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In vivo gene expression profiling of the entomopathogenic fungus *Beauveria bassiana* elucidates its infection stratagems in *Anopheles* mosquito

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The use of entomopathogenic fungi to control mosquitoes is a promising tool for reducing vector-borne disease transmission. To better understand infection stratagems of insect pathogenic fungi, we analyzed the global gene expression profiling of *Beauveria bassiana* at 36, 60, 84 and 108 h after topical infection of *Anopheles stephensi* adult mosquitoes using RNA sequencing (RNA-Seq). A total of 5,354 differentially expressed genes (DEGs) are identified over the course of fungal infection. When the fungus grows on the mosquito cuticle, up-regulated DEGs include adhesion-related genes involved in cuticle attachment, Pth11-like GPCRs hypothesized to be involved in host recognition, and extracellular enzymes involved in the degradation and penetration of the mosquito cuticle. Once in the mosquito hemocoel, the fungus evades mosquito immune system probably through up-regulating expression of β-1,3-glucan degrading enzymes and chitin synthesis enzymes for remodeling of cell walls. Moreover, six previous unknown SSCP (small secreted cysteine-rich proteins) are significantly up-regulated, which may serve as "effectors" to suppress host defense responses. *B. bassiana* also induces large amounts of antioxidant genes to mitigate host-generated exogenous oxidative stress. At late stage of infection, *B. bassiana* activates a broad spectrum of genes including nutrient degrading enzymes, some transporters and metabolism pathway components, to exploit mosquito tissues and hemolymph as a nutrient source for hyphal growth. These findings establish an important framework of knowledge for further comprehensive elucidation of fungal pathogenesis and molecular mechanism of *Beauveria*-mosquito interactions.

insect fungal pathogen, fungus-insect interaction, fungal pathogenesis, RNA-Seq, vector control

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INTRODUCTION

Mosquitoes transmit a wide range of diseases (e.g. malaria, Dengue fever, yellow fever and Zika virus) which have devastating impacts on human health (Li et al., 2016b; Medlock et al., 2012). Malaria is one of the most deadly diseases, infecting 300–500 million people annually and causing about 1 million deaths every year (Murray et al., 2012). *Plasmodium*, the causative agent of malaria, is absolutely dependent on completing a complex cycle in the vector *Anopheles* mosquito for transmission (Wang and Jacobs-Lorena, 2013). Thus, eliminating the mosquitoes or interfering with its ability to support the parasite cycle will arrest malaria transmission (Wang and Jacobs-Lorena, 2013). Vector control via use of

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insecticides is a major tool to reduce disease transmission. However, intensive use of insecticides not only poses risks to human and environment, but also creates intensive pressure for mosquitoes to develop resistance. Therefore, alternative tools to control mosquitoes are urgently needed. Entomopathogenic fungi, such as *Beauveria bassiana*, one of the US Environmental Protection Agency (EPA)-approved biological control agents, offer an environmentally friendly alternative to chemical pesticides (Fan et al., 2012a; Kanzok and Jacobs-Lorena, 2006), and have been proposed as the next generation of agents for the control of mosquitoes (Knols et al., 2010).

B. bassiana is a well-known fungal pathogen of terrestrial insects and can kill adult *Anopheles* mosquitoes to reduce malaria transmission (Blanford et al., 2005; Enserink, 2005). The infection of mosquitoes by *B. bassiana* begins with conidial adhesion to the hydrophobic surface of the vector cuticle. Then, fungal conidia germinate to form germ tubes, the tips of which further expand and develop into infection structures that facilitate to penetrate the cuticle. Following reaching the mosquito hemocoel, fungal filaments switch into hyphal bodies through yeast-type budding for rapid proliferation, and evasion of the host immune responses. Fast propagation of hyphal bodies facilitates the deprivation of nutrients to expedite the death of mosquitoes. Eventually, hyphae emerge from the mosquito cadavers and produce asexual conidial spores for next infection cycle (Shang et al., 2015; Wang and Wang, 2017).

Extensive molecular and genetic studies have revealed that insect cuticle degrading enzymes including proteases (Zhang et al., 2009), chitinases (Fang et al., 2005) and cytochrome P450 enzymes (Zhang et al., 2012), genes involved in oxidative stress responses such as catalases (Wang et al., 2013) and superoxide dismutases (SOD) (Xie et al., 2012), and mitogen-activated protein kinase (MAPK) signaling cascades (Luo et al., 2012; Zhang et al., 2010; Zhang et al., 2009) are important for *B. bassiana* pathogenesis. Other virulence-related proteins like a neuronal calcium sensor BbCsa1 regulating extracellular acidification (Fan et al., 2012b), a GH73 family of β-1,3-glucanosyltransferase BbGas1 (Zhang et al., 2011a) maintaining cell wall integrity and a zinc-finger transcription factor BbMsn2 (Liu et al., 2013) have also been characterized in *B. bassiana*. *B. bassiana* also produces a large array of insect toxic secondary metabolites including polyketides (e.g., oosporein (Feng et al., 2015), bassianin (Jeffs and Khachatourians, 1997) and tenellin (Eley et al., 2007)) and non-ribosomal peptides (e.g., beauvericin (Xu et al., 2008), bassianolides (Xu et al., 2009) and beauveriolides (Namatame et al., 2004)) (Xiao et al., 2012). Especially, the oosporein could inhibit the PPO activity and down-regulate expression of antifungal peptide genes in insects (Feng et al., 2015). Although previous studies have identified many factors required for *B. bassiana* pathogenicity, it remains to provide a genome-wide viewpoint for fungal pathogenesis.

The availability of annotated draft genome and expression profiles in different environmental niches provide important information on *B. bassiana* gene expression involved in signal transduction, secreted proteins and metabolism (Xiao et al., 2012). However, the transcriptional responses of *B. bassiana* to locust hind wings, cotton bollworm hemolymph and corn root exudates (Xiao et al., 2012) do not fully represent infection processes or delineate fungal *in vivo* gene expression patterns during infection. The timing and sequential expression of *B. bassiana* virulence-related genes during infection of mosquitoes are still unknown. Better understanding of gene expression patterns is needed to comprehensively elucidate fungal infection processes and interactions with insect hosts.

In this study, we performed next-generation RNA sequencing (RNA-Seq) to provide the first *in vivo* transcriptomic profiling of *B. bassiana* during infection of *Anopheles stephensi* adult females at four distinct infection stages. We compared two neighboring time point data to document the stage-specific gene expression patterns and reveal gene modulations involved in infection structure differentiation, cuticle penetration, hemocoel adaptation, evasion of host immunity and hyphal growth. This work facilitates comprehensive understanding of *B. bassiana* pathogenesis and interaction with mosquito host, and underlines the importance of resolving the infection process to better understand fungi-insect interactions.

