observed. It is possible that light, cell damage and also nutrient starvation can modify the intracellular pH in natural (non-buffered) conditions, resulting in the induction of the expression of genes that lead to conidiation.

Influence of VOCs

As in many fungi, a diverse array of VOCs has been detected from cultures of *Trichoderma* (Fiedler *et al.*, 2001; Stoppacher *et al.*, 2010; Wheatley *et al.*, 1997). These compounds include the eight-carbon VOCs 1-octen-3-ol and its analogues, which are the end products of fatty acid metabolism (Schnürer *et al.*, 1999). These C8 compounds produced by *Trichoderma* and other fungi have been shown to stimulate conidiation in *Trichoderma* and likely provide a signalling system for synchronization of conidiation (Nemcovic *et al.*, 2008). Furthermore, there is evidence that volatile compounds can influence developmental processes such as conidiation in *Aspergillus parasiticus* (Roze *et al.*, 2010). However, the mechanism by which VOCs stimulate conidiation in *Trichoderma* is not yet known.

Active participants in regulation of conidiation

The blue light regulator complex BLR-1/BLR-2

In *Neurospora crassa*, all responses to blue light are mediated by two zinc finger transcription factors encoded by the white-collar genes (*wc-1* and *wc-2*) (Liu *et al.*, 2003). The orthologues of *wc-1* and *wc-2* in *Trichoderma* have been identified (*blr-1* and *blr-2*), and they are essential for photoconidiation in *T. atroviride* and *T. reesei* (Casas-Flores *et al.*, 2004; Castellanos *et al.*, 2010). Consistent with the action spectra of photoconidiation, BLR-1 contains a PAS/LOV (Per-ARNT-Sim/light, oxygen and voltage) domain, which presumably binds FAD. Based on the structure of the BLR proteins, and the phenotype observed in *blr-1* and *blr-2* mutants (Casas-Flores *et al.*, 2004; Castellanos *et al.*, 2010), it has been postulated that BLR-1 acts as the photoreceptor, in association with BLR-2.

Light influences, however, not only conidiation but also vegetative growth in *T. atroviride*, and *blr-1* and *blr-2* are clearly involved in this phenomenon (Casas-Flores *et al.*, 2004). Furthermore, there is an interesting link between conidiation and the synthesis of small non-ribosomal peptides (peptaibols), which is also significantly influenced by light. No peptaibols have been detected in *blr-1* or *blr-2* mutants of *T. atroviride* induced by light, but formation of the peptaibol atroviridin by mechanical injury is light-dependent but BLR-independent, indicating that these photoreceptors of *Trichoderma* do not play an important role in regulation of peptaibol production by light, under additional stress (Komon-Zelazowska *et al.*, 2007).

There is carbon source-dependence for conidiation and the BLR complex is involved in this process. In darkness, sources that favour conidiation in the wild-type strain

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support low levels of conidiation in the *blr* mutants (Friedl *et al.*, 2008). Interestingly, *T. atroviride* mutants in either of the *blr* genes do not conidiate in response to a sudden carbon deprivation (Casas-Flores *et al.*, 2004, 2006).

It is evident that the light signal transmitted by BLR-1 and BLR-2 is of high importance for conidiation, but these proteins also play regulatory roles both in the dark and in the light that are not completely related to this developmental process.

