Integration of dsRNA against host immune response genes augments the virulence of transgenic *Metarhizium robertsii* strains in insect pest species

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**Summary**

The slow lethality of fungal biopesticides to insects restrains their widespread application as a strategy of pest control. In this study, unary, binary and ternary transgenic *Metarhizium robertsii* were created by integrating genes that encode the scorpion neurotoxin *Bj*IT, the cuticle-degrading protease *Pr*1A, and a double-stranded RNA (dsRNA) that targets host *gnbp3*, individually or collectively under a constitutive promoter to enhance virulence. Compared with the parental wild type, all unary transgenic strains had increased virulence against four insect species, *Tenebrio molitor*, *Locusta migratoria*, *Plutella xylostella* and *Galleria mellonella*, whereas the binary transgenic strain expressing both *pr*1A and *Bj*IT had increased virulence to *T. molitor* and *L. migratoria*, with no change in virulence against *P. xylostella* and *G. mellonella*. Importantly, all ternary transgenic strains simultaneously expressing *pr*1A, *Bj*IT, and the dsRNA specific to host *gnbp3* exhibited the highest increase in insect-specific virulence. This finding highlights a novel strategy for genetic engineering of dsRNAs that target genes associated with the host immune response alongside virulence genes to maximize fungal virulence and lethality against insect pests.

**Introduction**

Entomopathogenic fungi play a crucial role in controlling insect populations and are an attractive alternative to chemical insecticides (Roberts and St. Leger 2004; Wang et al., 2004). To date, several entomopathogenic fungi, such as *Metarhizium* spp. and *Beauveria* spp., have been reported to function as mycoinsecticides (Maina et al., 2018). Unfortunately, the lethality induced by such fungal formulations against target pests is relatively slow, thereby restraining their widespread application. Extensive studies have revealed that fungal infection of insects starts with the adhesion of conidia to the host cuticle, followed by germination, cuticle penetration, and entry into the haemocoel, wherein hyphal bodies proliferate by yeast-like budding, leading to death and mummification of the host. Genetic engineering techniques have been developed to improve fungal virulence or accelerate the lethal action based on their infection strategies (Wang and Wang, 2017; Chen et al., 2018).

Fungal virulence can be improved by the overexpression of endogenous or exogenous virulence-associated genes in entomopathogenic fungi. Constitutive overexpression of endogenous subtilisin-like protease (*Pr*1A) in *Metarhizium anisopliae* has been shown to accelerate the lethal action by 25% (St Leger et al., 1996). In *Beauveria bassiana*, overexpression of exogenous chitinase or a hybrid chitinase resulted in increased virulence in aphids, and a strain engineered with the *Metarhizium pr*1A showed a substantial increase in fungal pathogenicity to *Dendrolimus punctatus* and *Galleria mellonella* (Fan et al., 2007; Lu et al., 2008). Mosquitoes treated with *M. anisopliae* strain expressing the salivary gland and midgut peptide 1 gene (*SM1*), a single-chain antibody that agglutinates sporozoites, and an antimicrobial toxin (scorpine) displayed reductions in vector sporozoites by 71%, 85% and 90%, respectively (Fang et al., 2011). Expression of a scorpion neurotoxin peptide, AaIT, in *M. anisopliae* resulted in a substantial increase in fungal pathogenicity towards *Manduca sexta* and *Aedes aegypti* (Wang and St. Leger 2007). The expression of an insect-specific toxin in *Metarhizium ping-shaense* led to increased lethal action towards and longer persistence in mosquito populations in an African...
malaria-endemic region (Lovett et al., 2019). Despite significantly increased virulence in target pests through cuticle infection, these transgenic fungi have not yet been able to compete with chemical insecticides; however, the reduced efficacy of chemical insecticides due to the development of resistance by the target pest has become a major problem (Georgiou, 1994). Other studies have demonstrated an increase in the virulence of entomopathogenic fungi by per os infections. A B. bassiana strain engineered with Vip3Aa1, which encodes a toxin that is active in the insect midgut, showed enhanced virulence against Spodoptera litura larvae through both cuticle and per os infections, and a M. anisopliae strain, normally non-pathogenic to S. litura, also showed high oral virulence against S. litura after integration of Vip3Aa1 in its genome (Qin et al., 2010; Zhang et al., 2014). A B. bassiana strain overexpressing Vip3Aa1, under the control of a promoter of an endogenous hydrophobin, exhibited high efficiency in the seasonal control of the cabbage insect pest complex in the field and was also environmentally safe (Liu et al., 2013; Wang et al., 2013).

