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# Construction of a Hypervirulent and Specific Mycoinsecticide for Locust Control

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**Locusts and grasshoppers (acridids) are among the worst pests of crops and grasslands worldwide. *Metarhizium acridum*, a fungal pathogen that specifically infects acridids, has been developed as a control agent but its utility is limited by slow kill time and greater expense than chemical insecticides. We found that expression of four insect specific neurotoxins improved the efficacy of *M. acridum* against acridids by reducing lethal dose, time to kill and food consumption. Coinoculating recombinant strains expressing AaIT1(a sodium channel blocker) and hybrid-toxin (a blocker of both potassium and calcium channels), produced synergistic effects, including an 11.5-fold reduction in $LC_{50}$, 43% reduction in $LT_{50}$ and a 78% reduction in food consumption. However, specificity was retained as the recombinant strains did not cause disease in non-acridids. Our results identify a repertoire of toxins with different modes of action that improve the utility of fungi as specific control agents of insects.**

Locusts and grasshoppers are among the worst pests of crops and grasslands worldwide. The USA, for example, has a permanent program of rangeland grasshopper control, principally directed against the red legged grasshopper, *Melanoplus femurrubrum* (DeGeer), the most widely distributed and abundant North American species, and a major pest of legumes and grain crops[1]. In many other parts of the world certain species of grasshoppers called locusts periodically undergo a phase change from solitary to gregarious and reproduce in overwhelming numbers that migrate, inundate and devastate entire countries rendering them unstable in terms of predictable agricultural production[2]. With global warming, this problem is likely to become worse because the conditions that trigger the phase change are related to transitions from dry to wet seasons. In future these dry and wet conditions are likely to be more severe[3].

The unpredictable nature of the weather-dependent phase change means that several years can go by between major locust plagues. Stockpiles of insecticides are maintained to spray immature locust stages before they become flying swarms. For many years, dieldrin was the mainstay of locust control because of its persistence[4]. However, dieldrin and other organochlorine insecticides have been banned in most countries because of their environmental and human health impacts. Replacement insecticides are much less persistent and need to be applied more frequently in blanket treatments and in larger volumes. So, even though they are less toxic than dieldrin, their environmental impact may be worse[5].

Until recently, these chemical insecticides have been the only tools available to deal with locust swarms, but the devastating plagues of locusts in the mid-1980s provided a sense of urgency to developing biopesticides that are more environmentally friendly. A specific acridid (grasshopper) pathogen *Metarhizium acridum* was identified as the most promising candidate agent[6]. Various research groups, including the international LUBILOSA Programme (which developed the product 'Green Muscle', based on *M. acridum*), have identified and addressed key technical challenges for exploitation of microbial control agents including isolate selection, mass production and delivery systems (formulation and application), quality control, environmental impact, safety testing and host-pathogen ecology[7]. Since its commercial release in 2000, Green Muscle has been tried in Madagascar, Niger, Senegal and Sudan, but its largest trial started in May 2009 when it successfully contained an outbreak of red locusts (*Nomadacris septemfasciata*) that were threatening crops in East and Southern Africa[8]. Most infected locusts died within 1–3 weeks, depending on temperature and humidity, and the insecticide had an 80% mortality rate. A similar product is used in Australia[9], and large scale trials have commenced in China against *Locusta migratoria orientalis*[10].

