INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0575/2018/20–12–2879–2890
DOI: 10.17957/IJAB/15.0850
http://www.fspublishers.org

Full Length Article

Molecular Identification of Pathogenicity Associated Genes in Honeybee Fungal Pathogen, *Ascosphaera apis*, by Restricted Enzyme-Mediated Integration (REMI) Constructed Mutants

Awraris Getachew[1†], Abebe J. Wubie[1,2†], Jiangli Wu[1], Jin Xu[1], Pengjie Wu[1], Tessema Aynalem Abejew[1,2], Yangyang Tu[1], Ting Zhou[1] and Shu-Fa Xu[1*]

[1]*Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture; Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, 100093, Beijing, China*
[2]*College of Agriculture and Environmental Sciences, Bahir Dar University, Bahir Dar, Ethiopia*
[*]For correspondence: xushufa@caas.cn
[†]These authors contributed equally to this work and listed as first authors

Abstract

Chalkbrood disease in honeybees, caused by *Ascosphaera apis* (Onygenales: Ascosphaeraceae), exclusively affects brood. Restricted Enzyme-Mediated Integration (REMI) was used to construct stable *A. apis* transformants to investigate the infectivity of transformed pathogens to honeybee larvae. PCR, Clustal W sequence alignment, and Southern blot analyses confirmed insertion of *hph* gene and successful integration into host chromosomes in 184 stable *A. apis* transformants, of these 12 were selected for further study to identify pathogenicity associated genes. *In vitro* bioassay confirmed that 10 of the selected mutants had notable differences in pathogenicity among themselves and with the wild-type strain. More specifically, transformed *A. apis* mutants were relatively less pathogenic to *in vitro* reared honeybee larvae than that of the wild-type with mutant-7 having the lowest pathogenicity. Following the *in vitro* bioassay result, the wild-type and non-pathogenic mutant-7 were selected to identify virulence related genes in *A. apis*. Real-time PCR validated a total of eleven virulence related candidate genes and tested for their expression levels. Six genes (*Omt*A, *Ski*3, T*OX*D, *Hts*1, *Nor*-1 and melanin) were highly expressed in wild-type *A. apis* strain samples compared to its corresponding non-pathogenic mutant-7, which had lost pathogenicity on honeybee larvae and had relatively the lowest expression levels of selected candidate genes compared to the wild-type. Furthermore, the expression of these virulence related genes suggests that they might have an important role as virulence factors in the pathogenicity of *A. apis* through their involvement in the secondary metabolism processes that support pathogenicity functions.

Keywords: Mutant; *hph gene*; *Ascosphaera apis*; Southern blot; Pathogenicity; qRTPCR

Introduction

Honeybees play a significant role in pollinator-dependent agriculture (Aizen and Harder, 2009), specifically in crop monoculture, by enhancing production and maintaining ecological balance around the world (Hedtke *et al*., 2011). However, they face serious challenges from pathogens that affect honeybee brood, like chalkbrood (Engelsdorp and Meixner, 2010). Chalkbrood is honeybee disease caused by *Ascosphaera apis* which entirely affects honeybee brood (Spiltoir, 1955). Like any other eusocial insect, transmission of this fungus in the colony is facilitated by physical contact, feeding, and food sharing (Aronstein and Murray, 2010). Ingested *A. apis* spores germinate in the mid-gut of 2–4 instar larvae, after which the mycelium penetrates the larval cuticle before the pupal stage in a manner facilitated by chitinase activity (Cornman *et al*., 2012). Infected larvae commonly decrease their food consumption or stop eating altogether, exhibit swelling inside the cell, shrink with excessive defecation, die and harden to become chalky with time (Flores *et al*., 2004; Aronstein and Murray, 2010). Vegetative growth of *A. apis* extends from hindgut to anterior part of larva, and finally covers whole larval body with a thick layer of white mycelium (Aronstein and Murray, 2010). Even though different factors influence development of fungus in honeybee colonies, chilled larval brood at the time of capping and high humidity have been reported to play significant roles in the infectivity of the larvae (Bamford and Heath, 1989; Flores *et al*., 1996). Furthermore, chalkbrood outbreaks in the summer have been reported to be related to *Nosema ceranae* infection in the preceding spring and to *Varroa destructor* infestation in the same season (Hedtke *et al*., 2011).

Chalkbrood disease reduces 5–37% of honey

To cite this paper: Getachew, A., A.J. Wubie, J. Wu, J. Xu, P. Wu, T.A. Abejew, Y. Tu, T. Zhou and S.F. Xu, 2018. Molecular identification of pathogenicity associated genes in honeybee fungal pathogen, *Ascosphaera apis*, by restricted enzyme-mediated integration (REMI) constructed mutants. *Int. J. Agric. Biol*. 20: 2879–2890

production and causes 12–92% brood death depending on *A. apis* strain (Aronstein and Murray, 2010; Vojvodic *et al*., 2011a; Lee *et al*., 2013). Since knowledge regarding the prevention of fungal infectivity and its consequent damage is limited, recent studies have focused on understanding the molecular biology, infectivity pathways, reproduction, and other important features of infectious fungi. Despite the wide range of research towards controlling chalkbrood disease worldwide, new approaches are reported to be risky, not only to the honeybees but also for humans and the environment (Xue *et al*., 2015).

Major advances in genomics and proteomics have stimulated significant interest in the application of protoplasts (Rehman *et al.*, 2016) as a readymade toolkit in genetic manipulation and mutagenic transformation (Olmedo-Monfil *et al*., 2004). Transformation implies the introduction of foreign DNA molecules into the whole genome of an organism (Xu *et al*., 2005).

Restriction enzyme-mediated integration (REMI) is a technique involving mutagenesis, and has been used to generate random insertion mutations in different pathogenic fungi and other organisms (Xu *et al*., 2005; Lopes *et al*., 2008), including the insertion of *hph* gene as a selective marker (Hanif *et al*., 2002). In REMI technique, a restriction enzyme is added into transformation mix to give the enzyme access to genomic DNA in the nucleus of transformation recipient and introduce a foreign linearized plasmid DNA into a random locus at its restriction site to induce mutation (Thon *et al*., 2000). Employing this technique, various mutants have been generated. Southern blot hybridization is employed to validate proper integration of a specific insert and copy numbers of its integration in genetic manipulations aimed at establishment of transformants from a specific wild type. Furthermore, tagging and cloning of mutant genes, investigating mutant pathogenicity, plasmid rescuing, and some other activities have been achieved through this technique (Leem *et al*., 2003; Amaya and Kroll, 2010). To the best of our knowledge, REMI has not been employed in *A. apis*. Application of REMI in mutagenesis studies is useful for identification and isolation of significantly important genes and in establishment and maintenance of *A. apis* mutants for future use.

It has been reported that pathogenicity of *A. apis* varies between different strains (Lee *et al.*, 2013). Hence, it is essential to better understand differences in pathogenicity of wild and mutant strains in order to comprehend their functional implications. Several pathogenicity studies have been conducted by using artificial methods, such as spore inoculation and *in vitro* larval rearing techniques (Flores *et al*., 2004). To understand the infectivity of engineered pathogens in honeybees, this study isolated viable *A. apis* protoplasts for transformation, investigated REMI-based successful and stable mutant construction and evaluated the pathogenicity of *A. apis* mutants in honeybees. One approach to better understand the processes that allow a fungus to be pathogenic and colonizes a honeybee larva is identification of genes that contribute to pathogenicity. Transcriptome analysis of cDNA libraries are used to identify candidate virulence associated genes (Cornman *et al*., 2012), no molecular determinants of pathogenicity have, so far, been isolated from *A. apis*. This research was therefore conducted to discover the pathogenicity associated genes from mutants of this fungus constructed by REMI and the wild type strain based on relative gene expression levels using quantitative real time reverse transcriptase PCR (qRTPCR).

Materials and Methods

PDA, liquid regeneration medium (20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 12 g/L maltose, 5 g/L potassium phosphate dibasic anhydrous, pH of 5.8), solid regeneration medium (10 g/L glucose, 5 g/L yeast extract, 10 g/L agar, 6 g/L maltose and 44.73 g/L KCl), sodium buffer (1 *M*/L disodium hydrogen phosphate and 1 M/L of sodium di-hydrogen phosphate), DF (0.8 M/L of citric acid monohydrate with NaCl), Liquid II (0.2 M/L disodium hydrogen phosphate dodecahydrate, 0.8 M/L of KCl), and Driselase enzyme 50 mg/mL were prepared and used. All these growth mediums and reagents were autoclaved at 121°C for 20 min. All chemicals used in the study were purchased from Sigma Chemicals Co. Ltd. (Beijing, China).

