Analysis of Potential Molecular Targets in *Monochamus alternatus* (Coleoptera: Cerambycidae) Inoculated with *Beauveria bassiana* (Deuteromycotina: Hyphomycetes)[1]

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Abstract *Monochamus alternatus* Hope, the Japanese pine sawyer (Coleoptera: Cerambycidae), is a longhorn beetle that is a known vector of the pinewood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickel. *Beauveria bassiana* (Balsamo) Vuillemin is an entomopathogenic fungus that is widely used as a microbial control agent because of its ease of mass production and safety to most vertebrates. To identify molecular targets that are potentially associated with *B. bassiana* toxicology, differentially expressed gene (DEG) libraries of *M. alternatus* contacted with *B. bassiana* have been prepared. The transcripts are sequenced using the Ion Proton platform; We identify 5,637, 9,181, and 1,787 sequences that involved cellular components, molecular functions, and biological processes, respectively. Fifty DEGs are enriched in the metabolism of xenobiotics by the cytochrome P450 pathway, and 33 DEGs are enriched in insect hormone biosynthesis by a Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the DEGs. The pathways associated with these unique candidate targets yield new insights that will lead to an improved understanding of their functions and relationships. Artificial utilization of *B. bassiana* may be beneficial to the biological control of *M. alternatus* and other pests.

Key Words Japanese pine sawyer, differentially expressed genes, pathways, entomopathogenic fungus

The Japanese pine sawyer *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is a longhorn beetle that is recognized as a vector of the pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickel, which causes pine wilt disease. This beetle is widely distributed in East Asian countries including Japan, China, South Korea, and Taiwan, where it acts as a vector of pine wilt disease (Aikawa et al. 2014).

The most common method to prevent pine wilt disease from spreading is to control *M. alternatus*. The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) is suggested as a promising control agent of *M. alternatus* larvae (Shimazu and Kushida 1983). *Beauveria bassiana* is an entomopathogenic fungus that is widely used as a microbial control agent in many countries because of its ease of mass production

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and safety to most vertebrates (Boucias and Pendland 1998). This fungus is cultured on nonwoven fabric strips and placed as bands around the trunks of infested trees and obtains relatively high mortality levels of the larvae (Shimazu et al. 1995). This method of application is thought to be the most convenient and effective method for using *B. bassiana* to control *M. alternatus* larvae (Shimazu 1994).

Various methods of application of this fungus have been attempted to prevent the prevalence of this disease by killing the *M. alternatus* that inhabits nematode-infested pine trees such as by implantation of wheat-bran pellets with *B. bassiana* in infested trees (Shimazu et al. 1992) and application of nonwoven fabric strips containing *B. bassiana* cultures onto infested trees (Shimazu et al. 1995). However, information of the effects of *B. bassiana* on *M. alternatus* at the molecular level is sparse. In the present study, a differentially expressed gene (DEG) library of *M. alternatus* after short-term exposure to a sublethal concentration of *B. bassiana* is created, which provides a first step toward understanding the profile of *B. bassiana* targets in *M. alternates*. The results may provide insight for further exploration of target gene functions in detoxification and resistance to *B. bassiana*.

Materials and Methods

Insects and insecticide. *Monochamus alternatus* larvae were reared on artificial diets in complete darkness at room temperature and at 55% relative humidity (RH) until they reached the third instar. *Beauveria bassiana* was obtained from a commercially available strain (Guangzhou Duoyuduo Biotechnology Co., Ltd., China), cultured on nonwoven fabric strips according to the method of Shimazu et al. (1995), and stored in a refrigerator at 4°C before use. The density of conidia on the strips was $1–2 \times 10^8/cm^2$. *Monochamus alternatus* larvae were placed on the strips in contact with *B. bassiana* for 5 d. The number of conidia on the larvae that crawled on the strips was estimated to be 7×10^5 per individual. Whether the insect was killed by *B. bassiana* was determined by the growth of aerial mycelia and sporulation (Shimazu 2004). A control group of larvae was generated using the same procedure with the exception that water was used instead of *B. bassiana*. The experiment was performed in triplicate with 30 larvae per replicate. At the end of 5 d of inoculation, the larvae were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed.