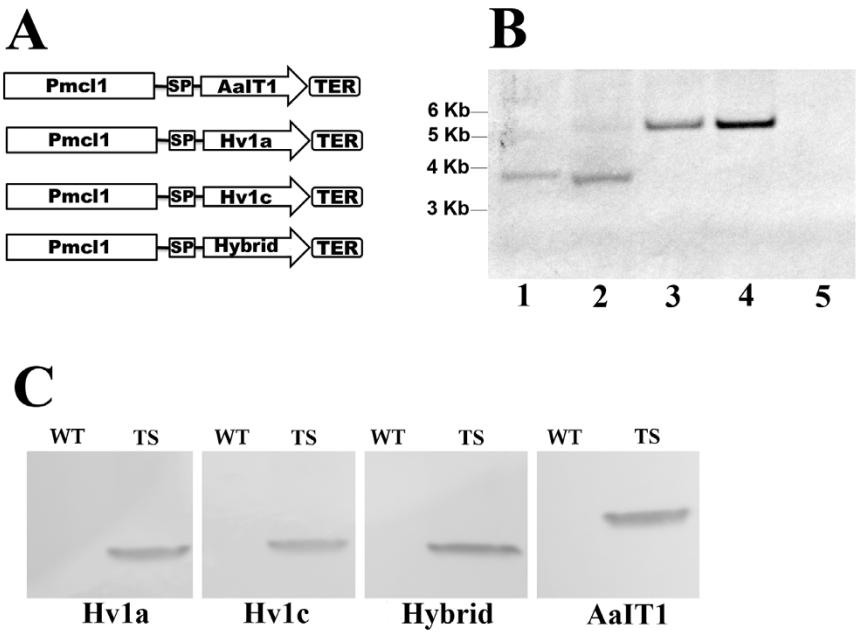


**Figure 1 | Construction of transgenic *M. acridum* ARSEF324 strains expressing four arthropod toxins.** (A) Schematic representations of the *Metarhizium* expression fragments cloned into the pFBARGFP plasmid. Pmcl1: *Mcl1* promoter that targets high level production of toxins into the hemolymph; SP: signal peptide of MCL1 that ensures secretion of the expressed toxins; TER: terminator region of the *TrpC* gene from *A. nidulans*; AaIT1: $Na_V$ channel blocker from *A. australis*; Hv1a: $Ca_V$ channel blocker from *A. robustus*; Hv1c: $K_{Ca}$ channel blocker from *H. versuta*; Hybrid: $Ca_V$ and $K_{Ca}$ blocker from *H. versuta*. (B) Southern blot analysis confirming insertion of genes encoding arthropod toxins into the genome of *M. acridum*. Genomic DNA was digested with *Eco*RI and *Spe*I. The ORF of the herbicide resistance gene *bar* was used as a probe. 1: a transformant expressing Hv1a; 2: a transformant expressing Hv1c; 3: a transformant expressing hybrid-toxin; 4: a transformant expressing AaIT1. 5: the wild-type strain. (C) Western blot analysis confirming expression of arthropod toxins by *M. acridum*. WT: the wild-type strain; TS: transgenic strains. Polyclonal antibodies to Hv1a, Hv1c or hybrid-toxin were used to detect their respective toxins. An antiserum to *A. australis* venom was used to detect AaIT1. All protein samples were run on 16% SDS-PAGE (Tris-Tricine) and blotted to nitrocellulose membrane (0.2 μm). Detecting target proteins with their respective antiserum was conducted with the standard Western blotting analysis. Pictures of different toxins were equally processed and combined with the image software Photoshop CS5.

However, the time lag from spraying to the locusts dying means that *M. acridum* is principally used as a preventative rather than for controlling outbreaks, and this requires accurate surveying and forecasting to detect hoppers in time[5]. If survival time could be reduced, *M. acridum* might be applied latter to prevent a sudden swarm outbreak. *M. acridum* is also more expensive than chemical insecticides. A reduction in effective conidial dose would mean that control could be achieved with less product that could translate into reduced costs. However, *M. acridum*'s comparative lack of efficacy is likely to be inbuilt because an evolutionary balance may have developed with their hosts so that quick kill, even at high doses, is not adaptive for the pathogen. Thus, cost-effective biocontrol will likely require transferring insecticidal genes to the fungi[11].

The remarkable extent to which fungal virulence can be increased was shown by expressing an ion channel blocker (the scorpion toxin AaIT1) in the broad host-range *M. anisopliae* strain ARSEF549[12]. The modified fungus achieved the same mortality rates in tobacco hornworm (*Manduca sexta*) at 22-fold lower conidial doses than the wild-type fungus, and survival times at some doses were reduced by 40%. Similar results were obtained with mosquitoes ($LC_{50}$ reduced 9-fold) and the coffee berry borer beetle ($LC_{50}$ reduced 16-fold)[12,13]. Strain ARSEF549's broad host range provided a suitable vehicle to test the effects of AaIT1 on several pests, but the risk of non-target effects suggest it would be safer to employ natural strains with narrower host ranges for insect pest control. Host-specific pathogens such as *M. acridum* in particular could benefit from this technology as they kill more slowly and produce fewer toxins than more generalist fungi[14]. In this study, we reduced the $LC_{50}$ of *M. acridum* by more than 10-fold by using it to express AaIT1 or ion channel blockers isolated from highly venomous Australian funnel-web spiders[15,16]. We also demonstrate that the aggressiveness of this pathogen can be increased while retaining specificity.