RESULTS

Infection processes of *B. bassiana* in *A. stephensi* female adults

To characterize the molecular pathogenesis of *B. bassiana* to mosquito *A. stephensi*, a dynamic infection process was investigated using both scanning electron microscopy (SEM) for observation of fungal growth on the mosquito's integument, and light microscopy for tracking fungal differentiation and interactions with mosquito immune responses. The spore suspensions of *B. bassiana* strain Bb252 (1×10^8 conidia mL^{-1}) were sprayed on *A. stephensi* female adults. As shown in Figure 1A, initially a large number of *B. bassiana* conidia adhered to the mosquito cuticles. At 36 hours post topical inoculation (hpi), the conidia germinated to form germ tubes, differentiated to infection structures and penetrated the cuticle. At 60 hpi, the fungus reached the mosquito's hemocoel, and triggered the host immune responses (encapsulation and melanization). Invasive mycelia transformed to the hyphal bodies phase (yeast-like cells) for rapid proliferation and evasion of mosquito immune responses around 84 hpi. At 108 h after infection, *B. bassiana* grew mainly as the multicellular hyphae form in the nutrient-rich hemolymph. Finally, abundant fungal mycelia grew

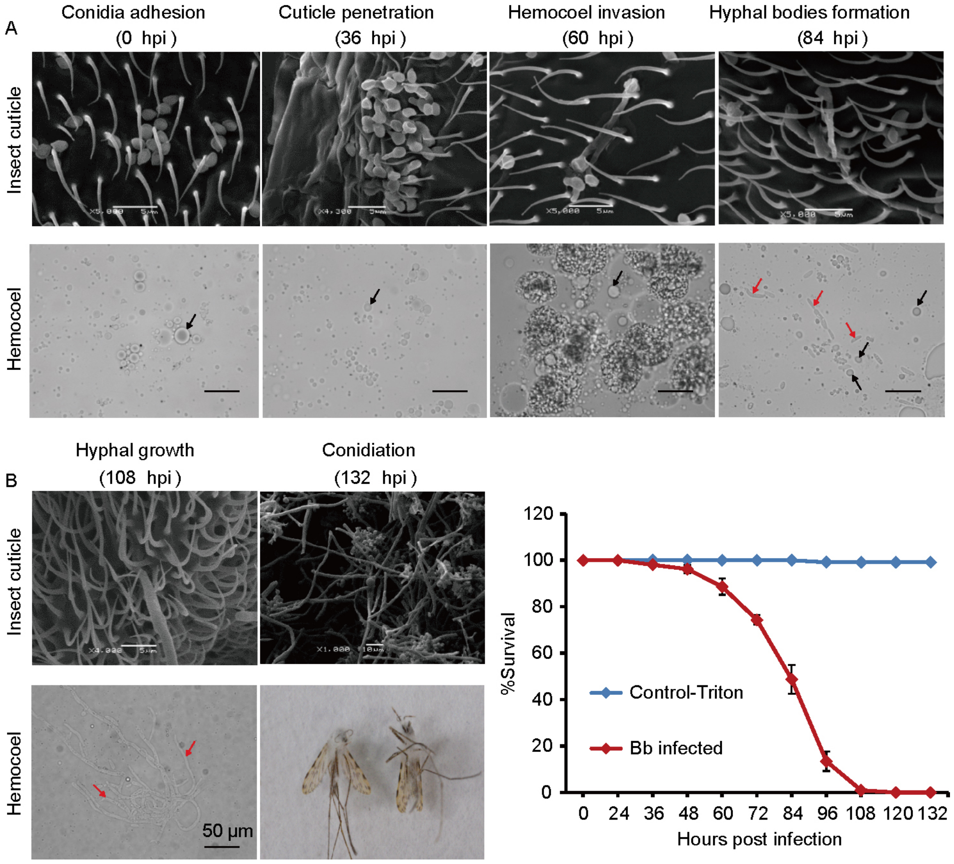

Figure 1 Tracking infection processes of *B. bassiana* Bb252 in *Anopheles stephensi* female adults. A, Infection process was monitored using both scanning electron microscopy (SEM) and light microscopy. Black arrow and red arrow in light microscopy indicate mosquito hemocytes and fungal hyphal bodies or mycelia, respectively. Bar, 50 μm. B, Survival of *A. stephensi* female adults following topical application of spore suspension of *B. bassiana* Bb252 (1×10^8 conidia mL^{-1}). Control mosquitoes were treated with 0.01% Triton X-100. Error bars indicate standard errors.

out of the mosquito cadavers and produced conidia for the next infection. The mosquito survival was high at 24, 36 and 48 hpi, but was dramatically decreased 60 h after infection (Figure 1B), reflecting that the formation of hyphal bodies coincides with the fatal aspect of infection (such as toxin secretion). Based on these distinct features, we defined four stages of infection: cuticle penetration (~36 hpi), hemocoel invasion (~60 hpi), hyphal body formation (~84 hpi) and hyphal growth (~108 hpi), respectively.

Identification and functional categories of differentially expressed genes in *B. bassiana* during infection of mosquitoes

To delineate the dynamic transcriptional reprogramming of *B. bassiana* infection, genome-wide transcriptome analysis was investigated by RNA-Seq in *B. bassiana* Bb252 during *in vivo* infection of *A. stephensi* female adults. The samples were collected at 36, 60, 84 and 108 hours after topical inoculation, respectively. The sample of conidia used for inoculation was used as the initial time point (Bb_0). Except for Bb_0 which obtained 25 million total reads, approximately 50 million reads were obtained at each follow-up time point. The reads obtained at different infection stages were aligned with *B. bassiana* genome to construct transcriptome maps during the progression of infection.

To gain insights into infection stage-specific transcriptional changes in *B. bassiana*, trancriptome at each time point was compared with the former time point to identify differentially expressed genes (DEGs). Over the 108 h course, a total of 5,354 genes were differentially regulated positively or negatively with ≥2-fold change (adjusted $P<0.05$) between two or more of the infection stages, with a core set of 290 (5.4%) genes being common to all time points of infection (Figure 2A, Figure S1 in Supporting Information), indicating that the gene transcription throughout the course of infection was highly dynamic. Relative to the former time point, more genes exhibited marked up-regulation at cuticle penetration (1,637 up-regulated) than other three distinct infection stages (Figure 2B), while more genes showed down-regulation at hemocoel invasion (2,007 down-regulated) (Figure 2C), showing a rapid modulation of the transcriptome at the early infection stages when growth on mosquito cuticle. Consistently, more genes were exclusively regulated at cuticle penetration (626 genes) and hemocoel invasion stage (552 genes) than those at hyphal body formation (259 genes) and hyphal growth stage (356 genes) (Figure S1 in Supporting