Complementary (cDNA) library. Total RNA was extracted from whole bodies of the third-instar larvae using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The mRNA was enriched using oligo (dT) magnetic beads mixed with the fragmentation buffer and fragmented into short fragments. The first strand of cDNA was synthesized using random hexamer-primed reverse transcription. Buffer, dNTPs, Rnase H, and DNA polymerase I were added to synthesize the second strand. End reparation was then performed. Adaptors were ligated to the ends of these fragments. Finally, the fragments were enriched by polymerase chain reaction (PCR) amplification, then purified by magnetic beads and dissolved in the appropriate amount of Epstein-Barr solution. The library products were sequenced via the Ion Proton platform.

Screening of differentially expressed genes. We developed a strict algorithm to identify DEGs between two samples (Yu et al. 2006). Briefly, the *P*-value corresponds to the differential gene expression test. The false discovery rate (FDR) is a method for determining the threshold *P*-value in multiple tests. We set the FDR to a number that is not greater than 0.01 (Benjamini and Yekutieli 2001) and used FDR <0.001 and the absolute value of $\log_2$ ratio ≥ 1 as the threshold to judge significance differences in gene expression.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs. We used WEGO software (Ye et al. 2006) for GO functional classification of the DEGs and to understand the distribution of the gene functions of *M. alternatus*. The calculated *P*-value underwent Bonferroni correction using a corrected *P*-value of ≤ 0.05 as a threshold. The methods used in the KEGG pathway enrichment analysis are the same as that used in the GO analysis. Pathways with a *P*-value <0.01 are considered significantly enriched.

Real Time Quantitative PCR (RT-qPCR). We verified the Ion Proton data by RT-qPCR using the comparative threshold cycle ($\Delta\Delta$CT) method. Cytoplasmic actin of *M. alternatus* was used as the endogenous control. The RT-qPCR data were acquired on a LightCycler Real-Time PCR instrument using SYBR Premix Ex Taq™ (TaKaRa, Japan). For each cDNA, three RT-qPCR reactions were performed. The threshold cycle (CT) and relative expression levels were calculated using LightCycler480 1.5 software (Roche Diagnostics).

Results and Discussion

Gene expression profiles after *B. bassiana* inoculation. The DEGs in surviving *M. alternatus* larvae inoculated with *B. bassiana* are introduced in this paper. The effects of *B. bassiana* on *M. alternatus* are complex, although most of the DEGs are not the primary targets of *B. bassiana*. The pathways associated with these unique candidate targets yield new insights that will lead to a better understanding of their functions and relations.

DEGs were analyzed by pairwise comparisons of control and *B. bassiana*-inoculated *M. alternatus*. The unigenes detected with at least 2-fold differences in the two libraries are shown in Fig. 1 (FDR < 0.001). We found that 1,204 and 7,297 unique genes were significantly up- and down-regulated, respectively.

Gene ontology functional classification of DEGs. We identified 5,637, 9,181, and 1,787 sequences that were involved in cellular components, molecular functions, and biological processes, respectively. The genes were distributed among 51 categories including the developmental process, enzyme regulator activity, immune system process, negative regulation of biological process, and negative regulation of biological process. Cellular process and metabolic process were the most abundant GO biological process categories. The most abundant GO molecular function categories were catalytic activity and binding, and the most abundant GO cellular components were cell and cell part (Fig. 2). The highest-rated GO terms for the three GO categories are shown in Table 1.

