

Review

Regulation of gene expression in entomopathogenic fungi in three different environmental conditions: A review

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The entomopathogenic fungi are subject to regulation system known as signal transduction that regulates the expression of the different genes required for each life stage, first comprising cuticle adhesion and degradation, later the survival in the hemolymph, and finally the fungal growth in soils. There are two signal-transduction mechanisms identified in the entomopathogenic fungi: mitogen-activated protein kinases (MAPK) and protein kinase A (PKA) dependent to cAMP pathway together with regulatory G protein signaling; both pathways participate in activation of genes linked to virulence for certain environments and hosts such as adhesion, formation of appressorium and penetration pegs, synthesis of cuticle-degrading enzymes, survival in hemolymph to evade insect immune responses, and osmolarity changes. Also, activating well-defined gene sets involved in entomopathogenic fungal survival on non-insect substrates as saprophytes or root colonizer utilized the nutrients present in root exudates, facilitating entomopathogenic fungi to act as endophytes.

Key words: Entomopathogenic fungi, signal transduction, mitogen-activated protein kinases (MAPK), protein kinase A (PKA).

INTRODUCTION

Entomopathogenic fungi are an important group of microorganisms that have been used as biological controls against insect pests in many agroecosystems that have caused several epizooties when the insects increase their populations and cause damage to the crops. The use of these microorganisms offers an ecological alternative to the use of chemical insecticides (Hajek and Delalibera, 2010). The entomopathogenic fungi are ascomycetes that produce asexual conidia, among which are *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii*, *Paecilomyces* spp. and

Isaria fumosorosea (Zimmermann, 2008). The pathogenic mechanism of these fungi has been extensively studied and begins with the adhesion of the conidia in an insect's exoskeleton surface, formation of an appressorium or a mucilaginous structure that remains adhered to a germinal tubule and penetration by mechanical pressure and enzymatic activity (Ali et al., 2010; Charnley, 2003). The enzymes degrade the insect's cuticle and help in the process of penetration by mechanical pressure. Once inside the insect, the fungi develop as hyphal bodies or blastospores to evade hemocytes and disseminate through the haemocoel and invade diverse muscle tissues, fatty bodies, Malpighian tubes, mitochondria and haemocytes, leading to death of the insect after infection (Pucheta et al., 2006; Wang and

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St. Leger, 2006), and begin to synthesize secondary metabolites that overcome the insect's immune responses and cause the insect's death.

Finally, the hyphae emerge after invading the insect's body with saprophytic growth, sporulate and restart the cycle, infecting other insects by contact (Khan et al., 2012). During pathogenesis, entomopathogenic fungi are required to express a variety of genes in different sequences staggered pattern (Wang et al., 2005), and this regulation process in general is known as signal transduction. This signal transduction is a very common mechanism in fungi, which use it as a response to extracellular stimuli (Lafon et al., 2006). The principals signal transduction pathways are Mitogen-activated protein kinases (MAPK), cAMP-PKA (AMP cyclic-protein kinase A) and regulatory G protein signaling (RGS), although G proteins are generally upstream of MAPK and cAMP-PKA pathways. In the infective process, the gene expression should be regulated according to three different microenvironmental conditions: first, the adhesion and germination of conidia and degrade the insect cuticle; later, the fungus invades the hemolymph; and finally, the fungus emerges from the cadaver after saprophytic growth (Khan et al., 2012). *M. anisopliae* and *M. acridum* are the best studied insect pathogenic fungi and thus serve as an excellent starting point for gaining a broad perspective of issues in insect pathology (Gao et al., 2011). In *M. anisopliae*, different numbers of genes are expressed; 1918, 1581 and 592 genes have been identified that are deregulated during the phases in the cuticle, in the hemolymph and during saprophytic growth, respectively (Wang et al., 2005). Comprehension of the regulation and deregulation processes of genes to understanding of fungal pathogenesis is necessary to improve biological control strategies using entomopathogenic fungi. The aim of this review is to discuss the progress of the regulation of gene expression in entomopathogenic fungi in different phases of the infection of insects and the saprophytic development.

SIGNAL TRANSDUCTION PATHWAYS

Microorganisms respond to physical and chemical environmental signals, such as nutrients or other compounds. Recognition can occur both at the surface and inside the cell. Such signals are perceived and processed through a mechanism called signal transduction, comprising a complex receptor system, enzymes and regulatory proteins that detect, amplify and integrate different external signals, resulting in the metabolic and morphogenetic changes necessary to invade host insects (Mantilla et al., 2012). The principals signaling pathway are heterotropic G protein that is coupled to MAPK and cAMP-PKA (Li et al., 2007).

