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Expression of scorpion toxin LqhIT2 increases the virulence of *Metarhizium acridum* towards *Locusta migratoria manilensis*

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Abstract LqhIT2 is an insect-specific neurotoxin from the venom of scorpion. In this study, the *LqhIT2* gene was introduced into the entomopathogenic fungus, *Metarhizium acridum*. The virulence of the genetically modified strain MaLqhIT2 was then evaluated against locusts (*Locusta migratoria manilensis*). Compared with the wild-type strain, the median lethal cell density (LC_{50}) for MaLqhIT2 was a 22.6-fold lower, and the median times to death (LT_{50}) for MaLqhIT2 were reduced by 30.3 and 29.6 %, respectively, after topical inoculation and injection. MaLqhIT2 also grew significantly faster in the hemolymph than wild-type strain. There were no significant differences in germination, appressorium formation and sporulation in locust carcasses between the MaLqhIT2 and wild-type strain. These results indicate that LqhIT2 increased the virulence of *M. acridum* towards locusts by shortening the in vivo infection period, without affecting cuticle penetration or conidia formation in the carcasses. LqhIT2 thus shows considerable potential for increasing fungal virulence against locusts.

Keywords *Metarhizium acridum* · Genetic engineering · LqhIT2 · *Locusta migratoria manilensis* · Virulence

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Introduction

Throughout history, the voracious appetites and huge numbers of locusts and grasshoppers have had significant impacts on agriculture worldwide [3, 33]. With increasing public concern over the continued use of insecticides, biological insect control has become an increasingly important part of integrated pest management [20]. Entomopathogenic fungi play a crucial role in controlling insect populations [5]. To date, 13 species or subspecies of insect pathogenic fungi have been formulated and registered [6]. Compared with bacteria and viruses, entomopathogenic fungi are particularly well suited to development as biological pesticides in dry grassland and desert habitats [5, 20]. The acridid-specific fungal pathogen *Metarhizium acridum*, previously classified as *M. anisopliae* var. *acridum* [4] or *M. flavoviride* var. *minus* [8], has been used for the effective biocontrol of locusts and grasshoppers [20]. Despite the ability of *M. acridum* to penetrate the cuticle, the relatively long time-to-death in *M. acridum*-infected insects has limited the commercial development of large-scale applications of *M. acridum* for locust control.

However, genetic engineering has significantly improved the insecticidal efficacy of certain fungi [29]. Entomopathogenic fungi infect insects by direct penetration of the cuticle, followed by proliferation within the insect hemocoel. Cuticle-degrading enzymes and exogenous virulence genes are of particular interest for the genetic modification of entomopathogenic fungi [13, 29]. Several endogenous genes encoding the cuticle-degrading protease Pr1 and chitinase have been introduced into the genomes of entomopathogenic fungi with the aim of enhancing their efficacy against pest insects [11, 28]. Fungal virulence has also been increased through the expression of certain insect molecules, such as diuretic hormone and trypsin-modulating oostatic factor [9],

and insecticidal toxins, such as Vip3Aa from *Bacillus thuringiensis* [25]. Studies of genetically modified fungi for biological insect control have primarily investigated the use of proteins of scorpion or spider venom [7]. The extent to which virulence can be increased in genetically modified fungi has been shown in the tobacco hornworm (*Manduca sexta*), the yellow-fever mosquito (*Aedes aegypti*), and the coffee-berry-borer beetle (*Hypothenemus hampei*) through the expression of the scorpion toxin, AaIT, in *M. anisopliae* [22, 31]. The expression of AaIT was also shown to increase the virulence of *Beauveria bassiana* against larvae of the Masson pine moth, *Dendrolimus punctatus*, and the wax moth, *Galleria mellonella* [21]. The expression of insect-specific toxins in genetically modified *M. acridum* might thus provide a biological alternative to the use of hazardous chemicals for locust control.

LqhIT2, a 61-amino-acid polypeptide, is an insect-specific neurotoxin and a component of the venom in the Israeli yellow scorpion, *Leiurus quinquestriatus hebraeus* [16, 35]. The toxin acts as a depressant by interacting with the voltage-dependent sodium channels of insect motor neurons [34]. The expression of the LqhIT2 protein increased the virulence of the *Autographa californica* nucleopolyhedrovirus (AcMNPV) against *Heliothis virescens* larvae [2], and LqhIT2 expressed in *Pichia pastoris* demonstrated insecticidal activity against *L. migratoria manilensis* via injection [18]. The expression of LqhIT2 in entomopathogenic fungi might thus increase their virulence against insect pests.