Ascosphaera apis was obtained from chalkbrood disease infected colonies from Institute of Apicultural Research, Graduate School of the Chinese Academy of Agricultural Sciences, Beijing, China and examined microscopically for the presence of ascomata and diseased larval mummies of *Apis mellifera.* Following the standard techniques (Spiltoir, 1955), this fungus was incubated (at 28°C), grown, and isolated on Potato Dextrose Agar (PDA) plates, which were supplemented 1% yeast extract, containing 50 μg/mL hygromycin B as an antibiotic to inhibit bacterial growth, and fungus was regularly transferred into new plates every month. Single layer growth medium plates were used to support sexual reproduction and sporulation. To minimize strain difference in pathogenicity, mutants were constructed from one isolate of *A. apis* to be used for the entire study. An empty comb was provided to a healthy and strong *A. mellifera* honeybee colony brood chamber to graft 1st instar larvae for *in vitro* larval rearing and pathogenicity test.

Protoplast Isolation

Protoplasts were isolated according to previously published techniques (Wubie *et al*., 2014) using liquid growth medium, shorter incubation period and 50 mg/mL driselase as the best enzymolysis agent. Then isolated protoplast was counted using a hemocytometer (Beyotime Institute of Biotechnology, Beyotime®, Beijing, China). Protoplast viability and regeneration rates were assessed using fluorescein diacetate (FDA)

treatment. The obtained protoplasts were then used in the transformation steps.

Plasmid DNA Preparation and Transformation

The plasmid pBluescript II KS-hph (4,334 bp) was provided by Ass. Prof. Boqiang Li (Institute of Botany Research, Chinese Academy of Sciences, Beijing, China). This plasmid contained hygromycin B (*hph*) and ampicillin (*amp*) resistant genes as selective markers (Fig. 1). For REMI transformation, the plasmid DNA was completely digested with *Hind*III (Takara Biotechnology Co. Ltd., Dalian, China) before use. Selective marker (*hph* gene) transformation into prepared *A. apis* protoplasts, suspended in DF, was performed in the presence of 60% PEG in an ice bath. The pelleted protoplasts were then re-suspended in liquid regeneration medium to a concentration of about 2×10^7 protoplasts/mL and were incubated on solid regeneration medium furnished with 50 μg/mL of hygromycin B for 7 days at 28°C. The grown transformants were screened and selected. Fungal colonies were sub-cultured seven times on PDA containing hygromycin B to obtain stable transformants. The transformants were then transferred back to the selection medium containing hygromycin B (50 μg/mL) to determine whether the colonies still retained the selection marker.

Screening of *A. apis* on Hygromycin B

The natural resistance of original *A. apis* to hygromycin B was tested by growing isolated protoplasts on PDA plates containing 0, 25, 35, 50, 65, and 75 μg/mL of hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany) following previously published protocol (Knight *et al*., 2009) and incubated at 28°C for about 7 days. An upper layer of the medium containing hygromycin B was overlaid after an overnight incubation (Blochlinger and Diggelmann, 1984), the fungal growth was observed after every 12 h and compared for different concentrations. The viability of stored *A. apis* spores was then evaluated following standard techniques (James and Buckner, 2004; Jensen *et al*., 2009) with slight modification. Freeze-dried spores were soaked overnight in 1 mL deionized water and 100 μL aliquots were transferred onto agar plates. Plates were incubated at 28°C with $\geq$ 20% CO_2 for 2 days to stimulate spore germination, and then up to 2 weeks at ambient CO_2.

PCR Amplification

Genomic DNA of *A. apis* was extracted using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-TekInc, Norcross, GA USA) following the manufacturer's protocol (Anderson *et al.*, 1998). The regenerated putative transformants were screened by PCR for the presence of the *hph* gene using specific primers; *hph*-F (5′-GTTATGTTTATCGGCACTTTG -3′) and *hph*-R (5′-GTTGGCGACCTCGTATT -3′). PCR analysis was performed under the following conditions: an initial denaturation at 94°C for 5 min followed by 35 cycles of amplification (with 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C for 1 min of extension at 72°C, and 10 min of further extension at 72°C). DNA from the untransformed fungus was also used, and a fungal DNA free mix was used as negative control and the transforming plasmid as a positive control in PCR analysis. Insert Check PCR Master Mix (Biomed, Beijing, China) was used. Amplified DNA of putative transformants was analyzed using ethidium bromide-stained 1% agarose gel electrophoresis. Detection of bands from the PCR product indicated the integration of the selective marker (*hph* gene) inside the transformants' DNA. The PCR products amplified from the putative transformants were then sequenced (Biomed, Beijing, China) and analyzed for sequence match using BioEdit Version 7.2.5 Sequence Alignment Editor (North Carolina State University). The sequence match (78.2 – 98.7%) of PCR products from transformants with the selective marker's sequence was considered an indicator of successful integration.

Southern Blot Hybridization

In this experiment, the *hph* gene (25μg) was digested with *Hind*III overnight at 37°C and probed following to the DIG High Prime DNA Labeling and Detection Starter Kit I manual (Roche Diagnostics GmbH, Mannheim, Germany). Genomic DNA (5–10 μg) from *A. apis* transformants were then digested with *Hind*III and fractionated using ethidium bromide free 1% agarose gel electrophoresis in 1X TAE buffer. Following denaturation (using 1.5 *M* NaCl, 0.5 *M* NaOH) for 30 min and neutralization (using 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.5) for 30 min, the digested products on the gel were equilibrated in 20X SSC buffer for 10 min. Then the products transferred onto a positively charged nylon membrane (Amersham Biosciences, UK Limited). Finally, hybridized with a DIG-labeled *hph* probe using the DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany).

***In Vitro* Larval Rearing and Pathogenicity Bioassay**

Various hurdles in carrying out uniform and controlled research studies in honeybee colonies has forced researchers to establish laboratory-based *in vitro* larval rearing techniques to create a uniform research environment and exclude the effects of social immunity for better and replicable experimentations in testing the effects of pathogens, toxins, and drugs on honeybees (Crailsheim *et al*., 2013). An empty comb was provided to a healthy *A. mellifera* honeybee colony brood chamber (Vandenberg, 1992). At the 84th h, 12-h-old 1st instar larvae were grafted using the Chinese larvae grafting tool to 50 μL freshly

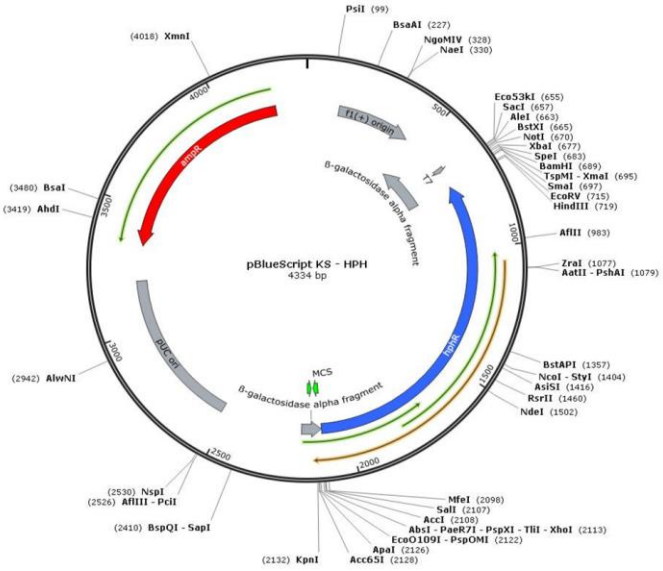


Fig. 1: pBluescript II KS-HPH (4334 bp) plasmid with its restriction enzyme sites. Here, the *hph* gene is embedded between *Hind* III and *Sal* I restriction enzymes