In several filamentous fungi, the RGS pathway plays integral roles for cell growth/division, mating, conidiation,

morphogenesis and pathogenesis and secondary metabolite production (Lengeler et al., 2000; Yu, 2006). The heterometric G protein is composed of α , β , and γ subunits, where the signal amplitude of G protein is activated by the balance of the rates of GDP/GTP exchange and deactivation by the rates of GTP hydrolysis (Fang et al., 2007). The RGS pathway senses environmental conditions and regulates the switch between vegetative growth and conidiation, where regulators of RGS interact with $G\alpha$ to produce GTP hydrolysis, leading to deactivation of $G\alpha$ and the cessation of G protein signaling (Chidiac and Roy, 2003). In entomopathogenic fungi, RGS is involved in conidiation and conidial thermotolerance of *B. bassiana*, and conidiation, virulence and hydrophobin synthesis of *M. anisopliae* (Fang et al., 2007, 2008).

MAPK are a family of serine/threonine protein kinases that are widespread (Wang and St. Leger, 2006) in eukaryotic cells and play crucial roles in the transduction of a variety of extracellular signals and the regulation of various developmental and differentiation processes (Schaeffer and Webber, 1999; Monge et al., 2006). The MAPK system is activated by a conserved signaling module that functions as a protein-kinase cascade; in this sense, the MAPK kinases (MAPKKK) acts on the MAPK kinase (MAPKK), and this enzyme is active on MAPK by phosphorylation (Salinas-Sánchez et al., 2012). This type of activation has been studied in many organisms. In *Saccharomyces cerevisiae*, at least five MAPK pathways have been identified; these pathways are designated as FUS3, KSS1, HOG1, SLT2 and SMK1 and are involved in mating, filamentous growth, response to high osmolarity, cell integrity and ascospore formation, respectively (Gustin et al., 1998). The MAPK cascade functions in different filamentous fungi change depending on the niche of each organism and show overlap of specificities and responses (Zhao et al., 2007). In *B. bassiana*, MAPK plays an important role in the regulation of various development and differentiation processes and pathogenicity in response to environmental cues. A MAPK-encoding gene was identified called *Bbhog1* that has three important functions: regulating spore viability by maintaining adequate levels of erythritol and glycerol contents in the conidia of entomopathogenic fungi to increase the germination rate, facilitating adhesion onto an insect cuticle with the expression of genes associated with hydrophobicity or adherence, and initiating appressorium development (Zhang et al., 2009). The gene *Bbsit2*, which encodes for a member of the Sit2 family corresponding of MAPK pathway, plays an important role in conidiation, viability, cell-wall integrity and virulence (Luo et al., 2012).

Fungi employ cAMP-PKA pathway in a variety of processes including the control of differentiation, sexual development, and virulence of a variety of human and plant pathogenic fungi, such as the human pathogen *Cryptococcus neoformans* (D'Souza et al., 2001) and the

rice-blast pathogen *Magnaporthe grisea* (Xu and Hamer, 1996). A central component of the cAMP signaling cascade is protein kinase A (PKA). PKA is a serine/threonine kinase which is conserved in eukaryotes. The PKA is a heterotetrameric complex consisting of two PKA catalytic subunits that are bound by two regulatory subunits; after binding two molecules of cAMP, the catalytic and regulatory subunits dissociate and the catalytic subunits are now able to phosphorylate proteins, such as transcription factors (Grosse et al., 2008). The catalytic subunit of PKA is the gene *MaPKA1* in *M. anisopliae*, and 24 genes have been identified that are down-regulated by this pathway, including components of the cellular machinery involved in translation, transport, stress response, metabolic functions, appressorium and penetration-peg formation, cuticle degradation and pH regulation (Fang et al., 2009), so cAMP-PKA pathway is an important regulator in the expression of many genes in the entomopathogenic fungi.