Locusta migratoria manilensis has been a major agricultural pest in China since 707 BC [33]. The commercial bioinsecticide strain of *M. acridum*, CQMa102, has been used for biocontrol of this species in China. However, a previous study found that CQMa102 took 11–15 days to kill 80–90 % of the locusts [23]. The CQMa102 strain has been used as a model pathogen to explore the relationship between the entomopathogenic fungus and the host [14]. In this study, we expressed LqhIT2 in *M. acridum* to investigate its potential for increasing the efficacy of entomopathogenic fungi for biological insect control.

Materials and methods

Microbial strains and media

The wild-type strain of *M. acridum*, CQMa102, was isolated from *Ceracris kiangsu* (Orthoptera: Acrididae), and stored in the China General Microbiological Culture Collection Center (strain no. 0877) [23]. Except where indicated, *M. acridum* was cultured on quarter-strength Sabouraud dextrose agar (SDA) medium, containing 1 % dextrose, 0.25 % peptone, 2 % agar, and 0.5 % yeast extract at 28 °C for 15 days before the conidia were harvested.

Gene synthesis and LqhIT2 expression vector construction

The DNA sequence of the *LqhIT2* gene was synthesized based on the codon preference of *M. acridum* and the LqhIT2 polypeptide sequence [35]. To ensure secretion, the *MCL1* signal sequence (*MCL1ss)* was incorporated at the 5′ end of the *LqhIT2* coding region, as described previously [31]. The *MCL1ss* and *LqhIT2* coding sequences were flanked by an *Eco*RI site at the 5′ end of the *MCL1ss* sequence and a *Bam*HI site and two consecutive stop codons (TAATGA) at the 3′ end of the *LqhIT2* sequence. The *MCL1ss-LqhIT2* gene fusion was cloned into pMD-18T (Takara, Takara Biotechnology Dalian LTD, Dalian, China), and transformed into *E. coli* DH5α for determination by TransGenBiotech (Beijing, China). The orientation and sequence of the expression cassette were confirmed by DNA sequencing. The *MCL1ss-LqhIT2* gene fusion and the pBarEx plasmid [17], which contains the phosphinothricin acetyltransferase gene, the *gpdA* promoter and *trpC* terminator from *Aspergillus nidulans*, were digested with *Eco*RI and *Bam*HI, and ligated using T4 DNA ligase to generate the pBarEx-LqhIT2 expression vector.

Fungal transformation and screening

The pBarEx-LqhIT2 plasmid was introduced into the CQMa102 strain of *M. acridum* using microparticle bombardment, as previously described [17]. The MaLqhIT2 transformants were screened for growth on Czapek medium, containing 3 % saccharose, 0.2 % $NaNO_3$, 0.1 % K_2HPO_4, 0.05 % KCl, 0.05 % $MgSO_4$, 0.001 % $FeSO_4$, 1.8 % agar, and 200 μg/mL glufosinate-ammonium (Sigma-Aldrich, St. Louis, MO, USA). The transformants were confirmed by polymerase chain reaction (PCR) amplification of the *LqhIT2* coding sequence using the P1 (5′-GTCGACAGAAGATGATATTGAAGG-3′) and P2 (5′-TCATCAGATCTCGGTGACGGGCAGG-3′) oligonucleotide primers.

To verify the expression of the*MCL1ss-LqhIT2* gene fusion, we quantified its expression using quantitative reverse transcription PCR (qRT-PCR) at 2, 4, and 6 days post-inoculation. Total RNA was extracted from infected insects using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The qRT-PCR was performed using *LqhIT2*-specific primers *LqhIT2*-F (5′-CGGCAGACGGTTACATCAAG-3′) and *LqhIT2*-R (5′-TTCGGACTTCCAGGTCTTGT-3′). The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as an internal control, using the P3 (5′-AGATG GAGGAGTTGGTGTTG-3′) and P4 (5′-GACTGCCCG CATTGAGAAG-3′) primers. The relative expression level of *LqhIT2* was determined using the comparative cycle threshold (CT) method [12].