## Results

**Expression of ion channels peptide blockers in *Metarhizium* specialist.** We compared the efficacy against *M. femurrubrum* grasshoppers of recombinant *M. acridum* expressing four arthropod toxins with different modes of action. Expression of AaT1, a blocker of voltage-gated sodium ($Na_V$) channels from the scorpion *Androctonus australis*, has already been tested in *M. anisopliae*[12]. ω-HXTX-Hv1a (Hv1a) from the Sydney funnel-web spider *Atrax robustus* is a blocker of insect voltage-gated calcium ($Ca_V$) channels[17]. κ-HXTX-Hv1c (Hv1c) from the Blue Mountains funnel-web spider *Hadronyche versuta* inhibits $Ca^{2+}$-activated $K^+$ ($K_{Ca}$) channels[18]. Hybrid-toxin is a self-synergizing peptide toxin from the venom of the Australian funnel-web spider *H. verusta* that targets both $Ca_V$ and $K_{Ca}$ channels[19]. This insecticidal peptide, also known as Versitude, was recently approved by the U.S. EPA for control of lepidopteran pests.

The toxin genes were chemically synthesized with the MCL1 signal peptide to ensure secretion, and they were cloned in a common transfer plasmid downstream of the *Mcl1* promoter (PMcl1) (Fig. 1A) to target high-level production of each toxin into the hemolymph (*Mcl1* is the most highly expressed gene in hemolymph)[20]. The four toxin genes were individually transformed into *M. acridum* strain ARSEF324. Real time RT-PCR was used to measure insecticidal toxin expression in transformants identified by Southern blot analysis as containing a single copy of the transgene (Fig. 1B). Transformants with ~1.6 ng of a specific toxin transcript in 1 μg of total RNA were subjected to Western blot analysis to identify

specific products of the toxin genes. Products with sizes similar to those expected for mature, processed toxins were observed (Fig. 1C). All transformants were morphologically stable and showed wild-type levels of growth and sporulation.

**Bioassay.** The virulence of each transformant was initially surveyed by calculating the median lethal time ($LT_{50}$, time taken to kill 50% of insects) using 1,000 conidia per grasshopper (*M. femur-rubrum*) (Fig. 2). Strains expressing each of the toxins killed significantly faster than the wild-type fungus ($P < 0.001$). Expressing AaIT1, Hv1a, Hv1c or hybrid-toxin reduced $LT_{50}$ values by 16%, 18.5%, 17% and 31%, respectively. Expression of hybrid-toxin killed grasshoppers significantly faster than expression of AaIT1, Hv1a or Hv1c ($P < 0.001$), while AaIT1, Hv1a and Hv1c killed at similar speeds ($P > 0.25$). Because AaIT1 blocks $Na_V$ channels and hybrid-toxin targets both $Ca_V$ and $K_{Ca}$ channels, we next looked for additive or synergistic effects between AaIT1 and hybrid-toxin by co-inoculating grasshoppers with conidia of 324-AaIT1 (transgenic *M. acridum* ARSEF324 expressing AaIT1. The naming system is also used for other transgenic strains) and 324-HYBRID. The wild-type fungus was lethal at 500 conidia per grasshopper ($LT_{50}$ 9.5 days) but 100 conidia failed to kill, whereas grasshoppers were killed by 100 conidia carrying genes for hybrid toxin ($LT_{50}$ 8.6 days) or AaIT1 ($LT_{50}$ 12.8 days), with hybrid-toxin killing significantly faster ($P < 0.001$). Grasshoppers co-inoculated with 50 conidia of both 324-AaIT1 and 324-HYBRID died significantly faster ($LT_{50}$ 7.5 days) than insects exposed to single toxins ($P < 0.01$). At 500 conidia per insect, $LT_{50}$ values were reduced by 20% (expressing AaIT1), 33% (expressing hybrid-toxin) and 43% (combining 324-AaIT1 and 324-HYBRID), compared to wild-type fungus ($LT_{50}$ 9.5 days). At higher inoculums (1,000 or 10,000 conidia per insect), the results show trends that are similar but toxin expression has comparatively less affect than with lower inoculums. Strains expressing either hybrid-toxin or AaIT1 killed significantly faster than the wild-type fungus ($P < 0.001$), and a combination of 324-HYBRID and 324-AaIT1 killed significantly more rapidly than the 324-HYBRID alone ($P < 0.001$). However, even though 10,000 conidia of a 1 : 1 combination of 324-HYBRID and 324-AaIT1 achieved an $LT_{50}$ value of only 3.9 days, the 11.4% reduction compared with 324-HYBRID alone is less than that seen with smaller inoculums (Fig. 2). This data indicates that 324-HYBRID and 324-AaIT1 have synergistic or at least additive interactions. A binomial test confirmed synergistic interactions 4–8 days post-inoculation with 100, 500 and 1,000 conidia per insect ($\chi^2 > 16.8$). With 10,000 conidia, synergistic effects were detected 4 and 5 days post-inoculation ($\chi^2 > 10.2$); however, only additive effects were observed at 6 and 7 days post-inoculation ($\chi^2 < 2.2$).