ENVOY, a tiny but nonetheless important blue light photoreceptor

A second blue light photoreceptor (ENVOY), the orthologue of the *N. crassa* VIVID (Heintzen *et al.*, 2001; Schwerdtfeger & Linden, 2003), was first identified in *T. reesei* (Schmoll *et al.*, 2004). ENVOY, a small protein that contains a single PAS/LOV domain, is also encoded in the genomes of *T. atroviride* and *T. virens*. In darkness, the corresponding gene (*env1*) is transcribed at a very low level, but upon illumination the abundance of its transcript increases up to 500-fold. This response requires the BLR-1/BLR-2 photoreceptor complex in both *T. reesei* and *T. atroviride* (Castellanos *et al.*, 2010; E. U. Esquivel-Naranjo and others, unpublished results). In agreement with its putative role in a negative feedback loop, as has been demonstrated for VIVID in *Neurospora* (Malzahn *et al.*, 2010; Chen *et al.*, 2010), mutants in *env1* in *T. atroviride* produce significantly more conidia under constant exposure to light and a blue light pulse (our unpublished data). In *T. reesei*, growth of the *env1* mutant is severely affected by constant light, with a reduced hyphal extension rate, loss of polar growth and reduced conidiation, but after a blue light pulse it conidiates to the same extent as the wild-type, indicating a function of *env1* in light tolerance (Castellanos *et al.*, 2010; Schmoll *et al.*, 2005). Interestingly, *T. reesei* mutants in *blr-1* and *blr-2* that do not express *env1* are not affected in growth under light (Castellanos *et al.*, 2010). ENVOY also has a role in gene regulation: it is required for turning off the expression of blue light-induced genes (Castellanos *et al.*, 2010). Recently, it was reported that ENVOY is involved in signal transduction via G proteins, acting positively in the feedback of *gna1*, and in the cAMP/protein kinase A pathway, controlling in a still unclear way the function of the corresponding phosphodiesterase (Tisch *et al.*, 2011). These data denote the important relationship between light and these signalling pathways.

Light is detected as a stress signal, and available data point to the involvement of ENVOY in the response to high light intensities with a role in photoadaptation. The precise mechanism by which ENVOY does this is not known, although it could be inferred from what is known in *N. crassa* (Chen *et al.*, 2010; Hunt *et al.*, 2010; Malzahn *et al.*, 2010) that ENVOY physically interacts with components of the BLR complex.

VELVET, a comprehensive regulator of morphogenesis

An important light-regulatory protein that is a relatively new player in the regulation of sporulation in *Trichoderma* is the orthologue of the *Aspergillus nidulans veA*, which encodes a conserved global regulator of morphogenesis and secondary metabolism in some filamentous fungi (Calvo, 2008). In *A. nidulans*, VeA physically interacts with VelB and the regulator of secondary metabolism LaeA to form a complex that regulates secondary metabolism and sexual reproduction (Bayram *et al.*, 2008a). In *A. nidulans*, transport of VeA into the nucleus is inhibited by light (Stinnett *et al.*, 2007). Deletion of the *veA* gene leads to an increase in asexual development, and reduced and delayed sexual reproduction (Kato *et al.*, 2003; Kim *et al.*, 2009). VeA is also required for the production of sclerotia and for aflatoxin biosynthesis in *A. parasiticus* (Calvo *et al.*, 2004). Deletion of the *ve-1* gene in *N. crassa*, like deletion of the *veA* gene in *A. nidulans*, results in deregulated conidiation (Bayram *et al.*, 2008b). In *T. virens*, deletion of the *Trichoderma velvet* orthologue (*vel1*) results in altered secondary metabolism, since *vel1* mutants are defective in the production of gliotoxin (Mukherjee & Kenerley, 2010). Morphogenesis is also affected: deletion of *vel1* results in a total loss of conidiation on solid medium in both *T. virens* and *T. atroviride* (Mukherjee & Kenerley, 2010; our unpublished data). Vegetative growth is also severely affected in a *T. atroviride vel1* mutant (our unpublished data). In contrast, *T. virens vel1* deletion mutants produce massive amounts of chlamydospores in submerged culture, suggesting that in *T. virens*, VELVET acts as a negative regulator of chlamydospore production (Mukherjee & Kenerley, 2010). It has been suggested that conidiation is associated with secondary metabolism in *T. atroviride* (Komon-Zelazowska *et al.*, 2007). Thus, VELVET may be a key regulator in this association. The secondary metabolites produced via VELVET, such as volatile compounds, can regulate conidiation in fungi, as noted in *veA* mutants of *A. parasiticus*, in which the volatiles produced via VELVET affect conidia and sclerotia formation (Roze *et al.*, 2010). So, a plausible explanation of the phenotypical alteration in *vel1* mutants of *Trichoderma* is that the correct production of secondary metabolites is required for the normal development of this fungus.