CONIDIATION AND ADHESION OF CONIDIA

In the entomopathogenic fungus, the conidium initiates pathogenesis and mediates disease transmission, however, the regulation of conidiation in *B. bassiana* is not yet known; whether it can be mediated by activation of MAPK or RGS. Although mutant of HOG1-type MAPK had significant reduction in conidiation in agar plates, it is not clear if it occurs through activation of MAPKs (Zhang et al., 2009). In addition, the regulatory G protein signaling gene *Bbrgs1* is also required for conidiation in *B. bassiana* (Fang et al., 2008) therefore; it is needed to elucidate the cross talk between MAPKs and G protein signal pathway for regulation of conidiation. Fang and Bidochka (2007) otherwise, found a RGS gene *cag8* that is, associated with conidiogenesis in *M. anisopliae*. Respect to conidia adhesion, it is mediated in part by spore-coat hydrophobins (Zhang et al., 2011); in the chestnut blight fungus *Cryphonectria parasitica*, the genetic expression of hydrophobin and hydrophobin-like proteins is regulated by RGS proteins (Segers et al., 2004) although in entomopathogenic fungi, it is not known. These proteins bind to the waxy epicuticle, which is the first barrier against fungal adhesion and consists of a heterogeneous mix of lipids (Howard and Blomquist, 2005).

The constitution of these lipids varies among insect species (Jarrold et al., 2007) and has an important effect on insect resistance to entomopathogenic fungal infection because the cuticle hydrocarbons can promote or inhibit fungal adhesion (Boucias et al., 1988; Lord and Howard, 2004). Therefore, entomopathogenic fungi must have the ability to degrade the cuticle lipids of insects in the initial process and provide nutrients for fungal growth (Napolitano and Juarez, 1997). Based on studies of *M. anisopliae* and *B. bassiana*, a hydrocarbon-degradation pathway was proposed to participate in the microsomal

cytochrome P450 enzyme system (Crespo et al., 2000). The cytochrome p450 monooxygenases constitute a large family of heme-thiolate proteins induced for alkane hydroxylation, a major hydrocarbon component of epicuticle lipids (Pedrini et al., 2007). In *B. bassiana*, seven different families of cytochrome P450 genes have been identified, and the *p450-2*, *p450-6* and *p450-7* genes have been shown to be deregulated and induced by insect-derived extracts (Pedrini et al., 2010). Additionally, the protein CYP52X1 has been chemically characterized as a member of a novel cytochrome P450 subfamily in *B. bassiana*, expressed during its growth on *Galleria mellonella* (Zhang et al., 2012). The gene *MrCYP52* has been identified from *Metarhizium robertsii*, expressing a single cytochrome P450 monooxygenase induced by the alkanes present on the insect-cuticle surface; this gene is regulated by a trans-acting DNA-binding protein CRR1, which is a zinc-finger protein (Lin et al., 2011); this regulation is similar to that of protease PR1 by C-catabolite repression.

PRODUCTION OF CUTICLE-DEGRADING ENZYMES

After germination and appressorium formation, the entomopathogenic fungi produce cuticle-degrading enzymes; these are proteases, chitinases, lipases and lipoxygenases (Pucheta et al., 2006) that provide available nutrients. In *M. anisopliae*, the hydrolytic enzymes are subject to regulation by a generalized mechanism of a cAMP-dependent PKA signaling (Fang et al., 2009) wherever proteases have been considered as the main enzymes involved in the hydrolysis of insect cuticle (St. Leger et al., 1995). Already, subtilisin-like serine protease (PR1) and trypsin-like serine protease (PR2) have been identified (Tiago et al., 2002); these enzymes are regulated specifically for insect cuticle proteins (Paterson et al., 1994). Moreover, the carbon and nitrogen sources are external signals, and the physiological response is regulated by signal activation of transcription factors (Li et al., 2007). In the case of nitrogen sources, PR1 and PR2 proteases are subject to nitrogen de-repression by *M. anisopliae* nitrogen response regulator (NRR1), which is a protein-type single zinc-finger motif (Screen et al., 1998), that is homologous to *Neurospora crassa* NIT2 (Fu and Marzluf, 1990) and *Aspergillus nidulans* areA (Kudla et al., 1990). The NRR1 protein binds to GATA sites from the promoter region of the gene *pr1* of *M. anisopliae* during nitrogen regulation. Carbon-catabolite repression in entomopathogenic fungi is mediated by a C₂H₂-type DNA-binding binuclear zinc-finger protein, *M. anisopliae* CRR1 (carbon-response regulator) with a significant homology to the CREA proteins of *A. nidulans* (Screen et al., 1997). The promoter of the *pr1* gene from *M. anisopliae* has three union sites similar for the corresponding CREA protein of *A. nidulans* (Cubero and Scazzocchio, 1994); thus, the CRR1 protein is considered