RNAi-mediated pest management strategies trigger developmental disorders or death in insects by silencing host-specific genes. For examples, artificial diets containing double-stranded RNA (dsRNA) specific to host genes have shown to exhibit potential for controlling coleopteran pests (Wang et al., 2011). Transgenic expression of dsRNA and the spraying of or soaking in formulations of dsRNA are potential means to deliver dsRNA in plants for pest control (Baum et al., 2007; Bolognesi et al., 2012). Recently, studies have shown that fungal virulence can be improved via the expression of dsRNAs that target insect immune response-associated genes. For example, transgenic Metarhizium acriduum strains expressing dsRNAs that target genes encoding the α and β subunits of F1F0-ATP synthase in Locusta migratoria displayed a 3.7-fold increase in virulence (LC50) compared with that of the wild-type strain (Hu and Xia 2019). Moreover, transgenic Lecanicillium attenuatum expressing dsRNAs that target the genes encoding for prophenoloxidase-activating factor and lysozymes in Diaurceodes citri exhibited a 3.62- and a 2.91-fold increase in virulence (LC50) compared with that of the wild-type strain, respectively (Yu et al., 2019). These studies demonstrated that transgenic fungi expressing dsRNAs specific to insect immune response-associated genes serve as potential vectors to facilitate the application of dsRNAs for pest control in the field.

Gram-negative binding protein 3 (GNBP3), first discovered in Lepidoptera, recognizes β-1,3-glucans in fungal cell walls and activates insect immune responses against fungal infections (Ochiai and Ashida 2000; Gottar et al., 2006; Matskevich et al., 2010; Hughes, 2012). Indeed, gnbp3 exists in several insect genomes, and the injection of dsRNAs that target gnbp3 in fungus-infected locusts or Drosophila resulted in their reduced survival (Matskevich et al., 2010; Wang et al., 2013). These studies indicate that gnbp3 is a critical insect gene that should be targeted by engineered fungal strains.

In this study, multiple genetic engineering strategies to enhance fungal virulence were compared in M. robertsi. Genes, encoding for the cuticle-degrading protease Pr1A, which accelerates cuticle penetration, the insect-selective neurotoxin BjαIT, which works in the insect haemocoel, and GNBP3, which activates insect immune responses against fungal infections, were chosen for genetic modifications. Four transgenic M. robertsii strains that expressed dsRNAs against the gnbp3 gene in four insects, Tenebrio molitor, Locusta migratoria, Plutella xylostella and G. mellonella, were created. Furthermore, unary and binary transgenic strains were constructed to express either pr1A or BjαIT, or both of these genes. Finally, a ternary transgenic strain was generated to overexpress pr1A, BjαIT and dsRNA specific to gnbp3 of each insect. Bioassays of the transgenic strains on the target insects revealed a novel strategy for effective augmentation of fungal virulence against insect pests by genetic engineering of virulence genes in combination with dsRNAs that target host immune response-associated genes.

Results

Construction of transgenic strains

To increase the virulence of M. robertsii in different insects, various transgenic strains were constructed (Fig. 1). First, pr1A and BjαIT, under the control of the gpdA promoter, were integrated into the fungal genome, either separately or together, to generate three transgenic strains (Mr-pr1A, Mr-BjαIT and Mr-pr1A-BjαIT) (Fig. 1A). Second, four transgenic strains were constructed to express dsRNA targeting gnbp3 gene in P. xylostella (Mr-PXgnbp3), T. molitor (Mr-TMgnbp3), G. mellonella (Mr-GMgnbp3) and L. migratoria manilensis (Mr-LMgnbp3) (Fig. 1). Finally, four transgenic strains (Mr-pr1A-BjαIT-PXgnbp3, Mr-pr1A-BjαIT-TMgnbp3, Mr-pr1A-BjαIT-GMgnbp3 and Mr-pr1A-BjαIT-LMgnbp3) that expressed pr1A, BjαIT and gnbp3-specific dsRNA were constructed (Fig. 1A). The genotype of the transgenic strains was confirmed by polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) (Fig. 1B). In addition, real-time quantitative PCR (qPCR) results showed that pr1A expression was significantly upregulated under the gpdA promoter in Mr-pr1A
compared with that in the wild-type strain (Mr-WT) grown on potato dextrose agar (PDA) (Fig. S1).

Colony growth rate and sporulation capacity was unaffected in the transgenic strains

To test whether the integrated genes had an impact on the basic phenotypes of *M. robertsii*, growth rates and sporulation capacities of wild-type and transgenic strains were compared. There was no significant difference in either the colony growth rate (F_{df1,df2} = 0.59, P = 0.21) or the sporulation capacity (F_{df1,df2} = 0.41, P = 0.13) among the wild-type and the transgenic strains (Fig. 2). Thus, we confirmed that the insertion of exogenous genes had no impact on the growth rate or sporulation capacity of *M. robertsii*.