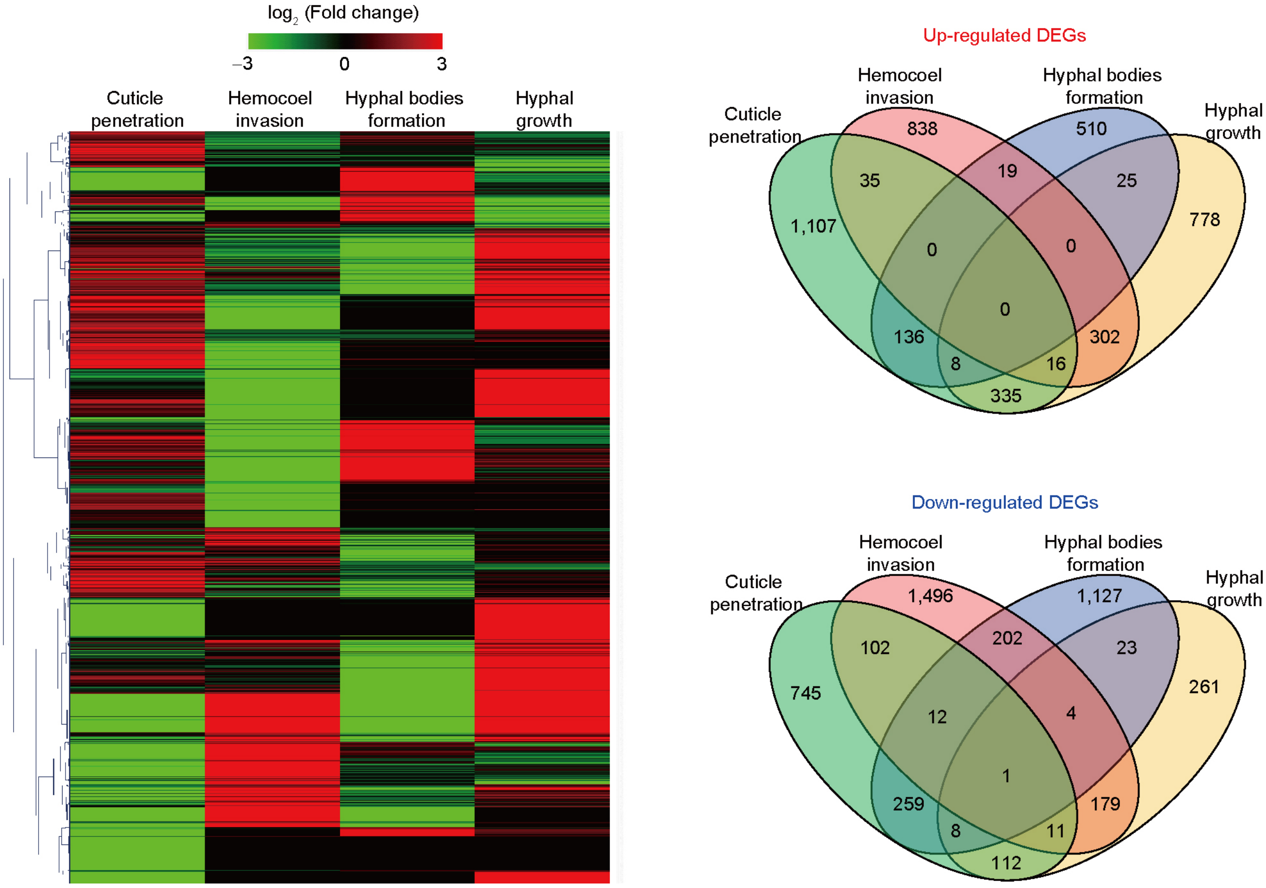

Figure 2 Transcriptional profiles for the whole transcriptome data. A, Heat maps and hierarchical clustering analysis of differentially expressed genes (DEGs) in *B. bassiana* Bb252 during infection in *Anopheles stephensi* mosquitoes. The expression of each gene was obtained by calculating fragments per kilobase per million (FPKM). DEGs in each infection stage were identified with $\log_2$(fold change) of ≥1 by comparison between neighboring time points. Colors from black to red indicate up-regulation; colors from black to green indicate down-regulation. B and C, Venn diagram showing the shared and unique DEGs among the four infection stages. The overlapping regions represent genes that were concomitantly regulated in two, three or four infection stages. B, up-regulated DEGs. C, down-regulated DEGs.

Information). To validate the differential expression of *B. bassiana* genes during the infection process against *A. stephensi*, twelve DEGs identified in the transcriptomic analysis were selected for quantitative PCR analysis (Table S1 in Supporting Information). Overall, 10 out of 12 genes (83.3%) were consistent in expression patterns between qPCR and RNA-Seq data (Figure S2 in Supporting Information, Table 1).

To explore potential biological functions of theses DEGs, Gene Ontology (GO) enrichment analysis was performed. At cuticle penetration stage (36 hpi), DEGs that were activated in response to mosquito cuticles were enriched for GO terms such as chitin synthase for cell wall modification of appressoria (Gao et al., 2011), threonine-type peptidase for degradation of insect cuticle (Gao et al., 2011), G-protein coupled receptor protein signaling pathway for transduction of extracellular signals leading to infection-specific development (Xue et al., 2008) (Figure 3, Table S2 in Supporting Information). In addition, DEGs that were repressed at the stage of infection structure formation were associated with actin filament organization, cellular membrane organization and coenzyme biosynthetic process. During hemocoel invasion (60 hpi), enriched up-regulated genes included those involved in biosynthetic process of amino acid, nitrogen compound and small molecule, intracellular transport and RNA binding. In contrast, GO enrichment analysis showed that enrichment of the down-regulated genes are involved in carbohydrate biosynthetic process, small molecule catabolic process, signaling pathways and oxidoreductase activity (Figure 3, Table S2 in Supporting Information). When *B. bassiana* colonized in the mosquito hemocoel (hyphal body formation stage, ~84 hpi), GO terms such as small GTPase mediated signal transduction, establishment of protein localization, protein transport and ncRNA processing were highly over-represented in the up-regulated genes, while biosynthetic process of amino acid, nitrogen compound and small molecule, vesicle-mediated transport and negative regulation of biological process were enriched in the down-regulated genes (Figure 3, Table S2 in Supporting Information). At the late stage of infection when the fungus grew in the mosquito