Bioassays and sporulation in locust carcasses

The virulence of strains was assessed by topical inoculation and injection, as described previously [24]. The pronota of adult male locusts (2–3 days after eclosion) were topically inoculated with 5 μL of conidia suspension with a cell density of 1.6×10^5, 8×10^5, 4×10^6, 2×10^7, 1×10^8, or 5×10^8 conidia/mL. A 5-μL aliquot of conidia suspension containing 5×10^5 conidia/mL in sterile water was injected into the hemocoel in the abdominal segments of the locusts. Cottonseed oil and sterile water were used as controls for the topical inoculation and injection experiments, respectively. The experiments were repeated five times using 30 insects per replicate. Survival was recorded every 12 h.

The virulence of the genetically modified MaLqhIT2 strain of *M. acridum* was determined based on the cell density causing 50 % mortality (LC_{50}) and the time at which 50 % of the infected insects died (LT_{50}). Dead locusts were weighed, and stored at 26 °C in a humidified container. Sporulated carcasses were minced, and suspended in sterile water containing 0.1 % Tween-80. The suspension was stirred for 0.5 h, and the conidia were counted using a hemocytometer.

Germination and appressorium formation

The conidia were suspended in sterilized water, and shaken vigorously. The suspension was filtered through a cotton cloth, and diluted to 1×10^7 conidia/mL. A 10-μL aliquot of the conidia suspension was mounted on a microscope slide, and covered with a 2-cm cellophane disc. The slide was dried naturally. The cellophane disc was removed from the slide, and placed on moist filter paper in a 9-cm Petri dish. The disc was incubated at 28 °C for 48 h or less, and microscopically examined at ×400 magnification. Germination and appressorium formation were evaluated in approximately 300 fields of view on five cellophane discs.

Quantification of MaLqhIT2 strain DNA in locust hemolymph

The growth of MaLqhIT2 in locusts topically inoculated with 2.0×10^7 conidia/mL was quantified using quantitative real-time PCR (qPCR) to detect the sequences of the internal transcribed spacers (ITS1-5.8 s-ITS2) of the ribosomal RNA gene (rDNA) of *M. acridum* in the hemolymph of the insect host, as described previously [24]. Genomic DNA was isolated from the hemolymph of infected locusts, and qPCR was performed using the P5 (5-TGGCATCTTCTGAGTGGTG-3′) and P6 (5′-CCCGTTGCGAGTGAGTTA-3′) primers. In addition, hyphae in the hemocoel were also examined using a Motic 2000 digital light microscope (Motic, Guangzhou, China), and digital images were recorded.

Statistical analysis

All treatments were performed five times, unless otherwise stated. The data were arcsine square-root transformed before being subjected to an analysis of variance for a completely randomized design. The means were analyzed using the Tukey multiple range test. The statistical analysis was performed using the SPSS, version 18.0, software (IBM, Armonk, NY, USA). The level of statistical significance was established at $\alpha = 0.05$.

Results

Characterization and identification of MaLqhIT2 transformants

The DNA sequence of the *Mcl1ss* (57 nt) and *LqhIT2* (192 nt) coding regions and the two stop codons was confirmed (Fig. 1a, b). The transformation of *M. acridum* was confirmed by the PCR analysis, which showed that the pBarEx-LqhIT2 vector was integrated into the fungal genome (Fig. 1c). After three rounds of subculturing, one putative transformant, MaLqhIT2, was found to express the *LqhIT2* gene during fungal growth in vivo, as determined by qRT-PCR analysis (Fig. 1d).

LqhIT2 improves the virulence of *M. acridum* against locusts

The virulence of the MaLqhIT2 strain against locusts was evaluated based on the LC_{50} and LT_{50}, and compared with that of wild type of *M. acridum* (CQMa102). In the topical inoculation and injection experiments, locusts infected with MaLqhIT2 had lower survival than locusts infected with the wild-type strain ($p < 0.05$) (Fig. 2a, b) at 4–7 days after inoculation. Locusts infected with MaLqhIT2 by topical inoculation had an LC_{50} of 3.4×10^5 conidia/mL, which was 22.6-fold lower than that of locusts infected with the wild-type strain (7.7×10^6 conidia/mL) ($p < 0.05$) (Table 1). The LT_{50} in locusts topically infected with MaLqhIT2 was 3.9 days, which was 30.3 % lower than in locusts infected with the wild-type strain (5.6 days) ($p < 0.05$). In the injection experiments, the LT_{50} was 3.8 days in locusts infected with MaLqhIT2, which was 29.6 % lower than in locusts infected with the wild-type strain (5.4 days) ($p < 0.05$) (Table 1). The LT_{50} values for locusts infected by topical inoculation and injection were 1.7 and 1.6 days shorter, respectively, than those of locusts infected with the wild-type strain (Table 1).The growth of conidia (3.21×10^9 conidia/g) in the carcasses of locusts infected by MaLqhIT2 was similar ($p > 0.05$) to that of locusts