At 5 days post-inoculation, too few insects infected with the wild-type fungus had died to allow meaningful calculation of effective conidial doses. However, $LC_{50}$ values for the transgenic strains were 12,468 conidia (expressing AaTI1), 3,982 conidia (hybrid-toxin) and 986 conidia (combining 324-AaIT1 and 324-HYBRID). At 6 days after inoculation, $LC_{50}$ values were reduced 2.2-fold by AaIT1, 8.4-fold by hybrid-toxin, and 11.5-fold by combining AaIT1 and hybrid-toxin, compared to the wild-type $LC_{50}$ of 4,649 conidia. Seven days after inoculation, $LC_{50}$ values were reduced 1.4-fold by AaIT1, 7.5-fold by hybrid-toxin, and 10.4-fold by combining AaIT1 and hybrid-toxin, compared to the wild-type $LC_{50}$ of 1,792 conidia. Thus, at all three time points, expressing hybrid-toxin reduced the $LC_{50}$ to a significantly greater extent than AaIT1 ($P < 0.001$), and combining AaIT1and hybrid-toxin further reduced effective conidial dose by a highly significant extent ($P < 0.001$) (Fig. 3).

Since prevention of feeding damage is of primary importance in assessing agronomic efficacy, we compared fecal production (dry weight) by grasshoppers as an estimate of food consumption. Compared to uninfected grasshoppers (controls), fecal production by grasshoppers infected by fungi started to decline 3 days after inoculation. As expected, the percent reduction compared to uninfected insects increased sharply with time (Fig. 4). The transgenic strains all reduced feeding of grasshoppers to a significantly greater extent than the wild-type strain ($P < 0.05$). After 7 days, fecal production by grasshoppers infected by 1,000 conidia/insect of the wild-type, 324-AaIT1, 324-HYBRID and the 324-HYBRID/324-AaIT1 combination was reduced by 53%, 65%, 67% and 78%, respectively, compared to the uninfected control grasshoppers. These reductions are significantly different for the wild-type and transgenic strains, and between the 324-HYBRID and 324-AaIT1/324-HYBRID combination ($P < 0.001$). AaIT1 and hybrid-toxin reduced fecal production to a similar extent ($P = 0.52$), but differentially effected grasshopper behavior. The bodies of grasshoppers killed by the wild-type, 324-AaIT1, 324-Hv1a or 324-Hv1c were dispersed randomly in their containers. In contrast, within three days of infection, grasshoppers infected by 324-HYBRID or 324-

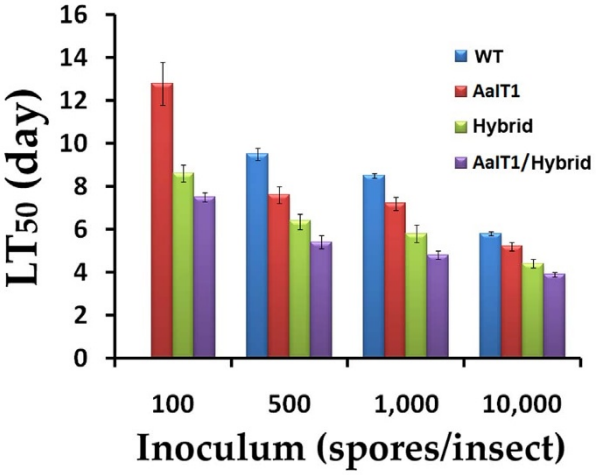


**Figure 2 | $LT_{50}$ values of the wild-type strain versus transformants at four inoculation doses (100, 500, 1,000 and 10,000 conidia per insect).** WT: the wild-type strain; AaIT1 and Hybrid are transformants expressing AaIT1 and hybrid-toxin, respectively. AaIT1/Hybrid: a 1 : 1 combination of transformants AaIT1 and hybrid-toxin. At inoculation dose of 100 conidia per insect, the $LT_{50}$ of WT could not be calculated because of its failure to kill.