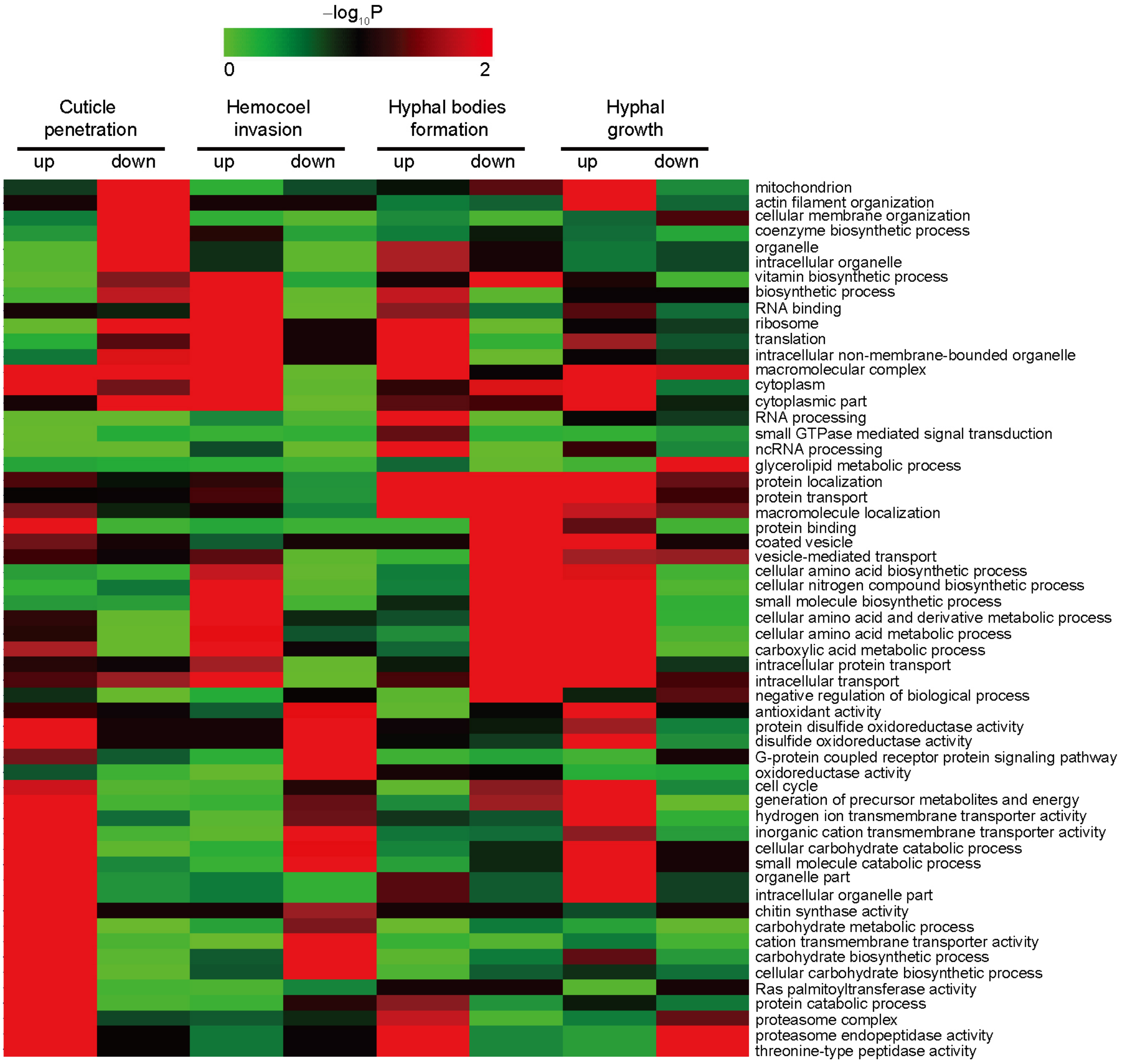

Figure 3 Heat maps and hierarchical clustering showing functional GO enrichment of differentially expressed genes (DEGs) in *B. bassiana* Bb252 during infection in *Anopheles stephensi* mosquitoes. Colors from black to red indicate significant enrichment (P<0.05); colors from black to green indicate non-significant enrichment.

tissue (108 hpi), the majority of DEGs were involved in cellular and metabolic process of carbohydrate, amino acid and nitrogen compound, generation of precursor metabolites and energy, protein transport, actin filament organization and antioxidant activity (Figure 3, Table S2 in Supporting Information). KEGG classification was also used to analyze the corresponding pathways involved in fungal infection. The up-regulated genes at the stages of cuticle penetration (36 hpi) and hyphal growth (108 hpi) were enriched in carbon metabolism, biosynthesis of amino acids, proteasome, and signaling pathways, while these pathways were over-represented in the down-regulated genes at stages of hemocoel invasion and hyphal body formation (Figure 4, Table S3 in Supporting Information). Other notable functional groups were Cytochrome P450, protein processing, fatty acid metabolism and oxidative phosphorylation in the up-regulated genes at stages of hemocoel invasion and hyphal body formation.

Expression profiling of *B. bassiana* during infection of *A. stephensi* mosquitoes

Cuticle penetration stage

B. bassiana conidia must adhere to the mosquito cuticle to initiate infection. One adhesin (BBA_02419) homologous to MAD1 in *Metarhizium anisopliae*, another entomopathogenic fungus used in biocontrol (Wang and St. Leger, 2007a), was up-regulated by 47-fold change at 36 hpi compared to the initial state (Table 1). Cell wall proteins in *M. anisopliae* (Li et al., 2010) and hydrophobins in *B. bassiana* (Zhang et al., 2011b) were also identified to mediate conidial adhesion to insects. Besides adhesion, two cell wall proteins (BBA_05808 and BBA_03246) with homolog in human opportunistic fungal pathogen *Aspergillus fumigatus* encoding cell wall galactomannoprotein (Mp1) conferring spore adhesion to mucosal surfaces (Upadhyay et

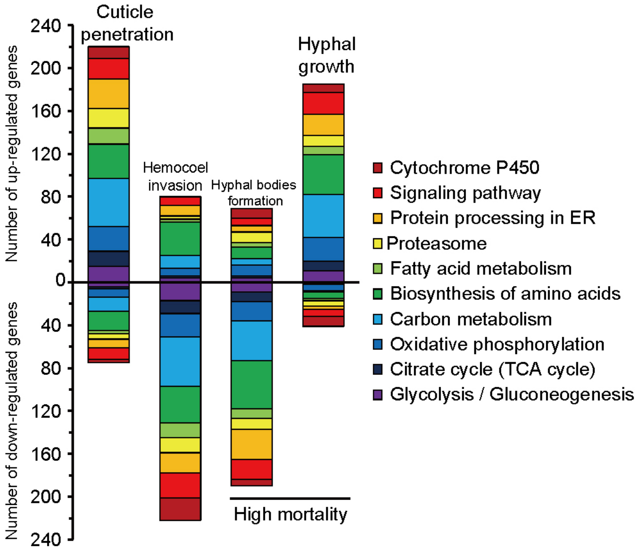

Figure 4 Distribution of selected KEGG functional groups within up- and down-regulated genes in *B. bassiana* Bb252 during infection in mosquitoes. The bar chart corresponds to the matched entries of the differentially expressed genes (DEGs) in their own functional category.

al., 2008) were also dramatically up-regulated (FC>20) (Table 1). Interestingly, three out of four genes encoding cell surface proteins (BBA_02353, BBA_03121, and BBA_09174) were dramatically induced at 36 hpi (especially for BBA_09174 with FC=4,717) and then gradually decreased to low levels. In contrast, two of four hydrophobins (BBA_03015 and BBA_06599) were highly expressed in spores, but down-regulated after spore adhesion (Table 1).