The enhanced virulence of transgenic strains

Mortality curves and median lethal times (LT50) of the four insect species infected with the wild-type and the transgenic strains are illustrated in Fig. 3. Compared with the Mr-WT, expression of Mr-pr1A produced a significant reduction (P < 0.05) of LT50 in *P. xylostella*, *T. molitor* and *L. migratoria*, but no significant change (P = 0.1214) in *G. mellonella* (Fig. 3). The LT50 in *P. xylostella*, *T. molitor*, *G. mellonella* and *L. migratoria* after Mr-Bj1IT infection (6.1 ± 0.3, 7.5 ± 0.5, 5.5 ± 0.4 and 7.1 ± 0.7 d, respectively) was significantly shorter (P < 0.05) than that after Mr-WT infection (7.8 ± 0.5, 9.1 ± 0.9, 6.6 ± 0.7, and 8.5 ± 0.9 days, respectively). Importantly, all dsRNA-expressing strains induced a larger reduction in LT50 (18.6–25.3%) than those induced by Mr-pr1A (12.5–17.1%) or Mr-Bj1IT (15.4%–22.0%), relative to the LT50 of Mr-WT in the four insects. These results suggest that the expression of insect *gnbp3*-specific dsRNA in *M. robertsii* could be an effective strategy for enhancing virulence against insect pests.

Compared with the unary transgenic strains, the binary transgenic strain Mr-pr1A-Bj1IT was more virulent in the tested insects. The LT50 was reduced by 32.3% for *P. xylostella*, 31.1% for *T. molitor*, 21.7% for *G. mellonella* and 31.9% for *L. migratoria*. The four ternary transgenic strains showed a higher virulence than both the binary strains, as demonstrated by the reduction in LT50 by 43.3%, 45.2%, 37.0% and 43.4% in *P. xylostella*, *T. molitor*, *G. mellonella* and *L. migratoria*, respectively.

The insects infected with Mr-pr1A and Mr-pr1A-Bj1IT were highly melanized with very little sporulation on cadavers, similar to insect larvae infected with *M. anisopliae* or *B. bassiana* overexpressing pr1A (Fig. 3B) (St Leger et al., 1996; Lu et al., 2008). However, the insects killed by the ternary transgenic strains were found to have a dense layer of conidia on the cadaver surfaces, suggesting that lack of the GNBP3-mediated activation of prophenoloxidase in the haemolymph leads to the melanization of the insect body infected with ternary transgenic strains.

Enhanced protease activity in pr1A overexpressed strains

A previous study showed that wild-type *B. bassiana* secreted Pr1A protein in a cuticle-inductive medium; however, the protein was not secreted in the insect haemocoel or in a non-inductive medium. In contrast, studies have reported that pr1A-overexpressing strains produced the protein in both cuticle-inductive and non-inductive media, with a much higher level of secretion in the inductive medium, and that the endogenous pr1A is
expressed along with the transgenic pr1A by the transgenic strain in the cuticle penetration stage (Lu et al., 2008). Cuticle penetration and haemocoel colonization, which are both essential for fungal infection, resemble cuticle-inductive and non-inductive environments, respectively. Therefore, enzyme activity and Western blotting assays were performed for the Pr1A protein in the different transgenic strains under different environments. Pr1A enzyme activity of different transgenic strains was assayed with the substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Fig. 4A). Compared with Mr-WT, all pr1A-overexpressing strains showed considerable increases in proteolytic activity when grown in Sabouraud dextrose broth (SDB) or a cuticle-inductive

Fig. 3. Survival trends and LT_{50} of the different transgenic strains in four insect species. A. Time-mortality trends of four insect species infected with the different transgenic strains. Third-instar Plutella xylostella, fourth-instar Tenebrio molitor, and fifth-instar Galleria mellonella and Locusta migratoria larvae were used to assess the virulence of the fungal transgenic strains. Conidia were applied by immersing larvae into an aqueous 10^5 conidia ml^{-1} suspension. Each treatment consisted of three replicates (60 larvae per replicate). B. LT_{50} values of the transgenic strains in four insect species. Different lowercase letters marked on the bars in each graph denote significant differences (P < 0.05). Inset in each graph are images of insect cadavers maintained at 28°C and high humidity for 12 days. Error bars, standard deviation from six replicate assays.