ROS drive the conidiation process

Oxygen is a weak reactant with a tendency to form radicals, either by energy or by electron transfer reactions, forming incompletely reduced ROS. By the energy transfer reaction, singlet oxygen ($^1O_2$) is formed, whereas electron transfer results in the sequential reduction to superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) (Heller & Tudzynski, 2011). ROS promote modification of cellular proteins by oxidation, for subsequent degradation by the proteasome (Reinheckel *et al.*, 1998), but can also react with DNA and lipids, causing cellular damage (Neill

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et al., 2002). In fungi, ROS have been proposed to be a critical component in growth and differentiation (Hansberg & Aguirre, 1990). In zebrafish, it has been shown that light induces the production of H_2O_2 (Hirayama *et al.*, 2007), and in *T. atroviride*, there is production of ROS upon mechanical injury (M. Hernández-Oñate and others, unpublished results). NADPH oxidases (Nox) have been characterized as enzymes of higher eukaryotes responsible for the production of ROS (Malagnac *et al.*, 2004). The best-studied mammalian $gp91_{phox}$ (Nox2) requires for its activity the assembly of a multi-subunit complex formed by the cytosolic regulatory component Rac, $p40_{phox}$, $p47_{phox}$, $p67_{phox}$ and the integral membrane protein flavocytochrome b_{558}, composed of the catalytic subunit $gp91_{phox}$ and the adaptor protein $p22_{phox}$ (Scott & Eaton, 2008).

Three different Nox subfamilies have been found in the kingdom Fungi, two homologues of the human $gp91_{phox}$, NoxA (Nox1) and NoxB (Nox2), and NoxC, which contains a putative EF-hand calcium-binding domain (Aguirre *et al.*, 2005). Fungi also contain an orthologue of $p67_{phox}$, named NoxR, which is found in all fungal genomes that have NoxA (Takemoto *et al.*, 2007). Functional analysis of NoxA and NoxB has shown that these proteins play a key role in fungal cell differentiation and development. In *T. atroviride*, disruption of the *nox1* and *noxR* genes results in a clearly diminished sporulation response after a blue light pulse, and practically no production of conidia upon mechanical injury. However, deletion of the *nox2* gene shows no phenotype (M. Hernandez-Oñate and others, unpublished results). Consistently, *nox1*-overexpressing transformants in *T. harzianum* showed increase production of conidia during confrontation with *Pythium ultimum*, and clear differences in growth were observed (Montero-Barrientos *et al.*, 2011). These data indicate that in *Trichoderma*, the oxidative state generated by biotic stress involves high NADPH oxidase activity to regulate the conidiation process. Similarly, previous work in other fungi indicates that *nox1* orthologues play rather specific roles in fungal development, as observed in *N. crassa*, where ROS are generated at the start of each of the morphogenetic steps that occur during asexual development (Hansberg *et al.*, 1993), and correlate with the oxidation of proteins (Toledo *et al.*, 1994). Similar blockages in fruiting body development have been observed in *Podospora anserina* and *N. crassa* after *nox1* deletion, demonstrating that Nox enzymes are critical for the development of sexual structures in filamentous fungi (Aguirre *et al.*, 2005; Malagnac *et al.*, 2004). Disruption of *noxA* in *A. nidulans* blocks sexual development, since differentiation of fruiting bodies cannot be completed (Lara-Ortíz *et al.*, 2003). Deletion of *nox1* in *N. crassa* results in complete female sterility and a marked reduction in hyphal growth and asexual development (Cano-Domínguez *et al.*, 2008). In both *N. crassa* and *Botrytis cinerea*, NoxR is required for both NoxA and NoxB function in cellular differentiation (Cano-Domínguez *et al.*, 2008; Segmüller *et al.*, 2008). In contrast, *A. nidulans noxR* deletion mutants are defective in both asexual and sexual development (Semighini & Harris, 2008).

Central signal transduction pathways and crosstalk

All living organisms have to deal with the environment. To ensure correct cellular responses to any stimulus, they have developed a complex network of signal transduction pathways.

In fungi, cAMP-dependent protein kinase A (cAMP-PKA), mitogen-activated protein kinase (MAPK) and heterotrimeric G protein signalling cascades are part of the molecular mechanism that allows them to sense and adapt to the environment in response to diverse cues (Bahn *et al.*, 2007). In *Trichoderma*, these pathways play a pivotal role in conidiation.