equivalent to CREA (Screen et al., 2001), although it may be regulated differently. This protein is homologous with the repressor proteins produced in response to glucose by *Aspergillus* and *Trichoderma* spp. additionally, the *M. anisopliae* *pr1A* gene was demonstrated to have differential expression with different carbon and nitrogen sources, but *M. anisopliae* CRR1 and NRR1 are not expressed differentially during cuticle degradation (Screen et al., 1998). *B. bassiana* have another type of regulation of proteases PR1 and PR2 during the initial stages of germination; the phenomenon of C/N repression is not relevant, allowing rapid induction in first stages of infection, with an obvious advantage to pathogens in assimilate nutrients by cuticle degradation before the insect produce an immune response (Qazi and Khachatourians, 2008).

Regarding chitinases, the regulation is controlled by an induction/repression system by the products of chitin hydrolysis, such as N-acetyl-D-glucosamine. This substrate is also a specific inducer to N-acetyl- β -D-glucosaminidase that is affected to a lesser degree by catabolite repression in *M. anisopliae* (St. Leger, 1996). In *L. lecanii*, the N-acetyl- β -D-glucosaminidase is induced by shrimp shell in solid-state fermentation (Barranco-Florido et al., 2009), moreover the expression levels of *L. lecanii* *chit1* gene depend on the carbon source present and basal levels were detected in solid-state cultivation (Mayorga-Reyes et al., 2012). Chitinase production by *B. bassiana* in a liquid culture medium was suppressed by the presence of an easily assimilable carbon source and induced by colloidal chitin or yeast extract (Dhar and Kaur, 2010). The *M. anisopliae* *chit1* gene was characterized, and its promoter contains the sequences CAAT and TATAA, which are recognized by transcription factors (Bogo et al., 1998). The CHIT1 protein is synthesized as a preproenzyme with signal peptides of 35 aminoacids, and the processed product is a 42 kDa protein with homology to chitinases from *Aphanocladium album* and *Trichoderma harzianum*. Baratto et al. (2006) characterized the *chit2* chitinase gene from *M. anisopliae* of 42 kDa; this gene has two introns, and the promoter region contains the control elements CAAT and TATAA, similar to CHIT1. In addition, a consensus motif was found for the CRR1 carbon-catabolism repressor, suggesting that chitinase CHIT2 is regulated by catabolic repression.

CELL DIFFERENTIATION AND SECONDARY METABOLITES IN THE HEMOCOEL

In the hemocoel, the insect entomopathogenic fungi grow as yeast-like propagules named blastospores or hyphal bodies to evade insect immune responses. The entomopathogenic fungi have highly complicated finely-tuned molecular mechanisms for regulating cell differentiations in response to insects hosts (Gao et al., 2011). In the hemocoel, the mode of regulation and signal pathway involved in regulation of cell differentiation has not been determined. However, the MAPK and cAMP-

PKA pathways of *M. grisea* coordinate to regulate aporesoria formation and pathogenic growth (Xu and Hamer, 1996). This suggests that cAMP-PKA cross talks with the MAPK pathway to regulate the cellular reorganization (Fang et al., 2009). In the hemolymph a large number of genes have been identified; *M. anisopliae* expressed *mcl1* gene of MCL1 protein collagen-type, which provides a hydrophilic antiencapsulation coat around the hyphal bodies that eliminate cell surface components associated with non-self recognition, thus avoid detection by hemocytes. The cellular reorganization allows the fungus to evade insect immune responses (Wang and St. Leger, 2006).

The hemolymph has a high osmotic pressure, with an interval from 300 to 500 mOsmol L⁻¹ (Chapman, 1998). *M. anisopliae* deregulates the gene *mos1*, which encodes the transmembrane protein MOS1 for the surveillance of osmotic conditions, and this deregulation implies that membrane permeability decreases, providing a barrier to solutes and cellular deformation (Wang et al., 2008). *M. anisopliae* uses the phosphoketolase pathway for pentose or fructose-6-phosphate catabolism in the hemolymph through deregulation of the gene *mpk1*. The *mpk1* gene is of bacterial origin and provides evidence that genes were horizontally transferred from prokaryotes to eukaryotes (Keeling and Palmer, 2008). The Mpk1 protein is activated to maintain redox balance for the anaerobic reoxidation of NADH (Duan et al., 2009), which is a strategy similarly employed by *S. cerevisiae* for pentose catabolism (Sonderegger et al., 2004). The secondary metabolites produced by entomopathogenic fungi in the hemolymph have been identified as depsipeptides with antibiotic, antifungal and insecticidal activity (Xu et al., 2009). It also produces toxin that causes insect death (Xiao et al., 2012).