In order to sense and rapidly respond to changes in nutrient availability (e.g. mosquito cuticle), *B. bassiana* needs G-protein coupled receptors (GPCRs) to transduce extracellular signals for nutrient sensing, stress response and infection-specific development such as infection structure formation (Xiao et al., 2012; Xue et al., 2008). Approximately half of *B. bassiana* GPCRs (22 of 43 GPCRs) are similar to the rice-blast fungus *Magnaporthe oryzae* Pth11-like proteins (Kulkarni et al., 2005; Xiao et al., 2012). Our transcriptome analysis showed that only two Pth11 homologs (BBA_00828 and BBA_05185) were highly induced on the mosquito cuticle (Table 1). However, other two Pth11-like protein genes (BBA_02401 and BBA_09882) were down-regulated when growth on mosquito cuticle, indicating that these two genes may not be involved in transduction of extracellular signals from mosquito host. In addition, STM1-like GPCR homolog (BBA_08155) required for triggering adaptation to nitrogen starvation in fission yeast *Schizosaccharomyces pombe* (Chung et al., 2001) and expressed in *Metarhizium* when growth on insect cuticles (Gao et al., 2011), was down-regulated (FC>5) in *B. bassiana* (Table 1). The up-regulation of G-protein alpha subunit (BBA_05207), protein kinase C (BBA_04438), two Calcium/calmodulin-dependent protein kinases (BBA_00215 and BBA_01650) and three mitogen-activated protein kinase (MAPK) cascade kinases (BBA_00937, BBA_01244 and BBA_07991) at 36 hpi compared to that at the initial state indicated that calcium-dependent pathway and MAPK pathway were strongly activated in *B. bassiana* during infection of *A. stephensi* (Table 1). The cAMP-dependent protein kinase catalytic subunit protein kinase A (PKA) is required for appressoria differentiation and full virulence in *M. robertsii* and some plant pathogenic fungi (Fang et al., 2009; Lengeler et al., 2000; Mitchell and Dean, 1995). However, down-regulation of adenylate cyclase (BBA_02117, FC>3) and no expression change of PKA (BBA_04346) (Table 1) were observed in *B. bassiana* during early infection.

To penetrate the insect cuticle, germ tubes of *B. bassiana* develops into mature infection structures which is achieved through accumulation of high concentrations of glycerol and glycogen in the infection structure (Wilson and Talbot, 2009). About 25% of the genes (73 out of total 283) involved in lipid and fatty acid metabolism as defined by Funcat were actively induced at 36 hpi. Especially, a perilipin-like protein (BBA_08759) that controls cellular lipid storage and therefore generates turgor pressure for infection structure penetration (Wang and St. Leger, 2007b) was significantly up-regulated by more than 40-fold at cuticle penetration stage (Table 1).

During penetration of the mosquito cuticles, *B. bassiana* needs to detoxify epicuticle compounds such as alkanes and lipids with antifungal activities that inhibit spore germination and hyphal growth (Pedrini et al., 2013). Several related genes were significantly up-regulated, including one member of Cytochrome P450 subfamily CYP52 (BBA_00965) (Table 1) which catalyzes the first step in the ω-oxidation pathway of alkanes (Zhang et al., 2012), and six secreted lipase enzymes required for detoxification of cuticular lipids (Table S3 in Supporting Information). Proteases and chitinases are secreted later at very high levels to digest protein-chitin rich procuticles (St. Leger et al., 1996). Although *B. bassiana* encoded 352 proteases, only about 20% of the genes were most highly expressed at the early stage of infection in a previous study (Gao et al., 2011) (Figure 5A). Different from *Metarhizium* spp. in which the most trypsins were expressed during the early infection (Gao et al., 2011), subtilisin type (seven genes) and carboxypeptidase type (five genes) serine proteases involved in insect cuticle degradation (Bagga et al., 2004) and eight aspartyl proteases which assisted the human pathogen *Candida albicans* to degrade cell surface molecules (Schaller et al., 2005) were over-represented in the up-regulated secreted proteases. Chitin constitutes up to 40% of the procuticle but is absent in the epicuticular layer (Merzendorfer and Zimoch, 2003). As expected, among 23 GH18 chitinases in *B. bassiana*, seven chitinases were rapidly induced at 36 hpi and five chitinases were highly expressed at 60 hpi to promote mosquito cuticle chitin digestion and procuticle penetration.

Hemocoel invasion and hyphal body formation stages

After *B. bassiana* penetrates into the insect hemocoel (60 hpi), the fungus has to colonize the mosquito hemolymph where acute immune responses, such as hemocyte ingestion, encapsulation, melanization, and expression of antifungal peptides occur (Gottar et al., 2006). Once inside the insect hosts, fungal hyphae undergo changes to form a yeast-like hyphal body phase for rapid proliferation and evasion of host immune responses. The osmosensor (BBA_08887) was significantly up-regulated (~2-fold) in *B. bassiana* at cuticle penetration stage but decreased to low expression level after colonization in mosquito hemocoel (60 and 84 hpi) (Table 1). The mitogen-activated protein kinase HOG1 (BBA_05209) decreased at hemocoel invasion stage (60 hpi), but was highly expressed at hyphal body formation stage (84 hpi) (Table 1).

Since mosquito cellular immune responses are triggered by detection of fungal cell wall structures (such as β-glucans and chitin), the remodeling of cell walls facilitates the fungus to evade host recognition. Two GH128 family of β-1,3-glucanases (BBA_03167 and BBA_04956), a GH55 β-1,3-glucanase precursor (BBA_03717) and four GH72 family of β-1,3-glucanosyltransferases (BBA_03082, BBA_03990, BBA_04640 and BBA_08600) including a previously known BBGAS1 (BBA_04640) (Zhang et al., 2011a) were significantly up-regulated before the fungus penetrating into the hemocoel (60 hpi) (Table 1). In addition, expression patterns of three chitin synthases (BBA_03590, BBA_04667, and BBA_06859) were significantly lower at 60 hpi, while one chitin synthases (BBA_03236), one chitin synthase activator (BBA_10281) as well as one chitin synthase chaperone-like protein Chs7 (BBA_03793) were down-regulated at hyphal body formation stage (Table 1). A previous study showed that a chitosanase gene in *B. bassiana* strain ARSEF 2860 was specifically highly induced in response to cotton bollworm hemolymph (Xiao et al., 2012). But both chitosanase (BBA_05880) and its precursor (BBA_06270) were not detected during *B. bassiana* Bb252 infection in *A. stephensi*. Moreover, we found that 63 proteases including 27 serine proteases (15 of which were up-regulated during cuticle penetration) were switched off to avoid triggering melanization response when *B. bassiana* grew in the insect hemolymph (84 vs. 60 hpi) (Figure 5A). Taken together, these data suggested that differentially regulated genes involved in cell wall remodeling and proteases enable *B. bassiana* to evade immune responses of *A. stephensi*.