KEGG pathway enrichment analysis of DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway enrichment

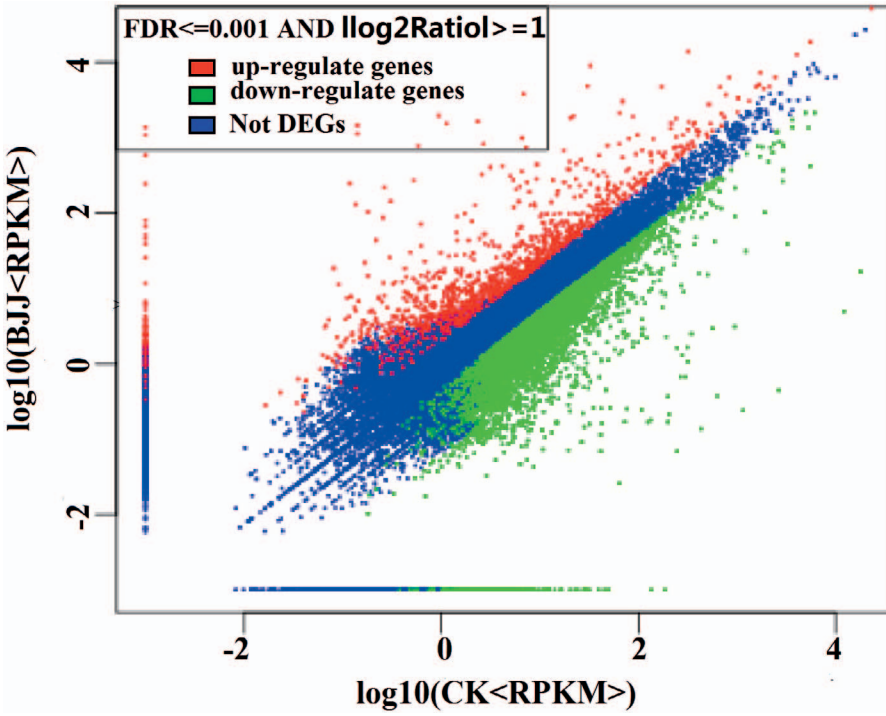

Fig. 1. Comparison of the gene expression levels between the control and *B. bassiana*-inoculated *M. alternatus*. The x and y axis represents $\log_{10}$ of the reads per kilobyte per million (RPKM) of the control and treated samples, respectively. The expression level of each gene is included in the volcano plot. The red, green, and blue dots represent transcripts that are more prevalent, present at a lower frequency, and did not change significantly in the *B. bassiana*-treated library, respectively.

analysis of DEGs showed that DEGs were enriched in 50 pathways with *P*-values <0.01. We generated a scatter plot of the KEGG enrichment results (Fig. 3).

Fifty DEGs were enriched in the metabolism of xenobiotics by the cytochrome P450 pathway (*P*-value = 0.005) including the DEGs encoding UGT (glucosyl/glucuronosyl transferases (0.45-fold), ADH1_7 (alcohol dehydrogenase 1/7), CYP1A1 (cytochrome P450, family 1, subfamily A), GST (glutathione S-transferase) (0.42-fold), CYP3A (cytochrome P450, family 3, subfamily A), fatty aldehyde dehydrogenase (0.1-fold), UDP-glycosyltransferase, and oxidoreductase (0.48-fold), all of which are down-regulated. The DEGs encoding CBR1 (carbonyl reductase 1), Allergen 5 (2.33-fold), hydroxybutyrate dehydrogenase type 2 (2.46-fold) and aldehyde dehydrogenase (NAD(P)+) are up-regulated.

Glutathione-S-transferases (GSTs) can be found in both humans and arthropods and can cause resistance to insecticides (Enayati et al. 2005) as well as protect against oxidative stress (Veal et al. 2002). GST activity is suppressed by five

Table 1. Significantly enriched gene ontology terms in differentially expressed genes (with *P*-values > 0.05).