Role of heterotrimeric G proteins in conidiation

The heterotrimeric G protein system is composed of a seven-transmembrane-domain G protein-coupled receptor (GPCR), the canonical heterotrimeric G protein consisting of α, β and γ subunits, and an effector (Yu *et al.*, 2006). The Gα proteins can be classified into three major subgroups: I, which inhibit adenylate cyclase; II, which have no homology with mammalian G proteins; and III, which in most fungi stimulate adenylate cyclase. In filamentous fungi, heterotrimeric G protein signalling pathways are involved in sporulation, mating, pathogenicity, secondary metabolite production, and vegetative incompatibility (Kays *et al.*, 2000; Rosén *et al.*, 1999; Horwitz *et al.*, 1999; Loubradou *et al.*, 1999).

A study of the GPCRs of *T. atroviride* revealed that the gene *gpr-1* is essential for vegetative growth, conidiation and conidial germination (Brunner *et al.*, 2008). However, the nature of the ligand activating this receptor is still unknown. All three genomes of *Trichoderma* species that have been sequenced encode three Gα subunits, one β and one γ subunit. In general, in *Trichoderma*, heterotrimeric G proteins have been shown to negatively regulate conidiation. *T. atroviride* transformants in which the gene encoding the Gα1 subunit (*tga1*) was silenced or interrupted showed intense conidiation (Rocha-Ramirez *et al.*, 2002; Reithner *et al.*, 2005). Conversely, transformants overexpressing the gene or expressing a constitutively active allele were unable to produce conidia in response to light (Rocha-Ramirez *et al.*, 2002). In contrast, in *T. virens*, knockout mutations of *tgaA* and *tgaB*, orthologues of *tga1* and *tga2* of *T. atroviride*, respectively, do not differ from the wild-type in conidial phenotype (Mukherjee *et al.*, 2004). In *T. atroviride*, loss of *tga3* (encoding the Gα3 subunit) also results in hyperconidiation and conidiation in the dark (Zeilinger *et al.*, 2005). Thus, both Gα proteins (Tga1 and Tga3) act as negative regulators of conidiation, perhaps by sharing downstream signalling components. Intriguingly, these effects on conidiation were not observed in *gna1* (*tga1*) and *gna3* (*tga3*) mutants of *T. reesei* (Schmoll *et al.*, 2009; Seibel *et al.*, 2009), shedding light on the different roles of this protein in each species. Consistent

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with the expected effect on adenylate cyclase activity of a subunit belonging to subgroup III, transformants expressing *gna3* in antisense showed reduced levels of cAMP. In *T. viride*, high levels of cAMP have been directly correlated with conidiation (Gresik *et al.*, 1988; Kolarova *et al.*, 1992). Nevertheless, transformants expressing a constitutively active allele of GNA3 showed a reduction in conidiation (Schmoll *et al.*, 2009). These observations led to the suggestion that a regulator of G-protein signalling (RGS) negatively controls the activity of GNA3, and that such a factor would be inactive on the constitutively active allele (Schmoll *et al.*, 2009). There are, however, no reports on the role of any RGS protein in the physiology and development of *Trichoderma*, although such an analysis would help us to further understand this signalling pathway in the different species.

In *Cryphonectria parasitica*, disruption of *cpg-1* ($G\alpha_i$ protein) abolishes asexual sporulation, reduces growth rate and leads to loss of virulence, while disruption of the *cpgb-1* gene ($G\beta$ subunit) leads to reduced pigmentation, conidiation, hyphal tip branching and virulence, while causing increased vegetative growth (Gao & Nuss, 1996; Kasahara & Nuss, 1997), implying that $G\beta\gamma$ may act as negative regulator of $G\alpha$ function in vegetative growth. In *N. crassa*, GNG-1 ($G\gamma$) and GNB-1 ($G\beta$) form a functional $G\beta\gamma$ heterodimer that is essential for normal asexual sporulation and female fertility; in addition, levels of GNG-1 and GNB-1 are decreased in the absence of the other subunit, and deletions in either of these subunits affect the levels of $G\alpha$ subunits (Yang *et al.*, 2002; Krystofova & Borkovich, 2005). These data suggest that $G\beta\gamma$ subunits can be the limiting factor in the signalling mediated by G proteins in some processes, and that an analysis of these subunits in *Trichoderma* is required in order to achieve a better understanding of the role of G proteins. Detailed studies have revealed that despite considerable sequence similarity among G protein subunits, their functions in some cases show variations between species. Thus, the specific role of a given G subunit cannot be predicted by extrapolating results obtained in another species, especially if their natural habitats and lifestyles are different, even if they are closely related.