prepared larval food containing 24-well larvae rearing cells with one larva per well and 4 rearing cells per treatment. The rearing cells were then incubated at 34°C and 85–90 RH in the dark for 10 days. The volume of larval food was increased according to larval stages up to the 6th day of rearing. Honeybee larvae were carefully transferred to new *in vitro* rearing cells on a daily basis with fresh larval food and appropriate cleaning of defecates if any were on the body of the larvae (Crailsheim *et al*., 2013). Royal jelly-based larval food was prepared as suggested by Genersch *et al*. (2005), Crailsheim *et al*. (2013) and Jensen *et al*. (2013) with following modifications (50% fresh Chinese royal jelly mixed with a solute of 6% D-Glucose (w/v), 6% D-Fructose (w/v), and 1% yeast extract (w/v) in 37% of sterile double distilled water pre-warmed to 34°C). Even though it has been suggested that all larval instars could be infected by *A. apis* (Jensen *et al*., 2013), a total number of 96 larvae (2nd instar) per treatment were separately fed with 10 μL of freshly prepared and activated 2.54×10^7 mL^{-1} original and transformed *A. apis* fungal spore suspensions mixed with fresh larval food. After 48 h post inoculation interval, grafted larvae were incubated at 22°C for 2 h to initiate or support fungal development and then returned to the previous incubation condition. Inocula were prepared separately from each treatment (Vojvodic *et al*., 2011b). Four negative control larval rearing cells with 96 larvae in total were used in this experiment and fed with freshly prepared larval food every day with continuous transferring of larvae to new *in vitro* cells. All the treatments were then replicated three times, resulting in a total number of 288 larvae per treatment. During the 10 incubation and observation days (normally around time of larval sealing), the diseased, survived, and infected larvae were counted daily and examined using a microscope (LEICA EZ4W). Infected larvae were distinguished by the presence of fungal hyphae on the lower abdomen (mummification). Larvae that died without the presence of any fungal hyphae were re-examined the next day, and, if the fungus was developing, were considered dead from fungal infection the preceding day. If the fungus was not detected on dead larvae, they were considered to have died for some other reasons.

The dead larvae were cut into pieces and inoculated individually onto solid PDA plates to allow growth of *A. apis* from ingested spores as a means of confirming presence of recovered spores in inocula, and testing spore survival (Heath and Gaze, 1987). Infected larvae samples were also collected and kept in sterile centrifuge tubes at -80°C for further use and subsequent examinations. Disease incidence and infectivity (index) level data were collected, summarized, and analyzed using MS Excel. ANOVA and mean comparisons were performed using GLM procedures (SAS Institute, 2002).

Total RNA Extraction and Quantitative Real Time PCR

Following pathogenicity test results, *A. apis* wild-type strain and the lowest pathogenic transformant were grown on Potato Dextrose Agar (PDA) for four days. Total RNA was isolated from powdered spores using Trizol kit following the manufacturer's protocol and used for Real time PCR. Fungal DNA was removed with DNAse I followed by the removal of rRNA with Ambion's Poly (A) Purist kit (Applied Biosystems, Austin TX). Then the absence of *A. apis* DNA following DNAse I treatment was confirmed by PCR analysis using *A. apis* actin species specific primers. Total RNA concentration and quality was

measured with a Nano-Drop ND-2000 spectrophotometer and brought the desired concentration of 600 ng/μL. The RNA integrity was determined using an RNA 6000 Nano Chip and was run on an Agilent 2100 Bioanalyzer (Agilent Technology Inc.). Then after, using an RNA Pico Chip, the rRNA removal was determined and the size of mRNA transcripts was visualized. The samples were diluted to 5 ng/μL concentration levels using RNAse-free H_2O, denatured for 2 min at 70°C and immediately placed on dry ice, 1 μL of each sample was run on the RNA Pico Chip and the remaining was kept at -80°C for further use. For RT-PCR, 6 μg of total RNA was reverse-transcribed into first-strand cDNA with oligodT primer using the SuperScript first-strand synthesis system (Invitrogen, Life Technologies, Carlsbad, CA) by following the manufacturer's protocol.

Validation of Virulence Related Genes Expression

A total of 11 candidate virulence related genes, namely *Omt*A, *Ski*3, *Cts1*, *TOX*A, *TOX*G, *TOX*D, *TOX*F, *Hts 1*, *Nor-1*, *Alta*7 and melanin biosynthesis gene, were selected for validation from a previous study conducted on *A. apis* pathogen transcriptome analysis (Cornman *et al*., 2012). Therefore, we compared the expression level of the selected candidate virulence related genes in wild-type strain against to the lowest pathogenic mutant. To validate the contribution of these genes in *A. apis* pathogenicity, the expression of each gene both in wild type strain and the lowest pathogenic mutant was analyzed using quantitative reverse transcriptase real time PCR (qRTPCR) in triplicates. The *A. apis* actin gene was used as a reference gene for normalizing qRTPCR validation since it has been reported to be among the most stable genes in *A. apis* (Aronstein *et al.*, 2007). Gene specific primers already constructed were used to generate specific PCR fragments in *A. apis* pathogenicity associated genes (Table 1). Here, all primer pairs were designed using PrimerExpress 3 Software (Life Technologies) following the standard procedure. The Relative quantification of a gene is defined as the change in expression of the target gene relative to the reference groups such as untreated control and/or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). The relative expression level of each gene was calculated by the formula ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). The mean differences between original and mutant strains in terms of relative gene expression were compared using student's t-test.

Results

REMI Transformation and Selection of Mutants

In this experiment, viable and regenerative protoplasts were successfully isolated from liquid media containing young *A. apis* mycelia (Fig. 2 and 3). REMI-based integration of *Hind*III digested and linearized pBluescript II KS-*hph* DNA plasmid into the *A. apis* chromosome resulted in a total of 215 transformed mutants. The transformants of *A. apis* were then screened on solid PDA plates containing 50 μg/mL of hygromycin B. Those transformants which could not grow well in this antibiotic concentration but could survive in lower concentrations were not selected for further investigation. After seven successive sub-cultures of hygromycin B resistant transformants, a total of 184 stable mutants that retained resistance to this antibiotic were obtained and maintained. Among them, best twelve stable mutants were randomly chosen for subsequent studies (Fig. 4a and c). In this process, the phenotypes of some transformants were observed to be different from those of the previous cultures (Fig. 4d), and the growth of some transformants at the 50 μg/mL concentration level were clearly much lower than of the others (Fig. 4b). Differentially responding transformants and those that were significantly affected by the hygromycin B were not considered to be stable and were removed from this experiment (Fig. 4b and d). These results indicated that 50 µg/mL of hygromycin B was adequate to select *A. apis* transformants.

PCR Verification

Hygromycin B resistant transformants were examined individually by PCR to verify the insert (selection marker,

Table 1: Primers used to identify virulence related genes

Target gene	Forward Primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size (bp)
*Omt*A	CTCAGGGTTCAGGCTATCCA	CTTGGTGCCCTCCACTAACT	186
*Ski*3	ACATGAACCCTAAGGTGGCA	GAGCGAACAATGGCTGGAT	372
*Cts*1	ATCCCAGCGGAGAAACTGAC	GCTTGACGCAACCGTAAACA	157
TOX A	CTTGCGGAGCGTTTATTGT	AAAGCAACACCCCCACTAGA	217
TOX G	AGGCAGGGATTATCTCAGCA	AGTTTCGTGGCTTCGTTTCA	101
TOX D	GCTTGAGAAGTATGGCGGGTA	TAGGGCGGCTGTAAGTGCT	127
TOX F	GACGGACTTATCATCGAGGACT	GCACAGTAACCGAATCCAAAA	265
*Hts*1	AGGCGTCCTTGGAGTGCTAA	AGAGTCGTGTCGGATGGTGC	255
Nor-1	TTGCCTTCCCTATTCACCCT	GCTTCACCGCC ATCATACTT	172
*Alta*7	CGCCGTTCAAAGATGCTG	GGGCTCAGCGATAGGGTAGT	276
Melanin	GGTCCATTTATCTACCCATCTACAC	ACCCGAGGGATTGAGATTTT	282
Actin A. apis	CATGATTGGTATGGGTCAG	CGTTGAAGGTCTCGAAGAC	

hph gene). As shown in Fig. 5, the anticipated specific *hph* gene bands (approximately 500 bp) were amplified in all twelve hygromycin B resistant transformants, whereas no specific *hph* gene band was amplified from the wild strains'