Except for evasion of host immunity, several virulence factors or effectors have been reported to play important roles in modulating host innate immunity (Oliva et al., 2010; Stergiopoulos and de Wit, 2009; Wang et al., 2016). In *B. bassiana* Bb252, thirty, six and 19 out of 220 small secreted cysteine rich proteins (SSCPs) were up-regulated since hemocoel invasion stage, which might function as effectors. Among them, we found a LysM (the Lysin Motif) domain-containing protein (BBA_08602) which binds chitin to suppress chitin-triggered immunity in *M. oryzae* (Mentlak et al., 2012), a CFEM (common in fungal extracellular membrane) domain-containing protein (BBA_09339) which functions in modulating iron metabolism during infection (Nasser et al., 2016), a blastomyces yeast-phase-specific protein (Bys1, BBA_04263) which regulates dimorphic transition in the human pathogenic fungus *Blastomyces dermatitidis* (Bono et al., 2001; Krajaejun et al., 2010), a filamentous hemagglutinin (BBA_03909) and two GPI anchored glycoprotein (BBA_01335 and BBA_09144) were up-regulated in *Beauveria* after reaching the mosquito hemolymph (Table 1). A polyketide synthase (PKS, BBA_08179) required for synthesis of the oosporein, which functions as effector to inhibit the PPO activity and suppress antifungal peptide expression (Feng et al., 2015), was up-regulated by more than 2-fold during cuticle penetration (36 hpi) (Table 1). ATP-binding cassette (ABC) transporters are usually involved in protecting the pathogen against host-produced secondary metabolites (Morschhäuser, 2010). Eleven ABC transporters were highly expressed during fungal penetration and hyphal body formation stage (Figure 5B). Moreover, rapid defense responses of mosquitoes such as production of reactive oxygen play an important role in killing pathogens (Kumar et al., 2010; Sexton and Howlett, 2006). In total, two catalases, three superoxide dimutases (SODs), ten thioredoxin-like proteins and eleven glutathione S-transferases (GSTs) showed up-regulated expression when penetration and growth in mosquito hemolymph to suppress host defenses (Figure 5C).

Hyphal growth stage

After successful colonization in the mosquito hemocoel, *B. bassiana* uses the trehalose-rich hemolymph for hyphal growth (Xu et al., 2015). *B. bassiana* encodes two trehalase genes (GH37), and one of them (BBA_07623) was strongly induced in the mosquito hemolymph (hyphal growth vs. hyphal body formation) to degrade the disaccharide (Table 1). One aspartyl protease (A01 pepsin), eight cysteine peptidases, 15 metalloproteases including two M01 aminopeptidases and two M24 methionyl aminopeptidases, and 13 serine proteases (five S08 and S53 subtilisins, one S10 carboxypeptidase, one S14 Clp peptidase, two S28 lysosomal Pro-Xaa carboxypeptidases, and four S33 prolyl aminopeptidases) were over-represented in the up-regulated proteases during hyphal growth (Figure 5A). Especially, a serine protease S08 (BBA_08695) and an aspergillopepsin-2 heavy chain protein (BBA_08462) belonging to glutamic peptidases were only highly expressed at later infection stage (108 hpi) but were not detected in spores or cuticle penetration stage, indicating that they might be specific for degradation of protein-rich mosquito tissues.

Fungi express membrane proteins to transport amino acids and small peptides (2–5 aa) into the cell for use as nutrients, whereas larger peptides cannot be assimilated. The major facilitator superfamily (MFS) proteins are typically involved in the transport of a wide range of substrates and may function as nutrient sensors (Morschhäuser, 2010). Thirteen MFS transporters showed pronounced up-regulation at 108 hpi (Figure 5B). Similarly, amino acid and peptide transporters including 3 aa/auxin permease (AAAP) family genes, 7 aa/polyamine/organocation (APC) family genes, three oligopeptide transporter (OPT) family genes and seven ABC transporters were also induced. A vast array of metabolism-related genes were induced when fungal growth in mosquito hemolymph. These included 33, seven and six genes involved in carbohydrate, amino acid and fatty acid metabolism, respectively (Figure 4, Table S3 in Supporting Information). These data suggested that *B. bassiana* up-regulates a broad spectrum of genes such as nutrient degrading enzymes, related transporters and metabolism pathway components to make use of hemolymph and mosquito tissues as a nutrient source for rapid colonization, hyphal growth and sporulation.

DISCUSSION

B. bassiana, one of the best-studied and most widely used biological control agents, can efficiently kill adult mosquitoes and reduce malaria transmission (Kanzok and Jacobs-Lorena, 2006). The infection process of *B. bassiana* in *Anopheles* female adults and mechanisms behind the fungus-mosquito interaction are of particular interest and great importance for development of cost-effective mycoinsecticides. In this study, we described the first *in vivo* transcriptome profiles of *B. bassiana* during infection of *A. stephensi* to show dynamic gene expression patterns at a genome-wide level and to provide comprehensive understanding of *B. bassiana* pathogenesis and interaction with mosquito host.

Notably, we observed that the adhesins, cell wall proteins and cell surface proteins were dramatically up-regulated when *B. bassiana* growth on the mosquito cuticle. Especially, a previously unknown cell surface protein (BBA_09174) was induced with 4,717-fold change, indicating that it is required for host adhesion or recognition. Most of the adhesion-related genes were significantly decreased after spore adhesion, indicating that these extracellular proteins may facilitate *B. bassiana* conidia to attach to insect cuticle and thereby contribute to the fungal infection. Two homologs (BBA_00828 and BBA_05185) of the rice-blast fungus *M. oryzae* Pth11-like proteins (Kulkarni et al., 2005; Xiao et al., 2012) were the most up-regulated Pth11 type GPCRs, indicating their specific roles for early host recognition events. During cuticle penetration, *B. bassiana* also activated a proportion of genes encoding cytochrome P450s and lipases which target epicuticular alkanes and lipids (Zhang et al., 2012), and proteases and chitinases for procuticle degradation (Gao et al., 2011). Interestingly, although more than half serine proteases were up-regulated during cuticle degradation, *B. bassiana* switched off large amounts of serine protease genes when growth in mosquito hemolymph. Since fungal serine proteases can trigger the host melanization response via the proteolytic activation of prophenoloxidases (PPO) (St. Leger et al., 1996), reducing production of serine proteases could be considered as one mechanism of evasion of host immunity.

When growth in hemolymph, *B. bassiana* is subjected to increasing chemical (nutrient) and physical stress (osmotic) in mosquito hemocoel and has to transform into yeast-like cells (called hyphal bodies) for rapid proliferation. We found osmosensor (BBA_08887) mediating adaptation to the osmotic pressure of the insect hemocoel (Wang et al., 2008) and the MAPK HOG1 (BBA_05209) involved in response to osmotic/oxidative stress and heat shock (Jin et al., 2012; Zhang et al., 2009) were highly activated when growth in host hemolymph. Except a previously known β-1,3-glucanosyltransferases BBGAS1 (Zhang et al., 2011a), we also observed dynamic changes of several novel cell wall remodeling genes, including up-regulation of β-1,3-glucan degrading enzymes and down-regulation of genes involved in chitin synthesis, suggesting their potential roles in change of cell wall components (e.g. β-1,3-glucan and chitin) for evasion of host immune responses.