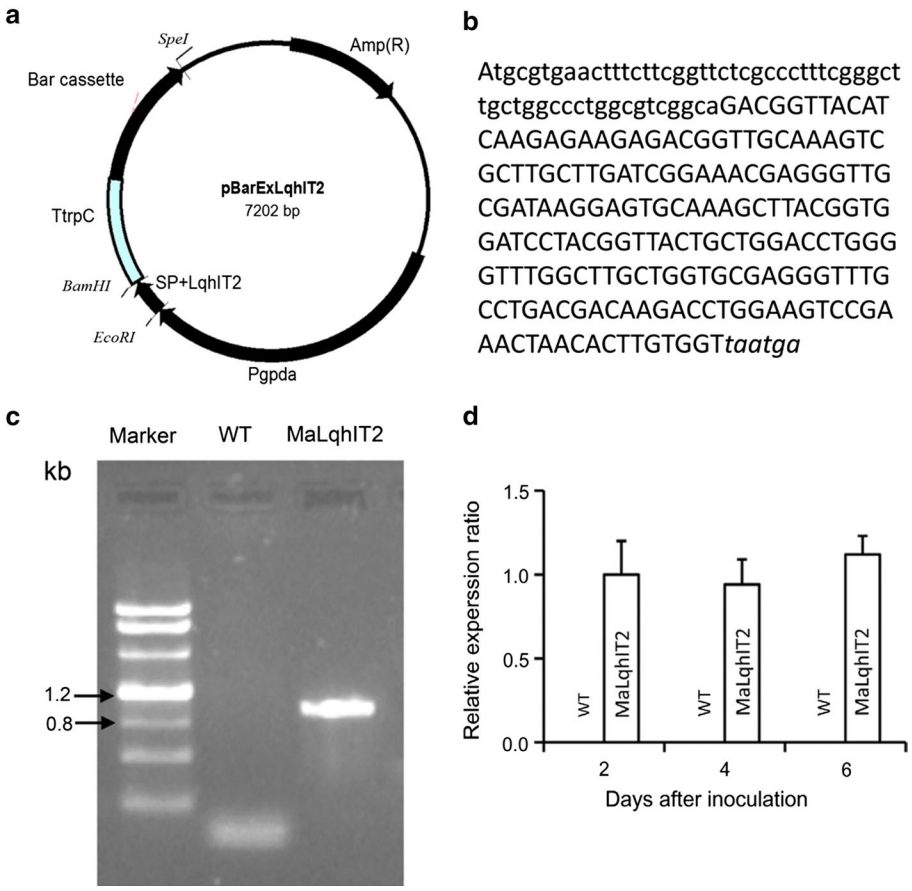

Fig. 1 The LqhIT2-expression constructs. **a** Diagrammatic structure of the LqhIT2-expression vector. SP: *Mcl1*signal peptide; **b** The nucleotide sequence of the *Mcl1ss-LqhIT2* gene fusion (*lowercase letters*: *Mcl1*signal peptide sequence; *capital letters*: LqhIT2 gene; *italic letters*: stop codons). **c** The expression of the *Bar* gene and (**d**) the *LqhIT2* gene was confirmed by quantitative reverse transcription PCR (WT, wild-type strain (CQMa102); MaLqhIT2, CQMa102 *Metarhizium acridum* expressing the LqhIT2 toxin). The *bars* represent the SE ranges

infected with the wild-type strain (3.62×10^9 conidia/g; Fig. 2c, d) after 6 days at 26 °C and high humidity, suggesting that LqhIT2 did not affect the formation of conidia.

Effects of LqhIT2 on germination and appressorium formation

There was no significant difference in germination rate between the MaLqhIT2 and wild-type strains ($p > 0.05$). Both strains achieved >95 % germination 36 h after inoculation (Fig. 3a). Similarly, there was no significant difference in appressorium formation between the MaLqhIT2 and wild-type strains ($p > 0.05$). The appressorium formation rates in both strains were about 60 % 36 h after inoculation (Fig. 3b). Furthermore, there were no morphological differences between the germ tubes or appressoria between the two strains (Fig. 3c). These results showed that transformation of *LqhIT2* into CQMa102 did not affect the germination rate or appressorium formation of the fungus, suggesting that LqhIT2 might not be related to the penetration of *M. acridum* through the insect cuticle.