cAMP-PKA

Biochemical changes detectable after light induction of conidiation include an increase in the activity of adenylate cyclase, protein phosphorylation, and transient increments in the levels of cAMP, all associated with the activation of a signalling pathway modulated by cAMP (Gresik *et al.*, 1988; Kolarova *et al.*, 1992). Further, addition of an analogue of cAMP (dibutyryl cAMP) to a *T. atroviride* colony growing in the dark triggers conidiation, while atropine, an adenylate cyclase inhibitor, blocks light-induced conidiation (Berrocal-Tito *et al.*, 2000). However, knockout mutants in adenylate cyclase (*tac1*) in *T. virens* are severely affected in growth, germination, mycoparasitism and secondary metabolism, but are able to conidiate in light (Mukherjee *et al.*, 2007), which suggests that at least in this species cAMP is dispensable for photoconidiation.

cAMP can overrule the function of BLR proteins in conidiation induced by carbon deprivation, but does not restore photoconidiation in the *blr* mutants (Casas-Flores *et al.* 2006), suggesting that there is a cAMP-dependent pathway for conidiation by carbon starvation in which the BLR proteins participate, although they are not an essential component as they are in photoconidiation.

As mentioned above, light induces protein phosphorylation; the addition of cAMP to a cell-free extract of *T. viride* (now *T. atroviride*) can mimic the action of light in this process (Gresík *et al.*, 1989). This modification of proteins is a common mechanism of post-translational regulation and is due to the action of protein kinases. cAMP-dependent protein kinases have been described in fungi; hence, this result suggests that phosphorylation is due to the action of cAMP-dependent protein kinases. In this regard, Casas-Flores *et al.* (2006) showed that transformants expressing an antisense version of *pkr-1*, a gene encoding the regulatory subunit of PKA, which have increased levels of PKA activity, did not produce conidia when a pulse of blue light was applied or under carbon deprivation. Some conidiation is observed if this strain is transferred to media without glucose and cAMP. In contrast, low levels of PKA activity achieved by overexpression of the *pkr-1* gene result in the production of conidia even in the dark. There is a clear increase of PKA activity levels upon blue light exposure in the wild-type and both *blr* mutants, suggesting the existence of an alternative blue light perception system. On the other hand, induction of a set of BLR-dependent blue light-regulated genes is also dependent on the activity of PKA, indicating that they are not necessary for conidiation. These molecular data suggest that complex mechanisms are involved in the cAMP signalling pathway that regulates asexual reproduction in *T. atroviride.*

The MAPK signal transduction pathway

MAPK pathways transduce a wide variety of signals, including those associated with cellular growth in a variety of eukaryotic organisms. There are three classes of MAPK pathway in filamentous ascomycetes: the pathogenicity MAPK typified by Pmk1 of *Magnaporthe oryzae*, the Slt2 MAPK pathway involved in maintenance of cell-wall integrity, and the Hog1 pathway involved in stress responses (Kumar *et al.*, 2010). All three pathways are present in *Trichoderma* spp. In filamentous fungi, MAPK genes are in some cases required but in other cases are totally dispensable for conidiation (Xu, 2000). Pmk1 homologues TmkA/Tvk1 (*T. virens*) and Tmk1 (*T. atroviride*), the Slt2 homologues TmkB (*T. virens*), as well as ThHog1 (*T. harzanium*) have been studied.