In plant pathogenic fungi and oomycetes, small secreted cysteine-rich proteins (SSCP) are used as effectors to undermine host innate immunity and enable fungal infection (Li et al., 2016a; Oliva et al., 2010; Stergiopoulos and de Wit, 2009). *B. bassiana* also encodes a large number of SSCPs, most of which are hypothetical proteins with unknown function. Different sets of SSCP-encoding genes were expressed at each infection stage, but the majority of these genes were strongly induced during hemocoel invasion. This suggests *B. bassiana* requires a maximum capacity for host manipulation during hemocoel invasion and that invasion hyphae provide a major interface for the delivery of effectors to the host cells. Especially, six previously unknown proteins with conserved functional domains were identified, including a LysM domain-containing protein (BBA_08602), a CFEM domain-containing protein (BBA_09339), a blastomyces yeast-phase-specific protein (Bys1, BBA_04263), a filamentous hemagglutinin (BBA_03909) and two GPI anchored glycoprotein (BBA_01335 and BBA_09144). In plant pathogen *M. oryzae*, secreted LysM protein 1 (Slp1) accumulates at the interface between the fungal cell wall and the rice

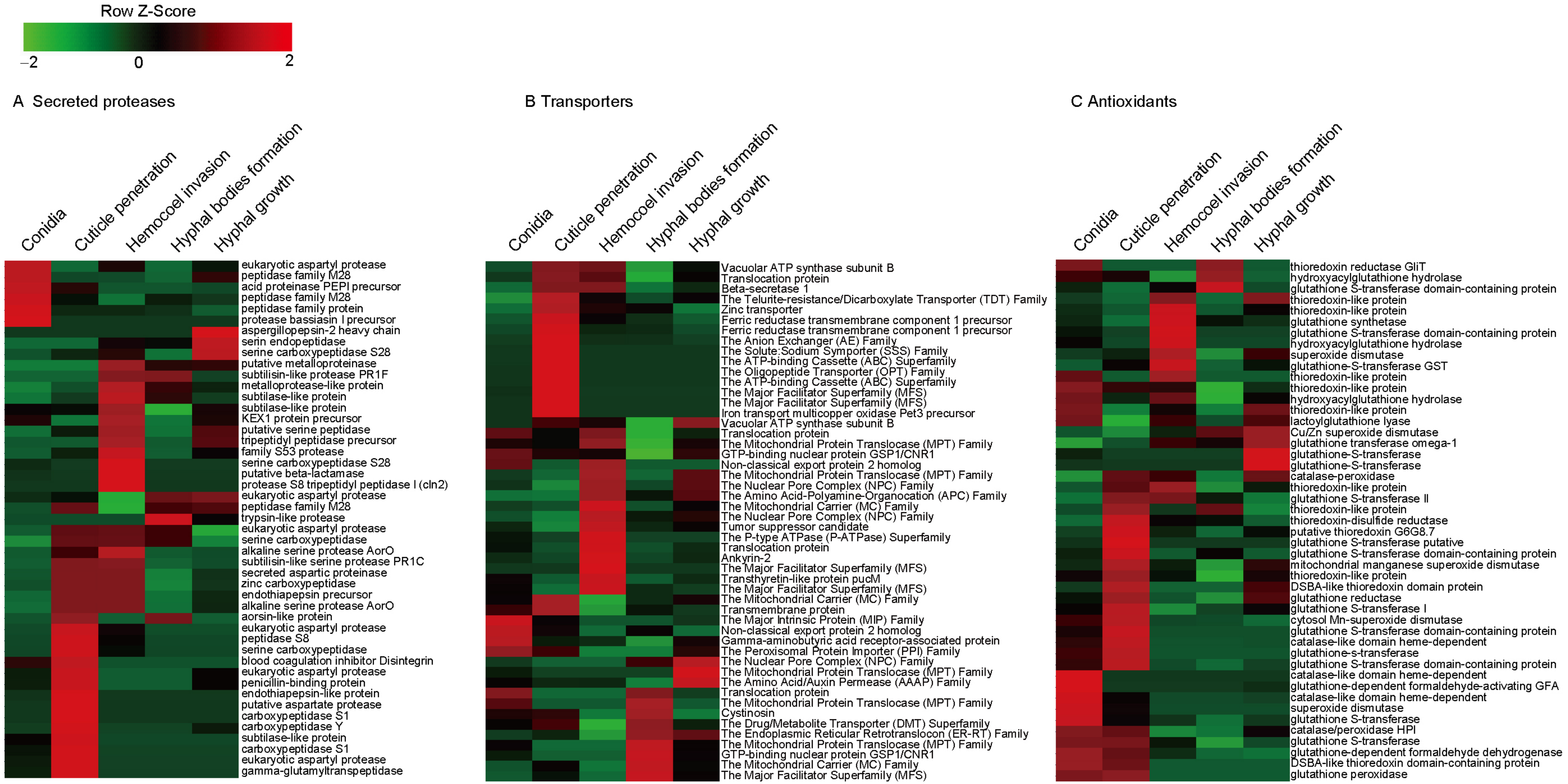

Figure 5 The detailed gene expression patterns of the top 50 differentially expressed genes from the selected functional groups in transcriptomic analysis. A, Secreted protease genes. B, Membrane transporter genes. C, Antioxidant genes.

plasma membrane, which can bind to chitin, and is able to suppress chitin-induced plant immune responses, including generation of reactive oxygen species and plant defense gene expression (Mentlak et al., 2012). CFEM proteins have been implicated in fungal haem-iron acquisition and pathogenicity, which bind haem, extract it from the host haemoglobin and deliver it to fungal cells (Nasser et al., 2016). Fungal pathogens use CFEM proteins to overcome host nutritional immunity (Nasser et al., 2016). Bys1 regulates dimorphic transition in a human pathogenic fungus *B. dermatitidis* and may play a role in cell wall formation or morphogenesis (Bono et al., 2001; Krajaejun et al., 2010). Taken together, these putative effectors may involve in *B. bassiana*-mosquito interaction, which require further functional verification and are being studied in our laboratory.

B. bassiana can produce a large array of biologically active secondary metabolites that might contribute to counter insect defenses (Xiao et al., 2012). The PKS required for oosporein synthesis was up-regulated by more than 2-fold during early infection. However, previous known virulence factors such as the non-ribosomal peptide synthetase (NRPS, BBA_09727) for beauvericin biosynthesis (Xu et al., 2008), BBA_02630 required for bassianolide (Xu et al., 2009), the PKS-NRPS hybrid (BBA_07338) required for tenellin biosynthesis (Eley et al., 2007) were not detected during *B. bassiana* infection in mosquitoes. Moreover, *B. bassiana* expressed large amounts of catalases, superoxide dimutases (SODs), thioredoxin-like proteins and glutathione S-transferases (GSTs) to counter host-generated exogenous oxidative stress when growth in mosquito hemolymph (Kumar et al., 2010; Sexton and Howlett, 2006).