Category	Gene Ontology Term	Cluster Frequency (%)	*P*-Value
Cellular component	Chromosomal part	3.0	0.00079
	Protein–DNA complex	0.9	0.00272
	Chromosome	4.0	0.00668
	Protein–carbohydrate complex	0.7	0.01137
	Organelle	49.3	0.04038
	Intracellular organelle	48.6	0.04626
Molecular function	Fatty acid synthase activity	1.4	2.46E-11
	Acyl-[acyl-carrier-protein] hydrolase activity	1.1	2.65E-10
	Hydrolase activity	29.0	8.00E-08
	Catalytic activity	63.6	4.20E-06
	DNA helicase activity	1.2	5.30E-05
	Helicase activity	1.7	0.00028
	Hydrolase activity, acting on glycosyl bonds	3.2	0.00261
	Thiolester hydrolase activity	1.6	0.00294
	Adenyl nucleotide binding	12.7	0.00320
	Adenylribonucleotide binding	12.7	0.00414
Biological process	DNA metabolic process	7.9	3.60E-15
	DNA-dependent DNA replication	1.7	8.38E-05
	Cell adhesion	3.1	0.00028
	Biological adhesion	3.1	0.00028
	Epithelial cell migration, open tracheal system	0.7	0.00054
	Epithelial cell migration	0.7	0.00054
	Epithelium migration	0.7	0.00054
	Aminoglycan catabolic process	0.8	0.00066
	Cell cycle phase	3.6	0.00189
	M phase	3.4	0.00246

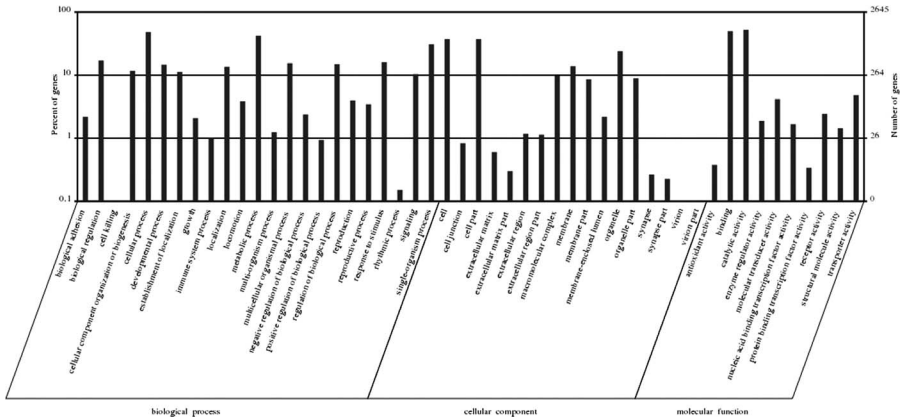

Fig. 2. Classification of differentially expressed genes (DEGs) ontology.

insecticides (beta-cypermethrin, fenpropathrin, phoxim, abamectin, and acetamiprid) (Tang et al. 2014). The gene encoding GST in *M. alternatus* is down-regulated in the current study. The GSTs may play a role in pyrethroid resistance in *Frankliniella occidentalis* (Pergande) populations (Thalavaisundaram et al. 2012). However, *M. alternatus* dose not confer any *B. bassiana* resistance.

Another pathway that is closely connected with *B. bassiana* is the insect hormone biosynthesis pathway (P-value = 0.005). There are 33 enriched DEGs in this pathway and these include up-regulated DEGs encoding juvenile hormone epoxide hydrolase (JHEH), juvenile hormone esterase (JHE), and down-regulated DEGs encoding methyl farnesoate epoxidase/farnesoate epoxidase (CYP15A1_C1), ecdysteroid 25-hydroxylase (CYP306A1), ecdysteroid 22-hydroxylase, ecdysteroid 2-hydroxylase, ecdysone oxidase, and ecdysone 20-monooxygenase.