In *T. virens*, null mutants of the MAPK-encoding gene *tvk1* are affected in several aspects of the life cycle, including growth, conidiation, conidial pigmentation, secretion of

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cell wall-degrading enzymes and biocontrol activity (Mendoza-Mendoza *et al.*, 2003). Interestingly, in *T. virens* (Gv28.9), deletion of *tvk1* results in a reduction in the production of conidia on solid medium, and profuse conidiation in liquid medium (Mendoza-Mendoza *et al.*, 2003). In contrast, loss of *tmkA* in *T. virens* (IMI304061) results in hyperconidiation (Mukherjee *et al.*, 2003). TmkA of *T. virens* seems to play a repressing function in conidiation in the dark. Mutants in the corresponding MAPK from *T. atroviride* (Tmk1) produce abundant conidia in a light-independent manner (Reithner *et al.*, 2007). In addition, *T. virens tmkB* mutants exhibit reduced growth and constitutive conidiation in the dark (Kumar *et al.*, 2010). Thus, the first two classes of MAPK in *Trichoderma* seem to repress conidiation. In contrast, analyses of MAPK null mutants of *Colletotrichum langenarium*, *Ustilago maydis* and *Cochliobolus heterostropus* have established that *tvk1* homologues are required for sporulation (Takano *et al.*, 2000; Müller *et al.*, 1999; Lev *et al.*, 1999), whereas mutants of the corresponding gene in *M. grisea*, *Fusarium oxysporum* and *B. cinerea* show no alterations in spore production (Di Pietro *et al.*, 2001; Zheng *et al.*, 2000; Xu & Hamer, 1996).

The role in conidiation of the MAPKs belonging to the Pmk1 and Slt2 classes is clear: mutants in these MAPKs share phenotypes that suggest an interaction and coordination between these two pathways. However, they must have unique upstream signal or downstream transcription factors that regulate diverse growth and differentiation processes such as conidiation. The identification of these factors is required for a further understanding of these signalling pathways.

Key actors in conidiophore development

In *A. nidulans*, the genetic mechanisms that control asexual reproduction have been addressed in detail. It has been proposed that the sequentially expressed activities of three regulatory genes, *brlA*, *abaA* and *wetA*, define the central regulatory pathway that controls conidiation-specific gene expression, and determine the order of gene activation during conidiophore development and spore maturation. *abaA* is essential for adequate phialide development, a common feature of conidiophores in all *Trichoderma* species. Together with these three genes, there are upstream regulators (*flbB*, *flbC*, *flbD*, *flbE*) required for the normal production of conidiospores. *stuA* and *medA* are considered developmental modifiers and are required for a restricted series of cell divisions that establish the spatial organization of the conidiophore (Adams *et al.*, 1998; Etxebeste *et al.*, 2010; Yu, 2010). We searched for orthologues of these genes in the genomes of three species of *Trichoderma*, to find that almost all the genes are encoded in these genomes. Interestingly, there is no orthologue of *brlA*, which determines the conidiophore vesicle, a structure apparently not formed in *Trichoderma* conidiophores (Clutterbuck, 1969). *flbE* is absent in *T. atroviride* and *wetA* is absent in *T. reesei*. The lack of conservation of some orthologues of the *Aspergillus* conidiation genes among three species of *Trichoderma* may suggest that different pathways determine conidiophore formation in the different species. Furthermore, promoter analysis of *abaA*, the direct target of *brlA*, showed that it has putative BrlA-binding sites; this may be interpreted as an indication that another C2H2-type transcription factor can exert its function. The absence of a *brlA* orthologue in *Trichoderma* denotes the apparent lack of conservation of key regulators of conidiation between *Aspergillus* and *Trichoderma*. These data also suggest that the pathway that involves *flbE* and *flbD* is not functional in *T. atroviride* (Fig. 4). In contrast, the conservation of the upstream signalling components (e.g. *flbC*) suggests a similar way of defining the entry into conidiation in these fungi.

Concluding remarks

The environment plays an important role in growth and differentiation of fungi. *Trichoderma* spp. respond to external