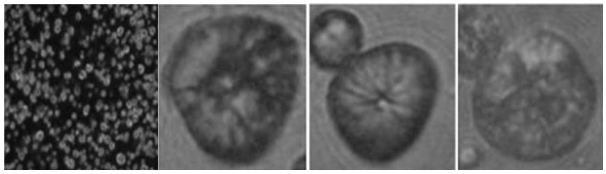


Fig. 2: Isolated viable *A. apis* protoplasts

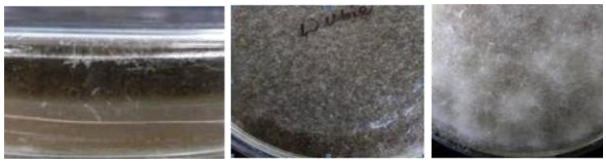
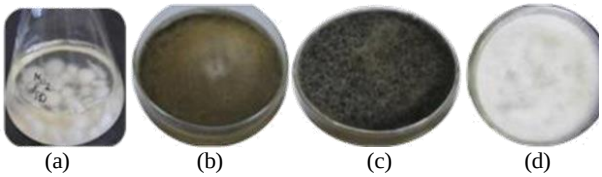


Fig. 3: *A. apis* fungus regenerated from protoplasts



(a) (b) (c) (d)

Fig. 4: Differences between antibiotic resistant and non-resistant mutation solid growth medium. (**a**) the *A. apis* mycelia shaken in liquid medium, (**b**) non-hygromycin B resistant transformant on solid PDA plates and (**c**) successful and hygromycin B resistant transformant grown on solid PDA medium and (**d**) physically affected transformant

(original fungus) DNA template (Ori) and the negative control (-ve ctrl). Results revealed that the *hph* gene was successfully integrated into the transformed mutants' chromosome. Furthermore, results of PCR product sequence alignment indicated that amplified product from the mutants had similar sequence with the anticipated *hph* gene sequence (accession no V01499) (Fig. 6). Despite detection through gel electrophoresis in mutant 12, the sequence of *hph* band did not match the anticipated sequence alignment, probably due to the incorporation of the insert into multiple loci in the chromosome. Consequently, this transformant was not selected for further study. These results suggested that transformed *A. apis* mutants were successfully generated by REMI technique.

Southern Blot Hybridization

To further examine an integration pattern and copy numbers of the foreign gene (*hph*), 11 *A. apis* mutants, confirmed by PCR and sequence alignment to reveal the presence of the *hph* gene, were subjected to Southern blot hybridization. The Southern blot hybridization confirmed that the foreign gene (*hph*) in the transforming plasmid had effectively been integrated into the chromosomal DNA of *A. apis* to generate effective transformants (Fig. 7).

The results indicated that hybridization bands were found in all eleven mutants, but there was no hybridization signal detected in the control sample (Fig. 7).

All the analyzed mutants contained a single copy of the *hph* gene in the genome except mutant 12, which produced two bands (Fig. 7, Lane 12), revealing that the transgene locus was stably maintained in the majority (90.9%) of fungal colonies. Therefore, the employment of REMI in this study resulted in an efficient transformation with single locus integration. In this analysis, 10 transformants (90.9%) had a single copy while two in the remaining transformant.

Generally, REMI integration of a plasmid into transformants leads to the appearance of a single band around the 1,500 bp mark, which was confirmed in our study. This indicated that *hph* gene insertion into a single locus of the transformant's genomic *Hind* III site was effective. Furthermore, the single hybridization signal in all lanes except lane 6 (the original fungus) and lane 12 (mutant 12) revealed that a single *hph* insertion event has occurred and the transformants are true mutants. Hence, mutant 12 was considered a false mutant and was not selected for further tests.

Larval Infection Rate (Pathogenicity) and Mortality

In this experiment, honeybees in the control group consumed food every day and were active, especially with light in the incubator during inspection, indicating that this group was negative for fungal infection throughout the entire study. Even though infected larvae also exhibited similar behavior until the 3rd and 4th day post infection, they were immobile and unreactive to any stimulus from the 5th day onwards. *In vitro* reared honeybee larvae infected with *A. apis* predictably grown chalkbrood disease in which the fungus caused a total mortality of 19.9% by the end of the experiment (Fig. 8).

Moreover, the vegetative growth of *A. apis* extended from the hindgut to the anterior part of the larva, finally covering the whole larval body with a thick layer of white mycelium (Fig. 8). However, analysis of the number of diseased, surviving, and infected larvae indicated significant differences in the pathogenicity among *A. apis* mutants themselves and the wild-type (original fungus). More specifically, chalkbrood incidence from transformed mutants in the challenged larvae was markedly lower than that noticed with the wild type. Mutant 7 had the lowest incidence and index of chalkbrood disease out of all transformed *A. apis* mutants on *in vitro* reared *A. mellifera* larvae, which may be because of the potential disintegration of the

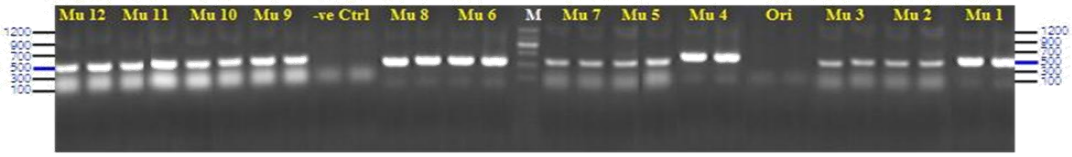


Fig. 5: PCR analysis result from 12 stably selected *A. apis* transformants for the presence of hph gene inside their genome. M denotes the DNA Marker. Ori denotes the wild strain; Lanes Mu1, Mu2, Mu3, Mu4, Mu5, Mu6, Mu7, Mu8, Mu9, Mu10, Mu11 and Mu12 denotes different independent transformants (mutants); and –ve ctrl denotes the negative control which is a sterile double distilled water and PCR mix template

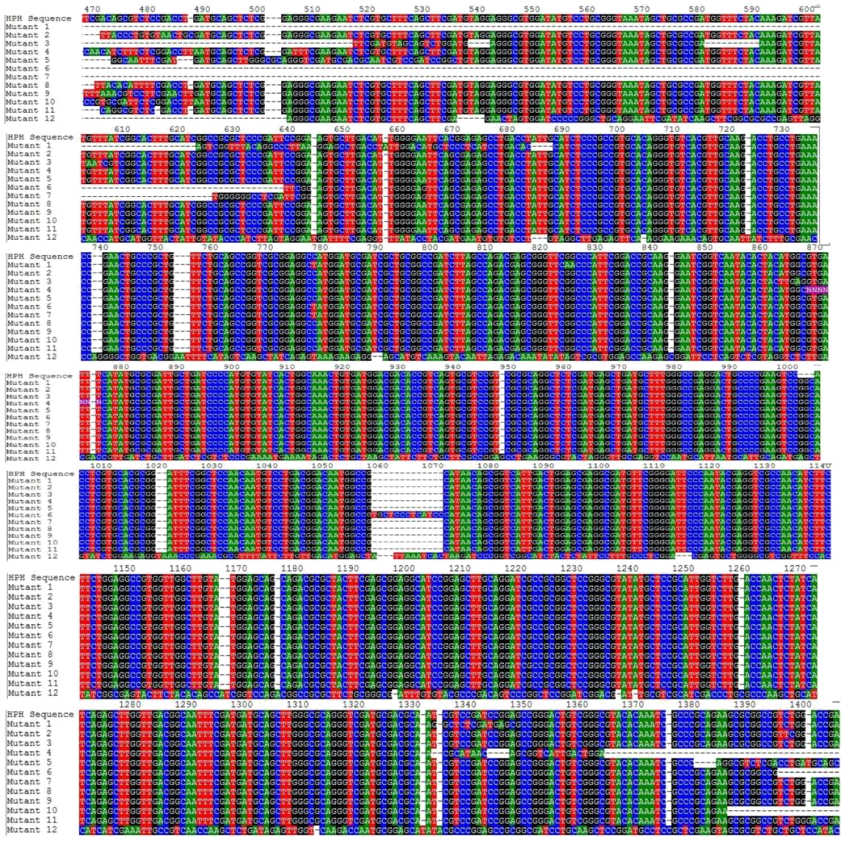


Fig. 6: Clustal W Multiple Alignment (BioEdit) based sequence match result as a confirmation of the anticipated *hph* gene integration into the transformed mutant's genome

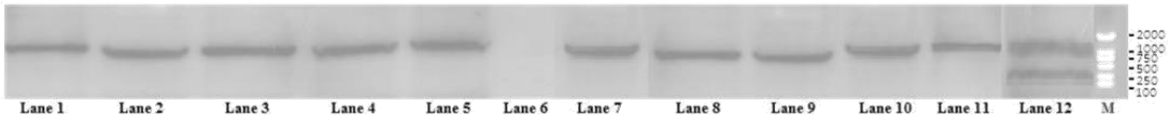


Fig. 7: Southern blot analysis of eleven selected stable *A. apis* transformants. Genomic DNA from the transformants and original fungus was digested with *Hind* III and probed with the *hph* gene and were used in the southern analysis. It shows the Southern blotting result with Lanes 1-12; these are: Lane1= mutant 1, Lane2 = mutant 4, Lane3 = mutant 10, Lane4 = mutant 9, Lane5= mutant 8, Lane6 = wild strain (original fungus), Lane7 = mutant 6, Lane8 = mutant 5, Lane9 =mutant 3, Lane10 = mutant 2, Lane11 = mutant 11 and Lane12 = mutant 7

pathogenicity-causing genes due to the insert.