The juvenile hormone (JH) is a type of epoxide-containing sesquiterpene ester secreted by a pair of corora allatum behind the brain of insects (Roller and Bjerke 1965); it controls the development of metamorphosis in insects (Marchal et al. 2010). Thus, the synthesis and degradation of JH are tightly regulated in different developmental stages (Hammock 1985). JH gradation is catalyzed by two hydrolases, JHEH and JHE. In conjunction with JHE, JHEH is a key player in the degradation of JH, which regulates both growth and development of insect larvae and reproductive functions of adults (Jindra et al. 2013). In many parasitoid–host systems, the activity of host JHE is inhibited after being parasitized (Beckage and Riddiford 1982; Dahlman et al. 1990; Hayakawa 1990; Strand et al. 1990; Zhang et al. 1992), but in diamondback moth, *Plutella xylostella* (L.), this is not the case (Lee and Kim 2004). Pyriproxyfen increases JHE activity of the diamondback moth by 50%, even when its concentrations are as low as 10^{-9} mol L^{-1} (Wei et al. 2010). In lepidopteran hemolymph, decreased JH titers are positively correlated with an increased abundance of JHE (Hammock 1985). In the current study, *B. bassiana* treatment is found to induce the expression of JHE, which indicates that fewer JH residues exist in the insect body after the treatment. JHEH expression was also up-regulated. The elevated JHEH activity stimulated by *B. bassiana* should accelerate juvenile hormone metabolism in *M. alternatus*. The larvae would be smaller than