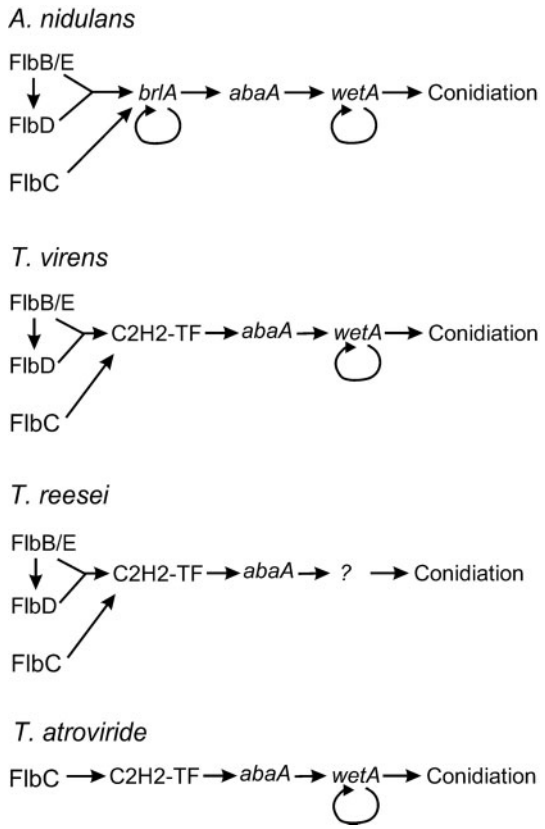

Fig. 4. Schematic representation of key regulators of conidiation in *Trichoderma* spp. compared with *A. nidulans*. The figure illustrates proposed pathways for the three *Trichoderma* species for which a genome sequence is available, and is based solely on bioinformatic analyses.

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stimuli during growth, conidiation and mycoparasitism. These three features are all important attributes that contribute to the development of these organisms as biofungicides.

The process of conidiation involves many common developmental themes, including sensing the stimuli, intracellular communication, temporal and spatial regulation of gene expression, and cell specialization (morphological changes).

Among the different ways in which *Trichoderma* perceives an environmental signal that triggers conidiation, the role of the BLR proteins in sensing light is well established. In the case of nutrient starvation, there are data that suggest the role of a GPCR (GPR-1). For mechanical injury, however, it is not completely clear what is the signal that triggers conidiation. It is plausible that damage of the cell wall and the release of the cytoplasmic contents generate the signal, but it is not clear whether a specific receptor is required.

Different stimuli can induce conidiation in *Trichoderma*; all of them can be considered as stress signals, so conidiation is a response to a stress situation in order to survive and disperse. Fungi have conserved signalling cascades to sense and respond to different type of stresses. Signal transduction cascades mediate communication between environmental signals and the cellular machinery that controls growth and differentiation. In this respect, heterotrimeric G proteins, MAPKs and the components of the cAMP-PKA signalling