Effect of LqhIT2 on *M. acridum* growth in locusts

Micrographs of locust hemolymph were inspected to evaluate the growth of *M. acridum* (Fig. 4a). There were more hyphal bodies in locusts infected with MaLqhIT2 than in those infected with the wild-type strain at 3.5 days after inoculation. This was confirmed by quantification of fungal growth in the locust hemolymph by analysis of *M. acridum* DNA concentration using qPCR (Fig. 4b). On day two post-inoculation, there was no significant difference in fungal growth (DNA concentration) between the MaLqhIT2 and wild-type strains ($p > 0.05$). However, the DNA concentration was significantly higher in MaLqhIT2-infected locusts than in wild-type-infected locusts ($p < 0.05$) at 2.5–3.5 days after inoculation. These results suggest that LqhIT2 promoted the proliferation of *M. acridum* during in vivo infection, leading to rapid death.

Discussion

As a key regulator of insect populations, entomopathogenic fungi have significant potential for applications as

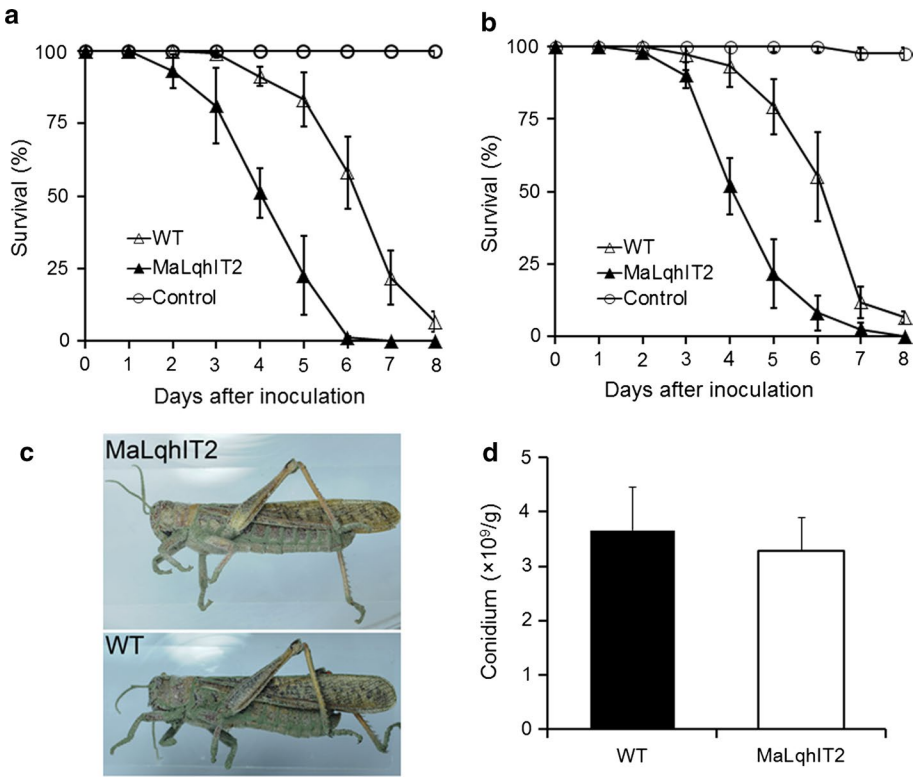

Fig. 2 Kinetics of insect survival based on bioassays and analysis of sporulation in *Locusta migratoria* carcasses. **a** The survival of *L. migratoria* topically inoculated with 5 µL of a 2 × 10^7 conidia/mL oil suspension of the MaLqhIT2 or CQMa102 (wild type) strain of *Metarhizium acridum* (mean ± standard error [SE] of five independent experiments). Control insects were inoculated with 5 µL of cottonseed oil. **b** The survival of *L. migratoria* following the injection of 5 µL of a 5 × 10^6 conidia/mL suspensions of the MaLqhIT2 or wild-type strain of *M. acridum* (mean ± standard error of five independent experiments). Control insects were injected with 5 µL of sterile water. **c** Sporulation photographs of the carcasses of *L. migratoria* infected with the MaLqhIT2 or wild-type strain of *M. acridum*, after the carcass was incubated at 26 °C and >90 % relative humidity for 6 days. **d** The number of conidia per gram of carcass of *L. migratoria* infected with the MaLqhIT2 or wild-type strain of *M. acridum* (mean ± SE of 30 carcasses). The *bars* represent the SE ranges