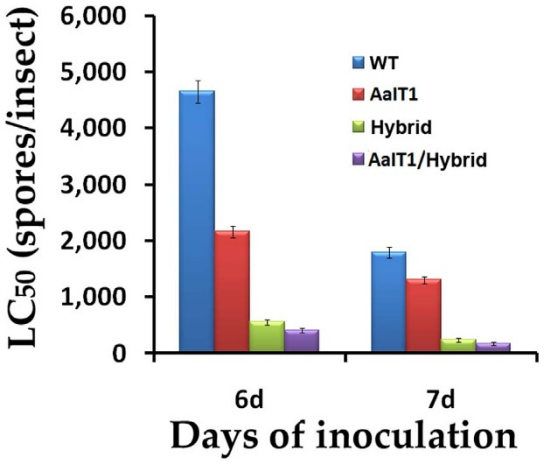


**Figure 3 | $LC_{50}$ values for the wild-type strain versus transformants at 6 and 7 days after inoculation.** WT: wild-type strain; AaIT1 and Hybrid are transformants expressing AaIT1 and hybrid-toxin, respectively. AaIT1/Hybrid: a 1 : 1 combination of transformants AaIT1 and hybrid-toxin.

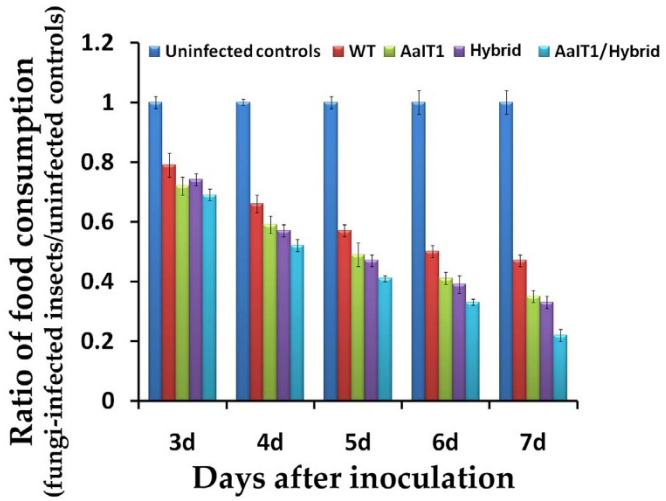


**Figure 4 | Food consumption by uninfected grasshoppers and grasshoppers infected by wild-type or transgenic *M. acridum*.** The food consumption of grasshoppers infected by a fungal strain was shown as the quotient (ratio of food consumption) achieved by dividing the amount of feces produced by infected grasshoppers by that of the uninfected controls, so the ratio of food consumption of the uninfected control is always 1 at different time points. Uninfected controls: uninfected grasshoppers; WT: wild-type strain; AaIT1 and Hybrid are transformants expressing AaIT1 and hybrid-toxin, respectively. AaIT1/Hybrid: a 1 : 1 combination of transformants AaIT1 and hybrid-toxin.

HYBRID/324-AaIT1 were located mostly on the wet cotton balls, and 43% died on these balls.

Conidial yields on insect cadavers killed by transformants expressing AaIT1 or hybrid-toxin were significantly reduced compared to those killed by the wild-type fungus ($P < 0.01$). Thus, a cadaver contained $3.3 \pm 0.5 \times 10^8$ wild type conidia, $1.98 \pm 0.28 \times 10^8$ 324-AaIT1 conidia and $2.34 \pm 0.31 \times 10^8$ 324-HYBRID conidia. The yield of 324-AaIT1 conidia was not significantly different from that of the 324-HYBRID strain ($P = 0.12$).

**Expression of arthropod toxins does not increase the host range of *Metarhizium acridum*.** The wild-type *M. acridum* ARSEF324 is a specific pathogen for acridids. To investigate if expressing toxins impacts host specificity we assayed transformants against the acridid *Schistocerca americana*, two other orthopterans (a katydid *Microcentrum rhombifolium*, and a gryllid *Acheta domesticus*), the giant cockroach *Periplaneta gigantea*, a caterpillar (*Galleria mellonella*), and the human malaria mosquito *Anopheles gambiae*. The wild-type fungus and all transformants killed locusts. However, none of the strains killed non-acridids. After two weeks, there was no fungus-induced mortality of katidid, crickets, cockroaches, wax worms or mosquitoes. Therefore, the expression of insect toxins did not change the host range of *M. acridum*.

## Discussion

The approximately 1,000 known species of entomopathogenic fungi are major mortality factors in insect pest populations and they offer many advantages as an alternative to chemical insecticides. Unlike bacteria and viruses that have to be ingested to cause disease, fungi infect insects by direct penetration of the cuticle. They therefore allow microbial control of sucking insects, as well coleopteran and orthopteran pests that have few known viral or bacterial diseases[21,22].