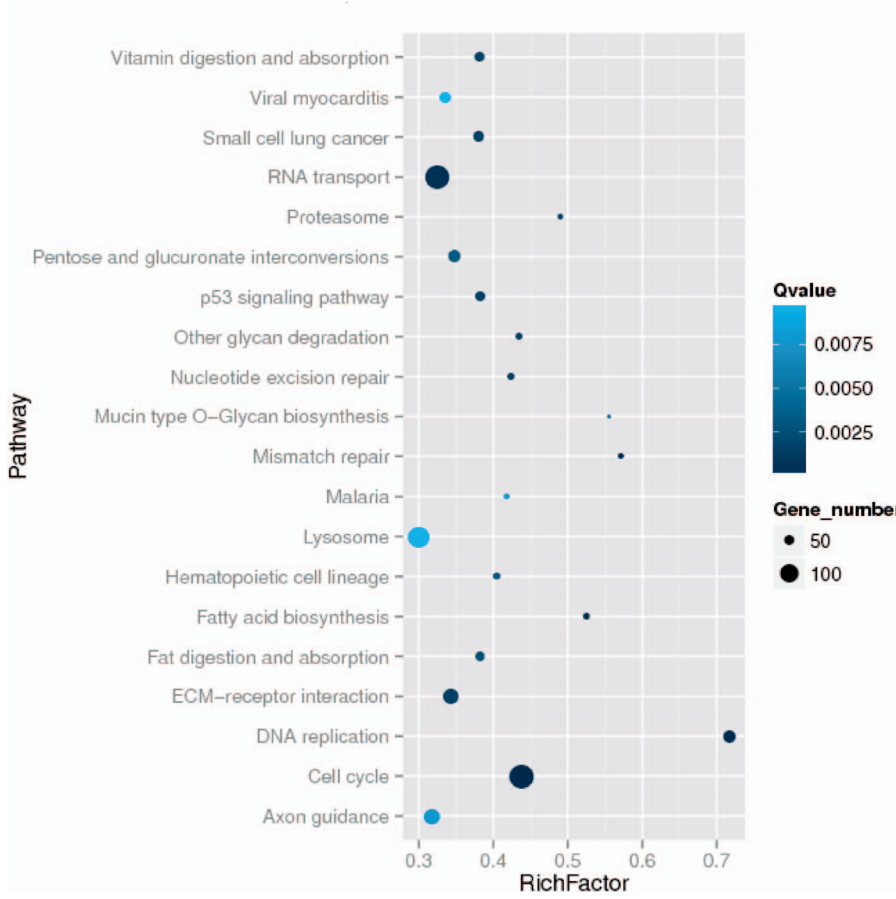

Fig. 3. Top 20 enriched pathways for *B. bassiana* treated with *M. alternatus* versus control. The RichFactor is the ratio of the differentially expressed gene numbers annotated in this pathway term to all gene numbers annotated in this pathway term. A greater RichFactor value means greater intensiveness. The Q-value is the corrected *P*-value ranging from 0 to 1, and a lower value represents greater intensiveness.

normal and the pupal period of *M. alternatus* would be shortened after *B. bassiana* treatment.

The last enzyme in the biosynthetic pathway to inhibit juvenile hormone III in the corpora allata of insects is methyl farnesoate epoxidase, a cytochrome P450 monooxygenase. *Beauveria bassiana* is a powerful inhibitor of the last step of juvenile synthesis in *M. alternatus*.

Molting hormone (ecdysteroid) is one of the most important hormones in insects. The synthesis and inactivation of ecdysteroid regulate the developmental process of insects. A major pathway of ecdysone inactivation is ecdysone conversion to 3-

Table 2. Comparison of the ratio of the gene expression values derived from Ion Proton sequencing to those verified by Real Time Quantitative PCR (RT-qPCR).

Gene ID	Blast Nr	RT-qPCR	Log2 Ratio	Ratio
Unigene13437	Calpain-C	0.15	−1.21	0.43
Unigene19549	Structural maintenance of chromosomes protein 2-like	0.79	−3.11	0.12
Unigene28267	Fat-like cadherin-related tumor suppressor homolog	0.82	−1.43	0.37
Unigene24658	Innexin inx2	0.33	−2.12	0.23
Unigene26853	Lysosomal alpha-mannosidase	0.54	−2.80	0.14
Unigene21455	Zinc transporter ZIP1	2.77	1.03	2.04
Unigene15755	Argininosuccinate synthetase	0.04	−2.30	0.20
Unigene18218	Minus strand unc-5	0.25	−1.64	0.32
Unigene56	Cytochrome oxidase subunit I	1.80	2.17	4.51
Unigene4812	Chymotrypsin-like proteinase 6D precursor	2.01	1.20	2.29
Unigene2372	Glycogen synthase	0.07	−1.56	0.34
Unigene5436	A disintegrin and metalloproteinase with thrombospondin motifs like	0.30	−1.57	0.34
Unigene23657	Tyrosine hydroxylase	2.13	1.43	2.69
Unigene25960	Carboxypeptidase A	1.21	1.65	3.15
Unigene3617	Aspartate 1-decarboxylase	1.47	1.15	2.22
Unigene20274	RNA-binding protein 19	0.14	−1.52	0.35

dehydroecdysone and then to 3-epiecdysone in insects. Ecdysone oxidase participates in this pathway, which is ecdysteroid responsive. Functional characterization of the enzyme participating in ecdysone inactivation in *M. alternatus* could provide hints for the artificial regulation of *M. alternatus* development.

The gene Cyp306a1, a member of the cytochrome P450 monooxygenase family, functions as C-25 hydroxylase and has an essential role in ecdysteroid biosynthesis during insect development (Ryusuke et al. 2004). Decreased expression of the ecdysone-inducible gene suggests that *M. alternatus* fails to produce a sufficient titer of ecdysone after *B. bassiana* infection. Because all insects require ecdysteroids for normal development, *B. bassiana* may be useful in the development of novel strategies for controlling *M. alternate* growth.

Confirmation of gene expression results. To validate the Ion Proton expression profiles, we randomly analyzed 16 genes by RT-qPCR. Table 2

demonstrates that the trend of the RT-qPCR-based expression profiles among the selected genes was similar to that detected by Ion Proton sequencing.

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