Table 1 Survival of *Locusta migratoria* infected with the CQMa102 (wild type) or MaLqhIT2 strain of *Metarhizium acridum*

Fungus	LC_{50} (conidia/mL)[a]	LT_{50} (days)[b]	LT_{50} (days)[c]
Wild type	7.7 (6.2–9.2) × 10^6	5.6 (4.7–6.5)	5.4 (4.8–6.0)
MaLqhIT2	3.4 (2.7–4.1) × 10^5	3.9 (3.4–4.4)	3.8 (3.3–4.3)

Data are presented as the median lethal cell density (LC_{50}) or the median time to death (LT_{50}), and the 95 % confidence intervals (shown in parentheses), based on probit and Kaplan–Meier survival analyses

[a] Dose-mortality response of *L. migratoria* infected with wild type or recombinant fungus 7 days after topical inoculation

[b] Topical inoculation using 2 × 10^7 conidia/mL

[c] Hemocoel injection using 5 × 10^5 conidia/mL

biocontrol agents. Enhancing fungal virulence by genetic manipulation would be a more cost-effective means of crop pest control, compared with the use chemical insecticides. The cuticle-degrading properties of the subtilisin-like protease, Pr1, and various chitinases have been shown to influence the virulence of entomopathogenic fungi [5]. Constitutive expression of the *Pr1* or hybrid chitinase gene caused a 23–25 % reduction in survival of the host, compared to the wild-type fungus [10, 28].

The AaIT protein is an insect-specific neurotoxin from the scorpion, *Androctonus australis*, that has been used to increase the virulence of entomopathogenic fungi. The LC_{50} for *M. anisopliae* expressing AaIT was 22-fold, 9-fold, and 16-fold lower in *M. sexta*, *A. aegypti*, and *H. hampei*, respectively, than that observed for wild-type *M. anisopliae* infections in those insect species [22, 31]. Likewise, *B. bassiana* expressing the AaIT demonstrated a 15-fold increase in insecticidal activity in *D. punctatus* [21]. In our current study, we genetically modified *M. acridum* to express the insect-selective neurotoxin LqhIT2. We observed 30.3 % and 22.6-fold reductions in the LT_{50} and LC_{50}, respectively, in topically-inoculated locusts. The expression of LqhIT2 increased the virulence of *M. acridum*, suggesting that LqhIT2 has great potential for increasing the virulence of entomopathogenic fungi.