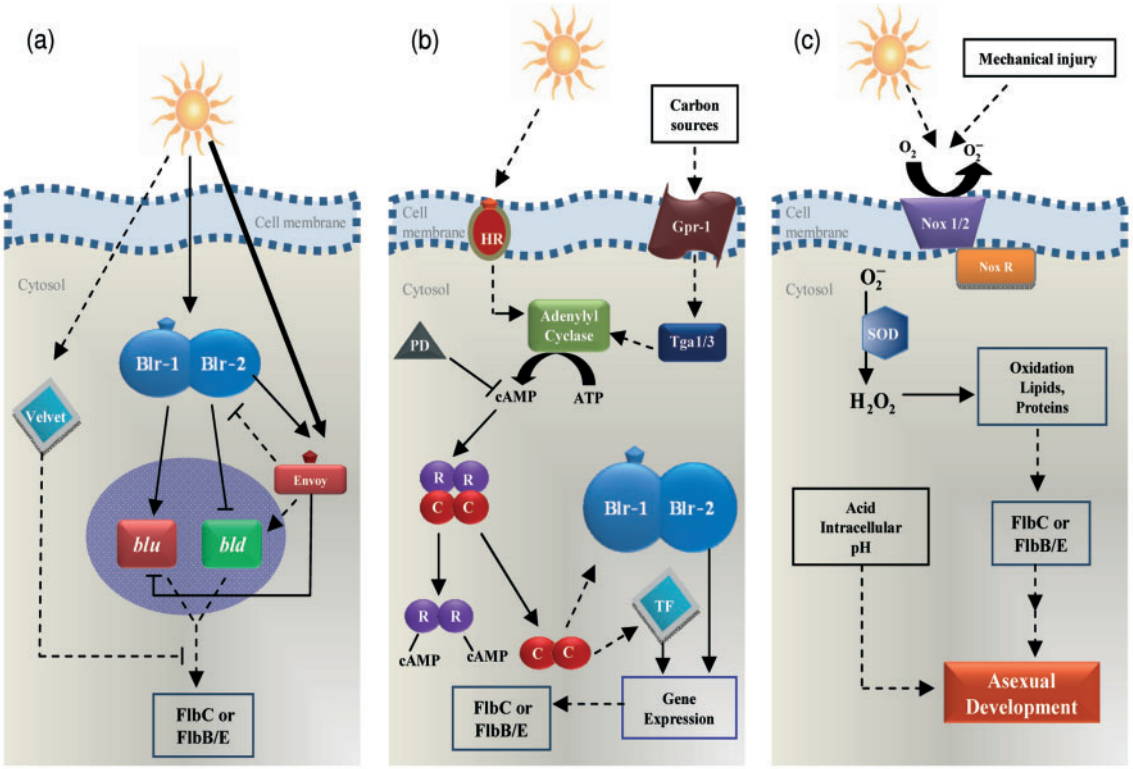

Fig. 5. Hypothetical models integrating the multiple signalling pathways that participate in the conidiation process of *Trichoderma*. Three conidiation inducers are presented. (a) The BLR-1/BLR-2 complex senses light, and it controls the expression of upregulated (*blu*) and downregulated (*bld*) genes. ENVOY participates in photoadaptation, controlling the expression of *blu* and, likely, *bld* genes, and may physically interact with the BLR complex. VELVET can be activated by light and, in a still unknown way, control conidiation. (b) A hypothetical receptor (HR) could activate adenylate cyclase, leading to the production of cAMP, which activates PKA, releasing the catalytic subunit (C); PKA activity may be involved in the phosphorylation of either BLR proteins or other proteins, probably transcriptional factors, whose modification is necessary for gene activation. A GPCR (GPR-1) that activates heterotrimeric G proteins senses different carbon sources, and Tga1 or Tga3 could activate adenylate cyclase. Phosphodiesterase (PD) would regulate cAMP levels, exerting a negative control on photoconidiation. (c) Light and mechanical injury activate the Nox complex for the production of ROS, leading to the oxidation of proteins and lipids. Intracellular acid pH favours asexual development. Finally, all pathways converge in the activation of intermediate genes (e.g. *flbC*), for the final activation of late genes, whose expression allows the formation of conidiophores and conidia in *Trichoderma.* Arrowheads indicate positive regulation and bars negative regulation. Solid lines indicate supporting evidence from experimental data and dotted lines indicate hypothetical steps. Thin lines indicate low light intensity and thick lines high light intensity.

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pathway are required for fungal development, and *Trichoderma* spp. are no exception.

Regulation of gene expression is of vital importance when the cell faces new environments, in order to adapt to and deal with the new conditions; initially, the response is broad, and seems to be general rather than specific (Gasch *et al.*, 2000; Chen *et al.*, 2003). In *Trichoderma*, there are many examples of this process, especially in response to light, which denote a wide response in the initial stages of the response because it is perceived as a stress signal. Although light influences different processes, such as primary and secondary metabolism, and morphogenesis, it is initially perceived as a stress signal, triggering a general stress response for immediate protection. Then, the cell expresses genes that are involved in some of these processes, among them those required for conidiation (e.g. *flbB–D*, *stuA*, *wetA*), thus ensuring survival. The fact that mutants in some light-regulated genes are not affected in conidiation is in agreement with the concept that these genes could be part of the general response to stress, although they are not among the genes required specifically for the conidiation process.

Cell specialization is a consequence of specific gene regulation; we suggest that most of the genes regulated in the first stages by light or mechanical injury are not directly involved in this process: they are part of the primary response to stress; and in a second cascade of gene expression, the result of the expression of the first set of genes, are the genes that directly govern conidiation. At some point, the cascades of gene expression regulated by the stresses that induce conidiation converge and turn on the gene or group of genes that directly controls the process of conidiophore formation (Fig. 5). To date this component has not been identified in *Trichoderma*, and more experimental effort is needed in order to achieve this goal. Nevertheless, the number of genes found to be involved in this process has increased significantly, covering different steps of the signal transduction pathway. Identifying all the components of the conidiation process and how it works in response to several developmental signals will help understand downstream signalling processes and generate improved strains for biological control.

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