At late infection stage, *B. bassiana* modulates the expression of enzymes including trehalase and proteases to use the nutrient-rich mosquito tissues for hyphal growth (Xu et al., 2015). The application of both endopeptidases (A01 pepsin, G01 scytalidoglutamic peptidase, cysteine peptidases, and S08 and S53 subtilisins) and exopeptidases (M01 aminopeptidase, M24 methionyl aminopeptidase, S10 carboxypeptidase, S28 lysosomal Pro-Xaa carboxypeptidase, S33 prolyl aminopeptidase) could enable highly efficient degradation of mosquito protein substrates (Martinez et al., 2012; Silva et al., 2006). In thermophilic filamentous fungus *Talaromyces emersonii*, aspergillopepsin is secreted for fungal growth, which is induced in the presence of an extracellular protein source but repressed by inorganic nitrogen (O'Donoghue et al., 2008). Consistent with this, aspergillopepsin was specifically expressed at hyphal growth stage. *B. bassiana* also activated genes encoding transport machineries to uptake extracellular nutrients, including the major facilitator superfamily (MFS) proteins involved in the transport of a wide range of substrates and nutrient sensing (Morschhäuser, 2010), amino acid and peptide transporters and the ATP-binding cassette (ABC) transporters.

This work provides evidence for *in vivo* transcriptional programming regarding the molecular mechanisms used by *B. bassiana* to confront mosquito host environments, including host recognition, cuticle degradation, cell wall remodeling, cell differentiation, immune evasion, stress resistance and detoxification. The elucidation of differential gene expression profiling during *B. bassiana* infection in mosquito establishes an important framework of knowledge for further investigations of the molecular interactions between *B. bassiana-Anopheles* mosquito, which may lead to novel approaches to control mosquitoes and intervene with the spread of vector-borne human diseases.

MATERIALS AND METHODS

Fungal culture and mosquito maintenance

Beauveria bassiana strain Bb252 was used for mosquito infection as it showed high virulence to mosquitoes in our previous screening (data not shown). Conidia of Bb252 were harvested from 7-day-old cultures on Sabouraud Dextrose Agar plus Yeast extract (SDAY) plates. Conidia suspensions were prepared in 0.01% (v/v) Triton X-100 and filtered through layers of sterile glass wool to remove hyphal fragments. *A. stephensi* (Dutch strain) mosquitoes were maintained on 10% sucrose at 26°C±1°C and 80%±5% relative humidity with a 12 h/12 h day-night cycle. Larvae were reared on cat food pellets and ground fish food supplement (Wang et al., 2012).

Fungal infection

To investigate infection process of *B. bassiana* Bb252, 5-day-old female adult mosquitoes were CO_2-anesthetized for 20–30 s and spread out on an ice-cold filter paper. The paralyzed mosquitoes were sprayed with 1 mL of fungal conidia suspension (1×10^8 conidia mL^{-1}) using atomizer, and subsequently maintained on 10% sucrose at 26°C and 80% humidity with a 12 h/12 h day-night cycle. Mortality was recorded every 12 h. Simultaneously, infection process in details was investigated using both scanning electron microscopy (SEM) for observation of fungal growth on mosquito's surfaces, and light microscopy for tracking of the fungal differentiation and interactions with mosquito immune responses.

Sample preparation, library construction and sequencing

Mosquitoes were collected after 36, 60, 84 and 108 hpi of *B. bassiana*, respectively. For each time course, about 50 infected female adults collected from two biological replicates were mixed. Mosquitoes were cold-anesthetized. The fungal conidia were used as control. The infected mosquito samples and fungal conidia were homogenized with beads in TRIzol reagent (Life Technologies, USA). Subsequently, total RNA was extracted using Direct-zol RNA Miniprep Kit (The Epigenetics Company, USA) followed by RNase-free

DNase I treatment. Messenger RNA (mRNA) was purified, and reverse-transcribed into cDNA libraries using TruSeq RNA Sample Prep Kit v2 (Illumina, USA) following manufacturer's instructions. Illumina sequencing was performed at Shanghai Biotechnology Corporation.

Assembly and annotation of transcriptomes

Before assembling the clean reads, the raw reads were preprocessed using filter-fq software. The raw reads containing only adaptor, sequences, reads with >5% unknown nucleotides, and low quality reads (reads containing more than 20% bases with Q-value$\leq$10) were removed. For the fungal transcriptome analysis, the obtained clean reads were aligned in paired-end mode to *B. bassiana* ARSEF 2860 genome (Accession No. ADAH00000000) using TopHat and assembled with Cufflinks with the default settings (http://cole-trapnell-lab.github.io/cufflinks/). The gene annotation was performed by FunCat (ftp://ftpmips.gsf.de/FGDB/). The potential secreted proteins were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The families of proteases were classified by full-length Blastp against the MEROPS peptidase database (E-value=1×10^{-4}) (http://merops.sanger.ac.uk/). Putative enzymes involved in carbohydrate utilization were identified by a combination of Blastp and HMMer searches against the carbohydrate-active enzymes database (http://www.cazy.org/). The cytochrome P450s were named according to the classifications collected at the P450 database (http://blast.uthsc.edu/). G-protein coupled receptors were selected from the best hits to GPCRDB sequences (http://www.gpcr.org/7tm/). Kinases were classified by Blastp against the KinBase (http://kinase.com/) with a cutoff E value of 1×10^{-30}. To identify the gene clusters and their proteins responsible for the biosynthesis of secondary metabolites, the whole genome data set was analyzed with the programs antiSMASH (http://antismash.secondarymetabolites.org/) with Hidden Markov model (HMM) signatures.

Differential expression genes, clustering and functional enrichment analysis

Differential expression genes (DEGs) were identified for each time point, which were compared to the data of the former infection time point using Cuffdiff. Differential expression was detected using $\log_2$(fold change)$\geq$1 and adjusted P<0.05. The combined transcriptomes were used as the background to search for GO terms enriched within the DEGs using http://bioinfo.cau.edu.cn/agriGO/ (Du et al., 2010) and a P<0.01 as the parameters for determining significantly enriched terms. Similarly, pathways significantly enriched with the DEGs were identified by mapping all DEGs to terms in the KEGG database using KOBAS2.0 (Xie et al., 2011) with a P<0.05.

Quantitative RT-PCR analysis

To validate the expression profile from RNA-Seq results, specific primers were designed to perform a quantitative RT-PCR analysis of twelve selected DEGs (Table S1 in Supporting Information). First-strand cDNA was synthesized from total RNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. The RT-PCR analysis was performed with the Thermo PikoReal 96 (Thermo, USA) using the AceQ® qPCR SYBR® Green Mix Master (Vazyme, Nanjing). A housekeeping gene *actin* was used as an endogenous control.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Venn diagram showing the shared and unique differentially expressed genes (DEGs) ($\log_2$(fold change) of ≥ 1) at four infection stages.

Figure S2 Quantitative RT-PCR analysis of twelve selected *B. bassiana* DEGs at different time points after infection in *A. stephensi*.

Table S1 Primers used in quantitative real-time PCR analysis

Table S2 Enriched GO terms in DEGs of each infection stage

Table S3 Enriched KEGG terms in DEGs of each infection stage

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