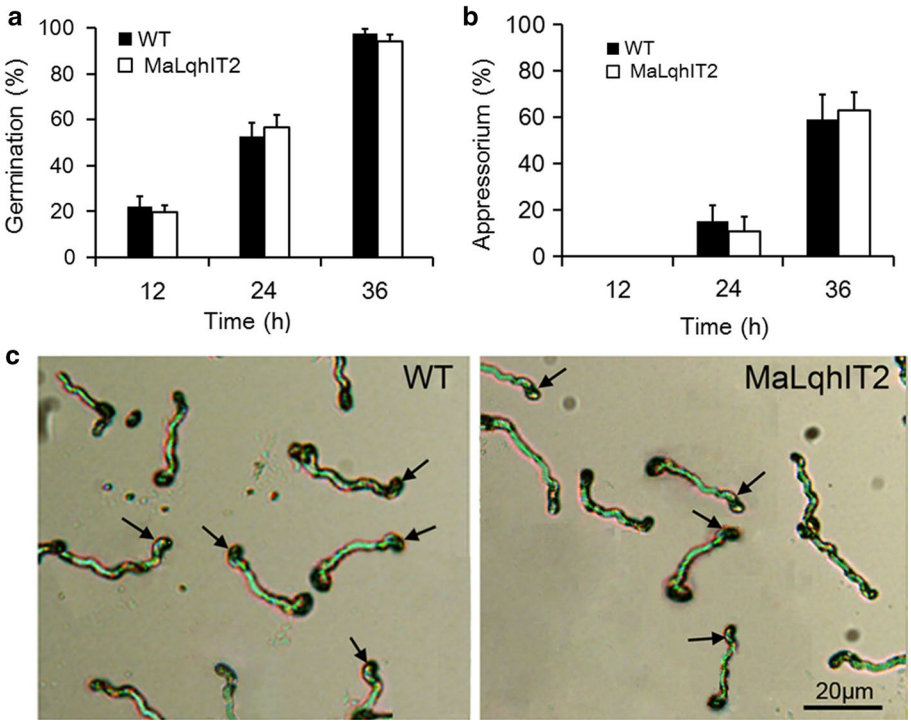

Fig. 3 The effects of LqhIT2 expression on *M. acridum* germination and appressorium formation in *Locusta migratoria*. **a** The germination rate of the MaLqhIT2 and CQMa102 (wild type) strains of *M. acridum*. **b** The appressorium formation rate of the MaLqhIT2 and wild-type strains of *M. acridum*. **c** Micrographs of the germ tubes and appressoria of the MaLqhIT2 and wild-type strains of *M. acridum*. The *bars* represent the standard error ranges

Entomopathogenic fungi infect the insect by penetrating the cuticle, and the fungus then kills the host through proliferation or the release of toxins in the insect's hemolymph [29]. Previous studies have shown that toxin production by *M. acridum* in insects is limited [27], which has been confirmed by the analysis of *M. acridum* genome [14] and studies of the effect of the *M. acridum* toxin in locusts [15]. Thus, the virulence of *M. acridum* is primarily dependent on the proliferation of the fungus in the insect hemocoel stage [23], and integration of toxin peptide genes such as *LqhIT2* into *M. acridum* could greatly increase the fungal virulence towards its locust host. As an insect sodium channel toxin, LqhIT2 causes paralysis and physiological disorders in insects [16]. Our study showed that the fungal transformant expressing LqhIT2 grew faster than the wild strain. This suggests that *Metarhizium* transformants synthesizing LqhIT2 may weaken host locusts by blocking the voltage-dependent sodium channels of insect motor neurons.

Although the virulence of the genetically engineered fungal strains is increased by the expression AaIT, the effects of AaIT expression on cuticle penetration in vivo remain unclear. One previous study was unable to assess the effect of AaIT on the germination and appressoria formation of an entomopathogenic fungus because the expression of AaIT was driven by the *Mcl1* promoter, which is active in insect hemolymph [30]. In our current study, the germination and appressorium formation rates of MaLqhIT2 in the locust were determined. No difference in germination or appressorium formation was observed between the MaLqhIT2 and wild-type strains, and the number of MaLqhIT2 and wild-type hyphal bodies in the hemolymph of locusts was similar on day 2 post-inoculation. The results indicate that the effects of LqhIT2 on virulence were not related to cuticle penetration. The expression of the *LqhIT2* gene accelerated the growth of *M. acridum* in the hemolymph of locusts on days 3 and 4 post-inoculation. This may have been the result of the suppression of the insect immune response caused by the LqhIT2 toxin, which affects voltage-gated sodium channels in insects [1].

Safety concerns regarding the use of genetically modified entomopathogenic fungi have been addressed in multiple studies [19, 32]. Overexpression of the *Pr1* gene prevented spore production in carcasses, thus blocking the ability of the fungus to reproduce in the field and decreasing the threat to non-target insects [28]. The threat to the environment posed by fungal strains expressing AaIT might be reduced by the use of tissue-specific promoters. However, the use of entomopathogenic fungi with a narrow host range would also serve to reduce the threat to non-target species [31]. The host range of *M. acridum* is limited to acridids, such as locusts and grasshoppers [26]. Thus, the expression of LqhIT2 in genetically modified *M. acridum* should not compromise environmental safety. Future studies of the effects of tissue-specific promoters, such as *Mcl1* promoter, on LqhIT2 expression in *M. acridum* are warranted to determine whether host specificity is affected.

In the natural environment, entomopathogenic fungi infect host insects primarily through sporulation in host