However, historically fungal pathogens of plant and insect pests have not met expectations as biocontrol agents. In many cases the reasons given for failure have included slow kill, failure to identify strains active at low doses, and inconsistent results compared with competing chemical insecticides[11]. In this study, we greatly enhanced the virulence of the acridid-specific pathogen by expressing insect-selective arthropod toxins. Although these transgenic strains would require eco-physiological testing to confirm increased efficacy and safety in field conditions, the current data suggests that they are safe. This safety results from several aspects: 1) the toxins are all insect-selective[23,24], so they are safe to human being and other animals; 2) The expression of these toxins does not alter the host range of the specialist fungus, so the transgenic strains do not infect non-host insects; and 3) the expression of the toxins was limited to the hemocoel of the target insects by using an insect-hemolymph-inducible promoter[12]. This precludes casual release of the toxins by *M. acridum* surviving saprophytically.

Most of our studies used the very abundant field pest *M. femurrubrum*. We also captured American locusts (*S. americana*), though not in sufficient numbers for replicated determinations of effective conidial dose. Nevertheless, we had sufficient numbers to determine that 100 conidia of 324-HYBRID or 324-AaIT1 killed locusts whereas this took a minimum of 500 wild-type conidia. This suggests that our transgenic strains have improved efficacy against different kinds of grasshoppers.

In spite of the environmental benefits of biological pesticides, costs of production and high application rates have frequently made control of insects by biocontrol agents economically impractical on a large scale basis. The current price of commercialized *acridum* (Green muscle) works out at US$20/ha for 50 g of conidia/ha, which is 1.5–2 times the price of conventional chemical insecticides[5]. The

Table 1 | Toxins information

| Toxin | Source | Function | Protein sequence (MCL1 signal peptide not included) | DNA sequence |
|---|---|---|---|---|
| Hv1a | *Atrax robustus* | $Ca_V$ channel blocker | SPTCIPSGQPCPYNENCCSQSC TFKENENGNTVKRCD | TCCCCCACCTGCATCCCCTCCGGCCAGCCC-TGCCCCTACAACGAGAACTGCTGCTCCCAG-TCCTGCACCTTCAAGGAGAACGAGAACGG-CAACACCGTCAAGCGCTGCGACTAA |
| Hv1c | *Hadronyche versuta* | $K_{Ca}$ channel blocker | AICTGADRPCAACCPCCPGTS CKAESNGVSYCRKDEP | GCCATCTGCACCGGCGCCGACCGCCCCTG-CGCCGCCTGCTGCCCCTGCTGCCCCGGCA-CCAGCTGCAAGGCCGAGTCCAACGGCGTC-TCCTACTGCCGCAAGGACGAGCCCTAA |
| Hybrid | *Hadronyche versuta* | Self-Synergizing $Ca_V$ and $K_{Ca}$ blocker | QYCVPVDQPCSLNTQPCCDD ATCTQERNENGHTVYYCRA | CAGTACTGCGTCCCCGTCGACCAGCCCTG-CTCCCTCAACACCCAGCCCTGCTGCGACG-ACGCCACCTGCACCCAGGAGCGCAACGA-GAACGGCCACACCGTCTACTACTGCCGCG-CCTAA |
| AaIT1 | *Androctonus australis* | $Na_V$ channel blocker | Ref. 12 | Ref. 12 |

Table 2 | Primers used in this study

| Primer | Sequence | Usage |
|---|---|---|
| PMcl1-5 | GGGAATTCCCTATGGGTTAAATGAGAG | Cloning the promoter of *Mcl1* |
| PMcl1-5 | GGATATCCCGGATCCGATGGTCTAGGGAACGG | |
| Bar-5 | ATGAGCCCAGAACGACGC | Cloning the ORF of *bar* as a probe for Southern blot |
| Bar-3 | GATCTCGGTGACGGGCA | |
| AaIT1-5 | AAGAAGAACGGCTACGCC | For real time RT-PCR analysis of *AaIT1* |
| AaIT1-3 | TTAGTTGATGATGGTGG | |
| Hv1a-5 | TCCCCCACCTGCATCCCC | For real time RT-PCR analysis of *Hv1a* |
| Hv1a-3 | TTAGTCGCAGCGCTTGAC | |
| Hv1c-5 | GCCATCTGCACCGGCGCC | For real time RT-PCR analysis of *Hv1c* |
| Hv1c-3 | TTAGGGCTCGTCCTTGCG | |
| Hybrid-5 | CAGTACTGCGTCCCCGTC | For real time RT-PCR analysis of *Hybrid* |
| Hybrid-3 | TTAGGCGCGGCAGTAGTAG | |