Depsipeptides, such as destruxins and beauvericin, are cyclic depsipeptides biosynthesized for multifunctional nonribosomal peptide synthetase (NRPS) (Pedras et al., 2002). The destruxins inhibit the DNA, RNA and proteins in the insects cells (Pucheta et al., 2006). Although in the entomopathogenic fungi, the regulation of genes responsible for the biosynthesis of destruxins are unknown. Wang et al. (2012) found a gene cluster in *M. robertsii* and *M. acridum* containing NRPS DtxS1, which is a multienzyme complex with six modules for the sequential addition of hydroxyl and amino acids during destruxin biosynthesis. Depsipeptides are able to suppress insect immune responses. The *bbBs/s* gene has been identified in *B. bassiana*; the gene encodes an NRPS for bassianolide, which is a cyclic depsipeptide secondary metabolite (Xu et al., 2008). However, the regulation mechanism is unknown, and likely it could be for cAMP-PKA or MAPK pathways.

SAPROPHYTIC GROWTH

The entomopathogenic fungi survived in the soil after

emerging from insect cadavers. They are presumably subject to selective pressures during soil survivability and establish a symbiotic relationship with plants, as some strains are endophytic or colonize the rhizosphere (Pava-Ripoli et al, 2011). Fungal survival is crucial to control insect-pest populations (Kerry BR, 2000). *M. anisopliae* has been shown to be highly rhizosphere-competent (Hu and St. Leger, 2002, 2008), and *B. bassiana* can persist in agrosystem soils for a long time until it infects another host insect (Meyling and Eilenberg, 2007). However, the persistence of *M. anisopliae* and *B. bassiana* depends more by their adaptability to soil rather than for their pathogenicity to insects (Quesada-Moraga et al., 2007). Therefore, the expression of a set of genes enabling a fungus to survive in these microenvironments is required.

Genes have been identified in *M. anisopliae* that are involved in the synthesis of cell-wall components, storage or mobilization of nutrients, and the production of antibiotics, that encode a variety of transporters and permeases for utilization of carbohydrates and amino acids or peptides that are present in root exudates and that are regulated by signal transduction (St. Leger, 2008). These genes facilitate the adaptation of entomopathogenic fungi to soil environments. Root exudates contain a mixture of compounds such as carbohydrates, lipids, aminoacids, organic acids and antimicrobial compounds that affect other microorganisms. *M. anisopliae* differentially expressed a set of genes that could be used to adapt to the nutrients present in root exudates (Pava-Ripoli et al., 2011). The mechanistic details of interactions with plant roots are unknown, but evidences show that *M. anisopliae* using two different proteins, MAD1 and MAD2, are differentially induced in insect hemolymph and plant roots, when the fungi have a saprophytic growth (Wang and St. Leger, 2007). Although the entomopathogenic fungi are used to kill an insect pest, other roles are also possible. For example, Ownley et al. (2008) have shown that *B. bassiana* plays a role against plant pathogenic fungi. Also, different genera of fungal entomopathogens have been reported as naturally occurring fungal endophytes (Vega, 2008); still the regulatory process is unidentified.

CONCLUSIONS

Together with analysis of the genes involved in germination, pathogenesis and adaptations in the soils of entomopathogenic fungi during its saprophytic and pathogenic life stages, it is necessary to know the mechanism that regulates the differential expression of the genes required for the different environments in which fungi are found. This knowledge will improve strategies for the efficient use of fungi as biological control agents as well as understand the coevolutionary processes between fungi and host organisms, including the development of the ability to overcome insect defenses and to prevent infection resistance hosts. Studies of pa-

thogenesis in entomopathogenic fungi have revealed that RGS, cAMP-PKA and MPKA signaling pathways all play essential roles in pathogenic morphogenesis. The interesting questions that remain are how the fungus perceives the environmental signals and specifies the signaling responses in different life stages. To address these issues, upstream components such as receptors and downstream targets of these pathways need to be identified and characterized. The entomopathogenic fungi are closer to plant endophytes and plant pathogens than animal pathogens and as they similitude between the regulatory processes of fungi that are human or plant pathogens, it may be possible to create drugs to prevent infections in humans or create defense alternatives in plants.

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