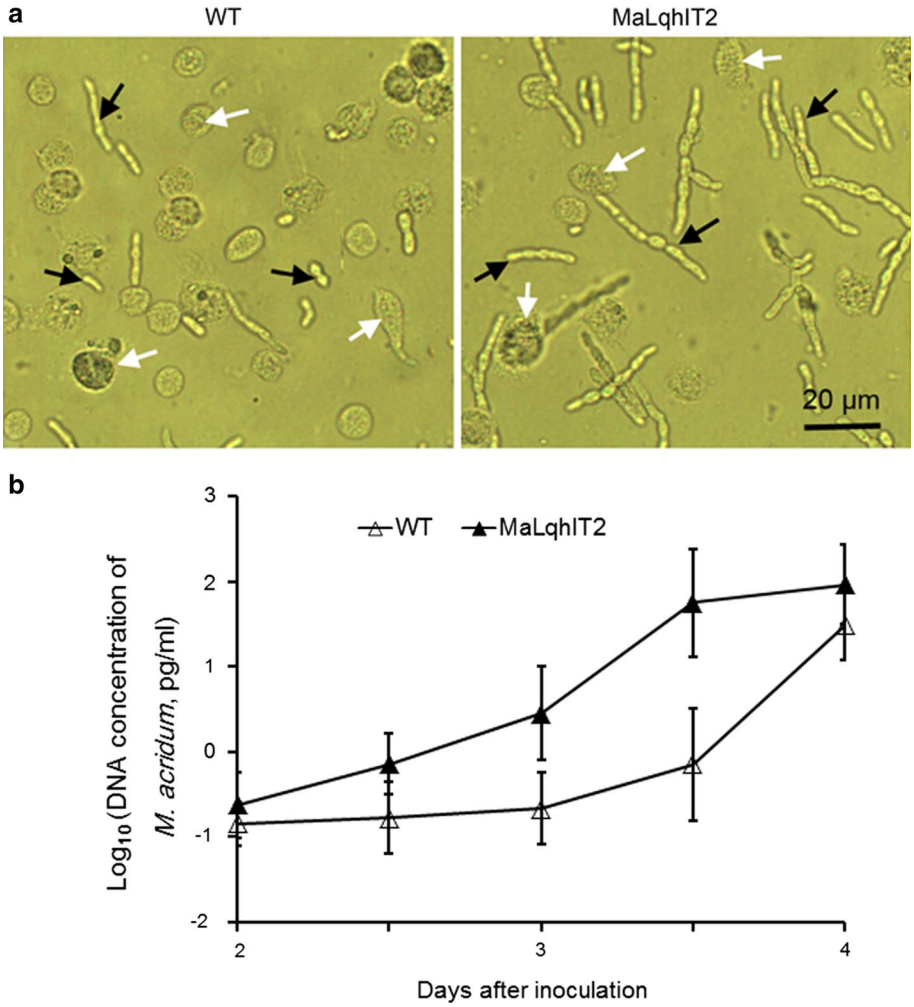

Fig. 4 The effects of LqhIT2 expression on the growth of *M. acridum* in *Locusta migratoria*. **a** Micrographs of the hemolymph of *L. migratoria* topically inoculated with 5 μL of a 2×10^7 conidia/mL suspension of the MaLqhIT2 or CQMa102 (wild type) strains of *M. acridum* on day 3.5 post-inoculation (*black arrow*: *M. acridum* hyphal bodies; *white arrow*: hemocyte). **b** The concentrations of *M. acridum* rDNA detected in the hemolymph of MaLqhIT2- or CQMa102-infected *L. migratoria* using quantitative real-time polymerase chain reaction. The data represent the mean ± standard error (SE) of four independent experiments, and the bars represent the SE ranges

carcasses, which plays an important role in the sustainable control of target pests in fields [5]. The expression of the AaIT toxin in *M. anisopliae* and *B. bassiana* blocked sporulation in carcasses, and suppressed fungal reproduction [21, 22], both of which would serve to reduce the sustainability of control efforts using these species. In our current study, LqhIT2 did not affect *M. acridum* sporulation in locust carcasses. The use of MaLqhIT2 to control locusts in the field should be highly successful, given that the LC_{50} of MaLqhIT2 was reduced 18.2-fold, compared with the genetically unmodified CQMa102 strain. Thus, fewer genetically modified MaLqhIT2 conidia would be needed to control locust populations in the field, compared with control efforts using the wild-type strain.

In conclusion, our results showed that expression of the insect-specific toxin LqhIT2 increased the virulence of *M. acridum* in locusts, without affecting cuticle penetration or conidia formation in the carcasses. These findings indicate that the expression of the LqhIT2 gene in genetically modified entomopathogenic fungi might provide a relatively safe and cost-effective method of biological insect control for crops affected by locusts and other acridids. Future studies of factors related to the practical application of the genetically modified MaLqhIT2 strain are warranted to determine the effects of mass production and storage of conidia and the viability of the MaLqhIT2 strain under field conditions, including exposure to high temperatures, solar radiation, and low humidity. Furthermore, LqhIT2 may represent a useful candidate for increasing the virulence of other entomopathogens as microbial insecticides.

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