Larval death during the pre-pupal stage was first detected in those experimental groups that were infected with the original (wild) fungal spores and chilled for 2 h at 22°C. Infected larvae were identified by the presence of fungal mycelium on the surface of the dead larvae and from the fungus grown on incubated dead larval bodies. Larvae that died without visible fungal symptoms were confirmed

(a) (b) (c)

Fig. 8: *In vitro* bioassay result. 2 days old (1st instar) honey bee larvae were fed with 10 µl of *A. apis* viable spores mixed with royal jelly based larval diet. (**a**) and (**b**) control bee larva during pupation; where (**c**) the infected and diseased bee larvae with developed chalkbrood disease (arrows denote infected larvae covered by white-colored fungal mycelium)

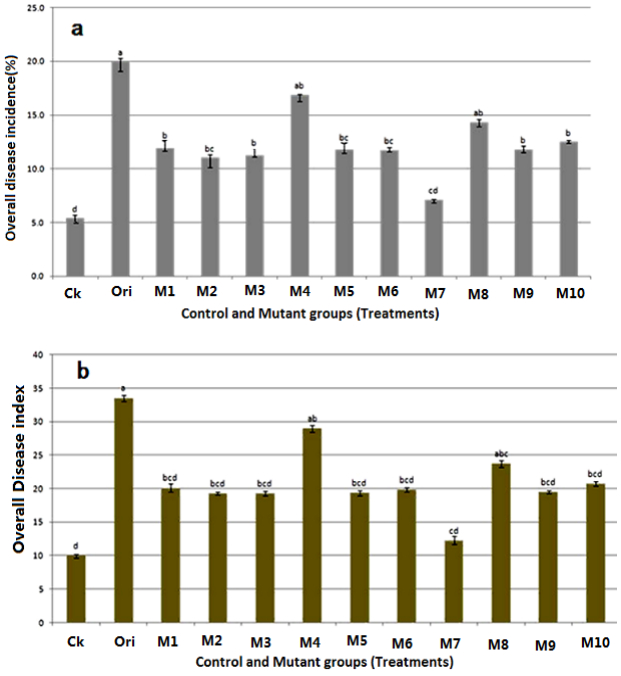


Fig. 9: Mean comparisons for overall disease incidence (**a**) and overall disease index (**b**) induced by the fungus *A. apis* from *in vitro* pathogenicity bioassay. Different letters in the figure shows significant pathogenicity differences among treatments

to be dead naturally or because of some other pathogens.

However, the growth of mycelium starting from the lower part of the dead larval body revealed if the larvae died by fungal infection. The infectivity data confirmed that the original (wild type) fungus caused the maximum (19.9%) amount of larval death starting from the 5th day post inoculation, and its disease index was 33.5%. Even though the infectivity of engineered mutants was not significantly augmented compared to that of the wild strain, mutants 4 and 8 had 16.8% and 14.3% larval death with 28.9% and 23.7% of disease index, respectively (Fig. 9). On the other hand, mutant 7 was not found to be pathogenic to honeybee larvae throughout the experimental period, and the number of diseased and dead larvae was much lower for mutant 7 than for other mutants and was not significantly different from the control group ($p < 0.0001$). In this experiment, we did not find mummified larvae from groups infected with mutant 7, whereas infection with the original fungus or mutants 4, 5, 6, 8, and 10 resulted in mummified, dead larvae. We were able to cultivate the fungus in most of the groups except the control group, and those of mutants 2, 3, and 7. In contrast, 78.75% of healthy larvae from the control group transformed to the pupal stage successfully. In summary, the pathogenicity of REMI-transformed *A. apis* mutants was lower than that of the wild-type.

Validation of Virulence Related Genes

A total of 11 virulence related candidate genes reported in previous studies (Cornman *et al.*, 2012) were selected for validation to understand their expression level in wild-type strain compared to its non-pathogenic mutant 7. Quantitative Real Time Reverse Transcriptase PCR analysis reveals, of the total 11 evaluated virulence related candidate genes, six genes (*Omt*A, *Ski3*, *TOX*D, Hts1, *Nor*-1 and melanin) were significantly expressed in the wild type compared to its non-pathogenic mutant 7 ($P < 0.05$; Fig. 10). The *Omt*A, *Ski3*, *TOX*D, Hts1, *Nor*-1 and melanin genes relative expression level in the wild type strain shows a 3.1, 1.4, 1.4, 1.9, 1.7 and 2.9 fold increase compared to the non-pathogenic mutant 7, respectively. However, the remaining five target genes (*Cts*1, *TOX*A, *TOX*G, *TOX*F and *Alta*7) had non-significant expression either in the wild type or in the non-pathogenic mutant 7 ($P > 0.05$; Fig. 10).

Discussion

There are various types of fungal species that are pathogenic and non-pathogenic to honeybees. Investigation for the differences in pathogenicity among transformed mutants and their wild types is important to understand and develop strategies for fighting honeybee diseases. Contrary to a previous notion (Aronstein and Murray, 2010; Hedtke *et al*., 2011) of chalkbrood being rather harmless since hygienic nurse bees can efficiently recognize and remove diseased larvae, allowing most colonies recover spontaneously, chalkbrood has emerged as an economically important disease affecting the colony. It has been reported that both strain of *A. apis* and colony of larval origin affected mortality rates; for example the strains from one phylogenetic clade caused 12–14% mortality, while those from other clade induced 71–92% brood mortality (Vojvodic *et al*., 2011a; Lee *et al*., 2013). Consistent with this view, we confirmed that the fungus had a significant effect on honeybee brood causing 20% larval mortality during *in vitro* assays.

Evaluation of different aspects of pathogenic fungi and their transformed forms is vital for designing effective strategies to control their effects. With the development of modern biotechnology, REMI insertions improve the

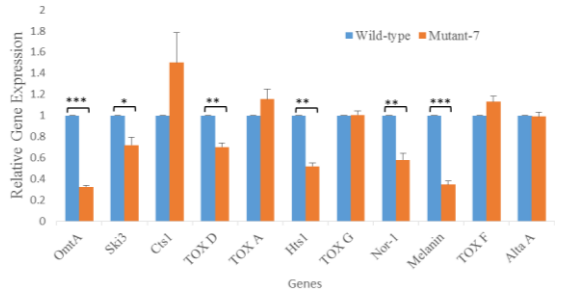


Fig. 10: Expression of 11 virulence related candidate genes in wild type and nonpathogenic mutant 7 strains of *A. apis* using quantitative reverse transcriptase PCR. Strains were grown for four days on Potato Dextrose Agar (PDA). RNA was isolated from spore and used for quantitative reverse-transcription polymerase chain reaction. The name of each gene is indicated at the bottom of each paired histogram. Relative expression levels were normalized to the mean of the expression of the reference gene actin. Y-axis indicates the relative expression level of each gene compared to the wild type strain where the expression of target genes in the wild-type was set to 1. The expression of target genes in wild-type strain was compared to nonpathogenic mutant 7. Data shown are the average of three independent experiments; standard errors are indicated. Means designated by *, **, *** are statistically different according to Student's t-test at < *0.05*, < *0.01* and < *0.001*, respectively

efficiency of genetic transformation (Lopes *et al*., 2008), which helps to identify fungal pathogenicity related genes from altered pathogen's genome. Similar to previous reports (Sato *et al*., 1998; Tunlid *et al*., 1999), PCR, sequence alignment, and Southern blot hybridization have confirmed that introduced foreign gene (*hph*) had effectively been integrated into the host chromosomes, signaling the establishment of stable transformants. Southern blotting is the most common method used to determine whether plasmid integration has occurred successfully. This technique is also used to determine whether REMI has integrated the target at multiple sites for any given mutants. More specifically, the results indicated that the presence of a restriction enzyme in the transformation mixture improved the transformation efficiency in *A. apis*. Although previous reports suggested that using pre-linearized plasmid did not increase the efficiency of transformation over that of the circular plasmids (Lopes *et al*., 2008), we utilized the linear plasmid and achieved successful insertions.