reductions produced by arthropod toxins in the effective conidial dose means that infection rates would be improved and equivalent control could be achieved with less product, providing scope for reduction of application rates and reduced costs. The effective persistence of the biopesticide should also increase; even if conidia of the genetically modified and wild-type fungi decay at the same rate there is a greater probability that an insect will come into contact with enough propagules to exceed the inoculum threshold[7].

In spite of their different targets ($Na_V$, $K_{Ca}$ and $Ca_V$ channels) all four toxins increased fungal virulence. The availability of multiple toxins reduces the potential for cross resistance, and $Ca_V$ and $K_{Ca}$ channels are previously unexploited insecticide targets[24], reducing the likelihood of pre-existing resistance. Major increases in agricultural productivity have resulted from pest control by chemical insecticides directed at neuronal $Na_V$ channels. However, this approach is threatened by resistance build-up among insects. It has not been determined whether more virulent fungi could impose selective pressures similar to those imposed by chemical insecticides but expressing multiple transgenes could reduce this possibility[11]. AaIT1, Hv1a and Hv1c were all similarly effective suggesting that targeting $Na_V$ channels is not preferable to targeting other ion channels for optimizing biopesticides. However, the hybrid-toxin produced the largest increase in efficacy, presumably because it has multiple sites of action for modifying channel activity. There was additionally a clear benefit in terms of effective conidial doses, speed of kill and the amount of feeding in mixing AaIT1 and hybrid-toxin, because these toxins likely interact synergistically. Such synergies suggest that optimizing the overall efficacy of the control strategy will require multiple transgenes.

Currently, *M. acridum* is used principally as a preventative. The reduction in survival time and feeding damage would obviously avoid important economic losses on agricultural crops and increase the possibility of applying *M. acridum* latter as a rescue treatment. However, rapid kill by fungal strains can reduce their ability to exploit host tissues for conidial production[25]. Thus, even though the modified strains are more virulent, they produce significantly less conidia on cadavers than the wild-type strain. This is an important environmental factor that could reduce rates of dispersal of the modified strains. It also illustrates that naturally occurring fungi are evolutionarily adapted to self preservation and propagation, not insecticide use. Thus, genetic engineering is required to improve the efficacy of fungi as insecticides. Our results demonstrate that a range of insect-specific neurotoxins with different ion channel targets can be used to improve the efficacy of fungal pesticides and increase their utility as specific control agents.

## Methods

**Vector construction and fungal transformation.** The coding sequences of the three spider toxins were synthesized commercially by GeneArt (Regensburg, Germany) using the preferred codon usage of *M. anisopliae* (http://www.kazusa.or.jp/codon/). The coding sequences of all toxins are shown in Table 1. The promoter region (2,000 bp), PMcl1, of *Mcl1* was amplified by PCR and inserted into the pGEM-T vector (Promega, USA) for sequencing. All primers and their respective usage are summarized in Table 2. The termination region of the gene *TtrpC* of *Aspergillus nidulans* was released from pBARGPE1 with *Not*I and *Bam*HI[26] and inserted into the corresponding sites of pGEM-PMcl1 to form pPMcl1. Synthetic genes encoding the spider toxins Hv1a, Hv1c and hybrid, were individually inserted into *Bam*HI and *Eco*RV sites of pPMcl1 to form pPMcl1-Hv1a, pPMcl1-Hv1c and pMcl1-hybrid, respectively. Each spider-toxin cassette was released from the pGEM-T vector with *Eco*RI and *Not*I, treated with T4 DNA polymerase, and inserted into the *Eco*RV-digested pFBARGFP[27], to form Ti plasmids, pHv1a, pHv1b and pHybrid. To construct the AaIT1 expression vector, the AaIT1 cassette was released from the plasmid pMcl1prAaIT with *Bam*HI[12], treated with T4 DNA polymerase, and inserted into the *Eco*RV site of pFBARGFP to form Ti plasmid pAaIT1. Fungal transformation was conducted as previously described[28].