Similar to the observations in fungal species other than *A. apis* (Xu *et al*., 2005; Wang *et al*., 2007), our results demonstrate the successful integration of the foreign *hph* gene in establishing transformants using REMI techniques. Unlike a previous study (Xu *et al*., 2005), where 5 stable mutants were generated from over 5,000 transformants in *Monacrosporium sphaeroides*, which is in the same phylogenic tree with *A. apis* indicating low mutant generation by REMI, we have successfully obtained 184 stable mutants from 225 *A. apis* transformants, which is a much higher yield than attained in previous REMI studies. The efficiency of REMI-based transformation depends on the quality and viability of protoplasts with a higher regeneration rate. In this study, of the selected 12 stable mutants for further study, all mutants but two contained single insert of the foreign gene (*hph*), which reveals that the mutants constructed by REMI technique could be used to identify the genes contributing to the pathogenicity of fungus as reported for *Aspergillus nidulans* (Sánchez *et al*., 1998), *Alternaria alternata* (Tanaka *et al*., 1999), and for *Coprinellus congregates* (Leem *et al*., 2003). Generally, these results have signified the importance of stepwise transformation- and/or insertion-based mutagenesis techniques not only in *A. apis* but also in other organisms.

Experimental replication in colony-based direct fungal spore induction is difficult (Hedtke *et al*., 2011) due to the uncontrolled factors inside the hive and varying hygienic behavior of the honeybees. Hence, establishment of a larval *in vitro*-rearing technique, by which this experiment investigated the differences in the pathogenicity of various engineered *A. apis* strains and the wild-type, was challenging. In conducting *in vitro* bioassays, contrary to previous reports (Flores *et al*., 2004), feeding a mixture of spores in a royal jelly-based larval diet has proved to be a potential avenue of inoculation and did not affect pathogenicity in this study. This work has made a possible and clear delineation of pathogenicity differences among transformed *A. apis* mutants. These variations in pathogenicity may depend on the type and locus of insertion, and disruptive ability of inserts on the genes responsible for pathogenicity.

Honeybee larvae found were to be susceptible to chalkbrood disease when they ingested *A. apis* spores during 3–4 days old and chilled at 22°C two days after they were sealed or close to sealing to pupal stage (Flores *et al*., 1996). However, present results, similar to previous reports (Bailey, 1967; Flores *et al*., 2004), verified that the infected larvae showed disease symptoms after 2 h of chilling at 22°C and/or 24 h before cell sealing periods. In all replicates, the original (wild-type) fungus caused maximum larval death beginning on the 5th day post inoculation. Furthermore, as previously reported (Bamford and Heath, 1989), artificial inoculation of honeybee larvae with the wild-type strain and the majority of its transformed mutants (4, 5, 6, 8 and 10) resulted with mummification of infected larvae in the incubator within 3–4 days after manipulation. This has explained further that random genetic engineering in this organisms could still give a chance for some pathogenicity associated genes to work together to cause a disease for life to survive. Hence, we should focus on targeted gene manipulation technologies for further and sustainable results to prevent honeybees from chalkbrood disease. Interestingly, our laboratory assay revealed that fungal mutants 2, 3 and 7 have failed to mummify the infected larvae after artificial inoculations. This might be due to the reason that basic genetic information has been

lost in these mutants during insertions. Moreover, these results indicated that genetic manipulation of *A. apis* could play a significant role in decreasing the pathogenicity of fungus through disruption of the genetic makeup of the genes associated with fungal pathogenicity. This successful insertion-based mutagenesis of the chalkbrood fungus by REMI, which is being reported for the first time, has led to the development of transformed mutants. These engineered and developed mutants could, thus, be used to explain various mechanisms of pathogenicity, investigate strategies to attenuate pathogenicity, and to better understand the genes associated with the virulence of this fungus. This method and the generated mutants could also be used in a variety of applications in the investigation and manipulation of genes in *A. apis*.

On the other hand, identification of pathogenicity or virulence associated genes in this fungus helps to suggest specific targets for appropriate control measures in chalkbrood. The Real Time PCR analysis in our study reveals that among the total 11 candidate virulence related genes selected and evaluated in this study, six genes: *Omt*A, *Ski*3, *TOX*D, *Hts*1, *Nor*-1 and melanin were highly expressed in *A. apis* wild type strain studied compared to its non-pathogenic mutant 7 (Fig. 10). The other five candidate genes: *Cts*1, *TOX*A, *TOX*G, *TOX*F and *Alta*7 did not have altered expression (Fig. 10).

Previous studies reported the ability of filamentous fungi to produce varieties of secondary metabolites, many of them are involved in virulence (Reichard *et al*., 2000; Ahn *et al*., 2002) including aflatoxins. Similarly, the six genes identified in our study are reported to be involved in critical functions related to Aflatoxins (AF)- Sterigmatocystin (ST) biosynthesis pathway (*Nor*-1 and *Omt*A), HC-toxins biosynthesis (*TOX*D and *Hts*1), a super killer protein 3 (*Ski*3) (Cornman *et al*., 2012) and melanin biosynthesis as a virulence factor in other fungal diseases (Nosanchuk and Casadevall, 2003, 2006).

The biosynthesis of HC-toxins is regulated by a single locus *TOX*2 (Scheffer *et al*., 1967). The *TOX*2 is reported to be a complex locus comprised of about seven genes (*Hts*1, *TOX*A, *TOX*C, T*OX*D, *TOX*E, *TOX*F, and *TOX*G) with reputable role in HC-toxins biosynthesis, exportation and regulation in *Cochliobolus carbonum* (Ahn and Walton, 1996; Cheng and Walton, 2000). Hence, in this work, the two genes *Hts*1 and *TOX*D, which are found to be highly expressed in wild-type strain compared to the non-pathogenic mutant-7 may explain their contribution in the HC-toxin biosynthesis pathway.

Notably, O-methyltransferase gene (*Omt*A) is reported to be involved in aflatoxins B1 biosynthesis pathway (Kannan *et al*., 2014). The deletion of the genomic region comprising the *Omt*A gene in *A. flavus* BFE311 has resulted with the construction of non-aflatoxigenic strains (Geisen, 1996) which validate its importance in aflatoxins B1 biosynthesis. In addition, the *Omt*A gene product is explained to catalyze the conversions of sterigmatocystin to O-methylsterigmatocystin and dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin in *A. parasiticus* (Yu *et al*., 1998). Accordingly, higher expression level of *Omt*A gene in the wild type strain in our work might explain that this gene is also contributing a similar function in *A. apis*.

Nor-1 gene which is highly expressed in our *A. apis* wild type samples has been reported to be involved in aflatoxin biosynthesis in filamentous fungus *A. parasiticus* which encodes a norsolorinic acid ketoreductase (Chiou *et al*., 2002), hence sharing its similar functions in *A. apis*. Nonribosomal peptides produced by nonribosomal peptide synthetases are involved in diverse biological activities, for example playing roles as fungal virulence effectors (Lee *et al*., 2005). For instance, it has been reported *Hts*1 gene controls production of peptide virulence effector HC-toxin by *Cochliobolus carbonum*, a maize crop pathogen (Scott-Craig *et al*., 1992).

Among several functions identified so far, melanin plays role as virulence factor in fungal pathogens by giving protection against to the environmental stressors, including intracellular killing by phagocytes (Nosanchuk and Casadevall, 2006). Recently, the effect of melanin biosynthesis on the virulence of fungal pathogen, *A. fumigatus* has been studied. As a result, it has been reported that *Aspergillus* cell wall melanin blocks LC3-Associated Phagocytosis (LAP) to stimulate pathogenicity on its hosts (Akoumianaki *et al*., 2016). Further, these researchers reported that LAP activation needs the genetic, biochemical or biological (germination) removal of cell wall melanin *in A. fumigatus*. Interestingly, melanin, as one of the highly expressed genes in our study samples, might explain that it is also contributing an important role as a virulence factor in *A. apis* pathogenicity.

Generally, there are eight nuclear genes collectively known as SKI for super killer, mutations in which enhance the production of extracellular toxin (Hougan *et al*., 1989). Among these, *Ski*2, *Ski*3, *Ski*4, *Ski*6, *Ski*7 and *Ski*8 are believed to overproduce killer proteins due to changes in the copy number of M1dsRNA (Ridley *et al*., 1984) to kill their hosts. It has been reported that yeast strains carry a 1.5 x10^6 Da dsRNA in virus-like particles secrete a protein toxin reported to be lethal to strains that do not carrying this species of dsRNA (Akio *et al*., 1978). More specifically, the higher expression of *Ski*3 gene in our *A. apis* wild type strain elucidates that this gene therefore might help the fungus to produce a protein toxin that is lethal to honeybee larvae as it has been explained that some fungi could produce toxins that able to disable the host cellular functions by killing host cells prior to infection or during the necrotrophic stage (Idnurm and Howlett, 2001).

At this point, even if it is explained that the ability of an entomopathogenic fungus to infect its insect host depends on the synchronized activity of virulence factors through mechanical pressure of the hyphae on the exoskeleton and/or gut peritrophic membrane to penetrate through (Cornman *et al*., 2012), the six specific genes,

which have been reported to be involved as virulent factors in other fungal pathogenic species, are evidenced for their highly expression in *A. apis* wild type strain and cause larval death. In this case, our laboratory bioassay experiment demonstrates that *A. apis* wild type strain was found to be highly virulent than all the mutants studied in this work. Accordingly, it has been validated that these six highly expressed genes: *Omt*A, *Ski*3, *TOX*D, *Hts*1, *Nor*-1 and melanin in our samples might have roles in contributing to virulence in *A. apis* pathogenicity. However, the molecular mechanisms that pathogenicity associated genes that govern *A. apis* fungus infectivity and host-pathogen interactions need further investigation.

Conclusion

REMI technique we used in this work has demonstrated the possibility of successful foreign *hph* gene integration into *A. apis* genome for the establishment of transformants. Furthermore, it enables to construct stable *A. apis* mutants which could be used for the identification and isolation of pathogenicity associated genes in this fungus. As a result, genes related to the virulence of the fungus were identified in this study. However, further study should be conducted focusing on characterizing pathogenicity associated genes, understanding genes functions, infection resulting host-pathogen interactions and to further uncover the molecular mechanisms of pathogenicity in *A. apis* that may reveal potential strategies to be developed against the disease caused by this fungus.

Acknowledgments

We would like to acknowledge Dr. Jiaxing Huang, Shimeng Zhang and Xiangfeng He for their valuable support during the study. We are pleased to acknowledge Ass. Prof. Boqiang Li, Institute of Botany Research and Huailei Song, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China for their generous provision of research materials. We are also grateful to acknowledge the support from a Special Fund of Technical Production System of the National Beekeeping Industry (CARS-45-KXJ6) and grant from Scientific and Technological Innovation Program of CAAS-2017 and National Natural Science Foundation of China (31072093) for their financial support.

References

Ahn, J.H. and J.D. Walton, 1996. Chromssomal Organization of TOx2, a Complex Locus Controlling Host-Selective Toxin Biosynthesis in *Cochliobolus carbonum*. *Plant Cell*, 8: 887–897

Ahn, J.H., Y.Q. Cheng and J.D. Walton, 2002. An extended physical map of the TOX2 locus of *Cochliobolus carbonum* required for biosynthesis of HC-toxin. *Fung. Genet. Biol*., 35: 31–38

Aizen, M.A. and L.D. Harder, 2009. The Global Stock of Domesticated Honey Bees Is Growing slower than agricultural demand for pollination. *Curr. Biol*., 19: 915–918

Akio, T., P. Guerry and R.B. Wickner, 1978. Chromosomal superkiller mutants of *Saccharomyces cerevisiae*. *J. Bacteriol*., 136: 1002–1007

Akoumianaki, T., I. Kyrmizi, I. Valsecchi, M.S. Gresnigt, G. Samonis, E. Drakos, D. Boumpas, L. Muszkieta, M.C. Prevost, D.P. Kontoyiannis, T. Chavakis, M.G. Netea, F.L. van de Veerdonk, A.A. Brakhage, J. El-Benna, A. Beauvais, J.P. Latge and G. Chamilos, 2016. *Aspergillus* Cell Wall Melanin Blocks LC3-Associated Phagocytosis to Promote Pathogenicity. *Cell Host Microb*., 19: 79–90

Amaya, E. and K. Kroll, 2010. Production of Transgenic Xenopus laevis by Restriction Enzyme Mediated Integration and Nuclear Transplantation. *J. Visual. Exp*., 200: 2–5

Anderson, D.L., A.J. Gibbs and N.L. Gibson, 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. *Mycol. Res*., 102: 541–547

Aronstein, K.A. and K.D. Murray, 2010. Chalkbrood disease in honey bees. *J. Invertebr. Pathol*., 103: 20–29

Aronstein, K.A., K.D. Murray, J.H. de Leon, X. Qin and G.M. Weinstock, 2007. High Mobility Group (HMG-Box) Genes in the Honeybee Fungal Pathogen *Ascosphaera apis*. *Mycologia*, 99: 553–561

Bailey, L., 1967. *Insect Pathology and Microbial Control*. North Holland Publishing Company, Amsterdam, The Netherlands

Bamford, S. and L.A.F. Heath, 1989. The Effects of Temperature and pH on the Germination of Spores of the Chalkbrood Fungus, *Ascosphaera Apis*. *J. Apicult. Res*., 28: 36–40

Blochlinger, K. and H. Diggelmann, 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. *Mol. Cell. Biol*., 4: 2929–2931

Cheng, Y. and J.D. Walton, 2000. A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis a eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. *J. Biol. Chem*., 275: 4906–4911

Chiou, C.H., M. Miller, D.L. Wilson, F. Trail and J.E. Linz, 2002. Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*. *Appl. Environ. Microbiol*., 68: 306–315

Cornman, R., A.K. Bennett, K. Murray, J.D. Evans, C.G. Elsik and K. Aronstein, 2012. Transcriptome analysis of the honey bee fungal pathogen, *Ascosphaera apis*: implications for host pathogenesis. *BMC Genomics*, 13: 285

Crailsheim, K., R. Brodschneider, P. Aupinel, D. Behrens, E. Genersch, J. Vollmann and U. Riessberger-Gallé, 2013. Standard methods for artificial rearing of *Apis mellifera* larvae. *J. Apicult. Res*., 52: 1–16

Engelsdorp, D.P. and M.D. Meixner, 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J. Invertebr. Pathol*., 103: 80–95

Flores, J.M., I. Gutierrez and F. Puerta, 2004. A comparison of methods to experimentally induce chalk brood disease in honey bees. *Span. J. Agric. Res*., 2:79–83

Flores, J.M., J.A. Ruiz, J.M. Ruz, F. Puerta, M. Bustos, F. Padilla and F. Campano, 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. *Apidologie*, 27: 185–192

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Syst. Appl. Microbiol*., 19: 388–392

Genersch, E., A. Ashiralieva and I. Fries, 2005. Strain- and genotype-specific differences in virulence of paenibacillus larvae subsp . larvae, a bacterial pathogen causing american foulbrood disease in honeybees. *Appl. Environ. Microbiol*., 49: 7551–7555

Hanif, M., G.A. Pardo, M. Gorfer and M. Raudaskoski, 2002. T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. *Curr. Genet*., 41: 183–188

Heath, L.A.F. and B.M. Gaze, 1987. Carbon dioxide activation of spores of the chalkbrood fungus *Ascosphaera apis*. *J. Apicult. Res.*, 26: 243–246