**Characterization of the expression of toxins in fungi.** The insertion of foreign genes into the genome *of M. acridum* was confirmed by Southern blot analysis using the *bar* gene as a probe that was labeled by DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, USA). Transformants with one copy of the transgene were subjected to expression analysis. The expression levels of Hv1a, Hv1c, AaIT1 or hybrid-toxin were determined using real-time RT-PCR. PCR product (1 pg, 10 pg 100 pg, 1 ng and 10 ng) of each gene was used to create absolute standard curves for each gene. To prepare RNA, 0.1 g wet weight of a mycelial inoculum from Sabouraud dextrose broth (SDB) cultures was transferred into 10 ml of *M. sexta* hemolymph as previously described[29]. After being cultured at 27°C for 12 h, mycelium was collected for RNA extraction, and the filtrant was subjected to Western blot analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, USA) and genomic DNA was eliminated with the RNAase-free DNAase set (Qiagen, USA). One microgram of total RNA was used for cDNA synthesis (Roche, USA), and 10 ng of cDNA was used for real-time RT-PCR on a Rotorcycle480 (Roche, USA). The amount of mRNA encoding each toxin was calculated from their respective standard curves. Real-time RT-PCR was repeated three times.

For Western blot analysis, the filtrant (1 ml) from hemolymph cultures was concentrated using protein purification columns (Centriprep, USA). The proteins were resuspended in 200 µl Milli-Q water, and 15-µl aliquots were subjected to Western blot analysis on 16% SDS-PAGE (Tris-Tricine; Bio-Rad, USA). The toxins all contain a high percentage of cysteine (16% in Hv1a, 21% in Hv1c, 15% in hybrid and 11% in AaIT1) increasing formation of dimers or multimers[30]. To completely denature the toxins, the amount of the reducing agent (β-mercaptoethanol) in the sample buffer was 2-fold that recommended by the manufacturer (Bio-Rad, USA). The antibodies of Hv1a, Hv1c and hybrid-toxin are polyclonal rabbit antisera raised by injection of unconjugated peptide into rabbits (SA Pathology, Australia). Rabbit antiserum to *A. australis* venome was used to detect AaIT1 as previously described[12].

**Bioassays.** Acridid grasshoppers (*M. femur-rubrum* and *S. americana*) and a katidid (*M. rhombifolium*) were collected from a field adjacent the Comcast Center at the University of Maryland College Park. This field has not been treated with any insecticides. The gryllid *A. domesticus* and the giant cockroach (*P. gigantea*) were obtained from the Carolina Biological Supply Company (Burlington, NC). The caterpillar *G. mellonella* was purchased from Pet Solutions (Beavercreek, OH). The human malaria mosquito *A. gambiae* was kindly provided by Dr. David O'Brochta (Insect Transformation Facility UMBI - Center for Advanced Research in Biotechnology, University of Maryland). Bioassays were conducted over two years (2008 and 2009) with three repeats in each year.

Inoculation of 5th instar *M. femur-rubrum* involved pipeting conidia (100, 500, 1,000 or 10,000 conidia in 1 µl of 0.01% Tween 80) under the pronotum of each insect. Inoculated grasshoppers were held individually in plastic containers and fed on commercially available oats and sterile tap water from a wet cotton ball. Mortality was recorded twice daily. Thirty grasshoppers were used for each treatment. After death, cadavers were surface sterilized[31] and incubated in Petri dishes with a wet cotton ball to promote fungal emergence, and thus confirm cause of death. Mortality caused by other factors was not included in $LT_{50}$ and $LC_{50}$ analysis. $LT_{50}$ and $LC_{50}$

values were calculated with the *SPSS* program. To quantify food consumption, the feces from each grasshopper were collected daily, dried at 80°C under vacuum and weighed. Additive, synergistic and antagonistic interactions between AaIT1 and hybrid-toxins were identified with a binominal test that compared the expected ($P_E$) and observed mortalities ($P_o$)[32].

The locust, katydid and gryllid were inoculated as described for grasshoppers with fungal conidia being pipetted under the pronotum. Cockroaches, *G. mellonella* larvae and mosquitoes were inoculated with conidia as described[29,31,33], respectively. One hundred to 1,000 conidia were inoculated on each locust. To try to force infections, very high inoculums (700,000 conidia per insect) were applied to the katydids, gryllids and cockroaches. Wax worms were likewise immersed in very high conidial concentration ($5 \times 10^7$ conidia/ml). Anesthetized adult female mosquitoes were inoculated by spraying fungal conidia ($5 \times 10^7$ conidia/ml) as described[29].


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## Author contributions

W.F., R.J.S. and G.F.K. conceived the idea. W.F. and H.L. performed the experiment and data analysis. All authors reviewed the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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