Hedtke, K., P.M. Jensen, A.B. Jensen and E. Genersch, 2011. Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood. *J. Invertebr. Pathol*., 108: 167–173

Hougan, L., D.Y. Thomas and M. Whiteway, 1989. Cloning and characterization of the SKI3 gene of *Saccharomyces cerevisiae* demonstrates allelism to SKI5. *Curr. Genet*., 16: 139–143

Idnurm, A. and B.J. Howlett, 2001. Pathogenicity genes of phytopathogenic fungi. *Mol. Plant Pathol*., 2: 241–255

James, R.R. and J.S. Buckner, 2004. Lipids stimulate spore germination in the entomopathogenic ascomycete *Ascosphaera aggregata*. *Mycopathology*, 158: 293–302

Jensen, A.B., K. Aronstein, J.M. Flores, S. Vojvodic, M.A. Palacio and M. Spivak, 2013. Standard methods for fungal brood disease research. *J. Apicult. Res*., 52: 1–20

Jensen, A.B., R.R. James and J. Eilenberg, 2009. Long-term storage of *Ascosphaera aggregata* and *Ascosphaera apis*, pathogens of the leafcutting bee (*Megachile rotundata*) and the honey bee (*Apis mellifera*). *J. Invertebr. Pathol*., 101: 157–160

Kannan, K., S. Supriya, R. Adhithya and R. Velazhahan, 2014. *Aspergillus flavus* and aflatoxin contamination of poultry feeds in Tamil Nadu, India. *Int. J. Agric. Environ. Biotechnol*., 7: 361

Knight, C.J., A.M. Bailey and G.D. Foster, 2009. *Agrobacterium*-mediated transformation of the plant pathogenic fungus *Verticillium albo-atrum*. *J. Plant Pathol*., 91: 745–750

Lee, B.N., S. Kroken, D.Y.T. Chou, B. Robbertse, O.C. Yoder and B.G. Turgeon, 2005. Functional analysis of all nonribosomal peptide synthetases in *Cochliobolus heterostrophus* reveals a factor , NPS6, involved in virulence and resistance to oxidative stress. *Eukaryot. Cell*, 4: 545–555

Lee, G.M., P.A. McGee and B.P. Oldroyd, 2013. Variable virulence among isolates of *Ascosphaera apis*: Testing the parasite-pathogen hypothesis for the evolution of polyandry in social insects. *Naturwissenschaften*, 100: 229–234

Leem, Y.E., I.K. Ross and H.T. Choi, 2003. Tagging and localization of a phospholipase D gene in *Coprinellus congregatus* by restriction enzyme-mediated integration and pulsed-field gel electrophoresis. *FEMS Microbiol. Lett*., 225: 285–289

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25: 402–408

Lopes, F., M.V. De Queiroz and J.O. Lima, 2008. Restriction enzyme improves the efficiency of genetic transformations in *Moniliophthora perniciosa*, the causal agent of witches' broom disease in *Theobroma cacao*. *Braz. Arch. Biol. Technol*., 51: 27–34

Nosanchuk, J.D. and A. Casadevall, 2006. Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents Chemother*., 50: 3519–3528

Nosanchuk, J.D. and A. Casadevall, 2003. The contribution of melanin to microbial pathogenesis. *Cell. Microbiol*., 5: 203–223

Olmedo-Monfil, V., C. Cortés-Penagos and A. Herrera-Estrella, 2004. Three decades of fungal transformation: Key concepts and applications. *Meth. Mol. Biol*., 267: 297–314

Rehman, L., X. Su, H. Guo, X. Qi, and H. Cheng, 2016. Protoplast transformation as a potential platform for exploring gene function in *Verticillium dahliae*. *BMC Biotechnol.*, 16:57

Reichard, U., G.T. Cole, R. Rüchel and M. Monod, 2000. Molecular cloning and targeted deletion of PEP2 which encodes a novel aspartic proteinase from *Aspergillus fumigatus*. *Int. J. Med. Microbiol.*, 290: 85–96

Ridley, S.P., S.S. Sommer and R.B. Wickner, 1984. Superkiller mutations in *Saccharomyces cerevisiae* suppress exclusion of M2 double-stranded RNA by L-A-HN and confer cold sensitivity in the presence of M and LA-HN. *Mol. Cell. Biol*., 4: 761–770

SAS Institute Inc., 2002. Cary, North Carolina, USA

Sánchez, O., R.E. Navarro and J. Aguirre, 1998. Increased transformation frequency and tagging of developmental genes in *Aspergillus nidulans* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.*, 258: 89–94

Sato, T., K. Yaegashi, S. Ishii, T. Hirano, S. Kajiwara, K. Shishido and H. Enei, 1998. Transformation of the edible Basidiomycete *Lentinus edodes* by restriction enzyme-mediated integration of plasmid DNA. *Biosci. Biotechnol. Biochem*., 62: 2346–2350

Scheffer, R.P., R.R. Nelson and A.J. Ullstrup, 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. *Phytopathology*, 57: 1288–1289

Scott-Craig, J.S., D.G. Panacciones, J. Pocard and J.D. Waltonn, 1992. The cyclic peptide synthetase catalyzing hc-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-Kilobase Reading Frame. *J. Biol. Chem.*, 267: 26044–26049

Spiltoir, C., 1955. Life cycle of *Ascosphaera apis* (*Pericystis apis*). *Amer. J. Bot*., 42: 501–508

Tanaka, A., H. Shiotani, M. Yamamoto and T. Tsuge, 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. *Mol. Plant-Microb. Interact*., 12: 691–702

Thon, M.R., E.M. Nuckles and L.J. Vaillancourt, 2000. Restriction enzyme-mediated integration used to produce pathogenicity mutants of *Colletotrichum graminicola*. *Mol. Plant-Microb. Interact*., 13: 1356–1365

Tunlid, A.Y., J. Ahman and R.P. Oliver, 1999. Transformation of the nematode-trapping fungus *Arthrobotrys oligospora*. *FEMS Microbiol. Lett*., 173: 111–116

Vandenberg, J., 1992. Bioassay of the chalkbrood fungus *Ascosphaera aggregata* on larvae of the alfalfa leafcutting bee, *Megachile rotundata*. *J. Invertebr. Pathol*., 60: 159–163

Vojvodic, S., A.B. Jensen, B. Markussen, J. Eilenberg and J.J. Boomsma, 2011a. Genetic variation in virulence among chalkbrood strains infecting honeybees. *PLoS One*, 6: e25035

Vojvodic, S., A.B. Jensen, R.R. James, J.J. Boomsma and J. Eilenberg, 2011b. Opposing temperature-dependent mortality after infection with an obligate and facultative fungal pathogen of honeybees. *Vet. Microbiol*., 149: 200–205

Wang, Y., B. Guo, Z. Miao and K. Tang, 2007. Transformation of taxol-producing endophytic fungi by restriction enzyme-mediated integration (REMI). *FEMS Microbiol. Lett*., 273: 253–259

Wubie, A.J., Y. Hu, W. Li, J. Huang, Z. Guo, S. Xu and T. Zhou, 2014. Factors analysis in protoplast isolation and regeneration from a chalkbrood fungus, *Ascosphaera apis*. *Int. J. Agric. Biol*., 16: 89–96

Xu, J., M. Mo, W. Zhou, H. Xiao-Wei and K.Q. Zhang, 2005. Transformation and mutagenesis of the nematode-trapping fungus *Monacrosporium sphaeroides* by restriction enzyme-mediated integration (REMI). *J. Microbiol*., 43: 2005

Xue, F., W. Li, A.J. Wubie, Y. Hu, Z. Guo, T. Zhou and S. Xu, 2015. Biological control of *Ascosphaera apis* in honey bees using restricted enzyme mediated integration (REMI) transformed Trichoderma atroviride mutants. *Biol. Contr.*, 83: 46–50

Yu, J., P. Chang, K.C. Ehrlich, J.W. Cary, B. Montalbano, J.M. Dyer, D. Bhatnagar and T.E. Cleveland, 1998. Characterization of the critical amino acids of an *Aspergillus parasiticus* Cytochrome P-450 monooxygenase encoded by ordA that is involved in the biosynthesis of aflatoxins B1, G1, B2 and G2 characterization of the critical amino acids of an Asper. *Appl. Environ. Microbiol*., 64: 4834–4841

(Received 17 April 2018; Accepted 18 June 2018)