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Larvae immersed with conidial suspensions of different transgenic strains
medium, which is consistent with the increased Pr1A expression levels in the pr1A transgenics compared with Mr-WT in these medium (Fig. 4B). The toxin BjαIT was detected in Western blot analysis of BjαIT-overexpressing strains grown on SDB medium. The binary transgenic strain (Mr-pr1A-BjαIT) and all ternary transgenic strains showed a decreased expression of BjαIT compared with that in Mr-pr1A. However, BjαIT was detected in Mr-BjαIT but not in binary and ternary transgenic strains when grown on a cuticle-inductive medium, indicating that the Pr1A protease produced in Mr-pr1A and in binary strains degraded BjαIT completely in the cuticle-inductive medium and incompletely in the non-inductive medium. GNBP3 was detected in the haemolymph of different insects infected with dsRNA-expressing strains, and Western blot analyses showed an increase in GNBP3 protein levels in Mr-WT-infected insects. A reduction was observed in GNBP3 protein levels in insects infected with the gnbp3-targeting dsRNA-expressing strains, which confirmed a marked targeted interference of host–insect gene expression by integration of dsRNA into fungal cells.

**Reduced AMP expression in infected insects and off-target effects of dsRNA-expressing strains**

We also assessed the expression levels of antimicrobial peptides (AMP) of the Toll signalling pathway after inactivation of GNBP3 in insects infected with the dsRNA-expressing strains (Fig. 5). All AMPs were found to be significantly downregulated in the infected insects, indicating that the downregulation of gnbp3 blocked the Toll pathway.

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**Fig. 4.** Pr1A enzyme activity and Western blot analyses of protein expression in the different transgenics. A. Pr1A enzyme activity of the different transgenic strains grown in Sabouraud dextrose broth (SDB) or cuticle-inductive medium. Error bars, standard deviation from six replicate assays. B. Western blot analysis of Pr1A, BjαIT and GNBP3 expression. Pr1A expression in the different transgenic strains (1, Mr-WT; 2, Mr-pr1A; 3, Mr-pr1A-BjαIT; 4, Mr-pr1A-BjαIT-GMgnbp3; 5, Mr-pr1A-BjαIT-LMgnbp3; 6, Mr-pr1A-BjαIT-PXgnbp3; 7, Mr-pr1A-BjαIT-TMgnbp3) grown in SDB or cuticle-inductive medium; BjαIT expression in different transgenic strains (1, Mr-WT; 2, Mr-BjαIT; 3, Mr-pr1A-BjαIT; 4, Mr-pr1A-BjαIT-GMgnbp3; 5, Mr-pr1A-BjαIT-LMgnbp3; 6, Mr-pr1A-BjαIT-PXgnbp3; 7, Mr-pr1A-BjαIT-TMgnbp3) grown in SDB or cuticle-inductive medium; GNBP3 expression in the different insect species infected with different transgenic strains (1, Water; 2, Mr-WT; 3, Mr-x, x = PXgnbp3, TMgnbp3 or GMgnbp3; 4, Mr-pr1A-BjαIT-x, x = PXgnbp3, TMgnbp3 or GMgnbp3) at 72 h.

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signalling pathway and, hence, reduced AMP expression.

Mr-LMgnbp3 and Mr-TMgnbp3 were used to infect *P. xylostella*, *T. molitor*, *G. mellonella* and *L. migratoria* to assess the effects of dsRNA-expressing strains on non-target hosts (Fig. 6). Compared with the wild-type strain, insect-specific dsRNA-expressing strains showed no significant difference in virulence in the non-target host but did exhibit a significant reduction ($P < 0.05$) in $LT_{50}$ against target insects.

**Discussion**

Degradating insect cuticles, colonizing the host haemocoel and evading the insect's immune responses are essential steps for lethal actions of fungal pathogens against...
insect pests. In this study, unary transgenic strains overexpressing BjIT or pr1A showed increased virulence in different insect species in accordance with previous studies (St Leger et al., 1996; Lu et al., 2008). Furthermore, unary strains expressing dsRNA that targets host gnbp3 displayed increased virulence, supporting the conclusion that host gnbp3 is targeted during fungal infection (Hughes, 2012). Previously, it has been shown that the LT50 of M. acridum expressing dsRNA that target genes encoding the F1F0-ATPase subunit was reduced by 13–14% (Hu and Xia 2019). This reduction is clearly lower than the reduction in LT50 we observed in our transgenic strains expressing host gnbp3-specific dsRNA, suggesting that expression of gnbp3-specific dsRNA exhibits a greater enhancement of fungal virulence against different pests compared with the expression of F1F0-ATPase subunit gene-specific dsRNA in fungal cells.

Binary transgenics that have been engineered for the overexpression of scorpion AaIT and Metarhizium pr1A in B. bassiana showed an insignificant change in virulence in D. punctatus and G. mellonella due to speculated degradation of AaIT by Pr1A when expressed together (Lu et al., 2008). In this study, compared with Mr-BjIT, the binary transgenic Mr-pr1A-BjIT showed a significant improvement in virulence in T. molitor and L. migratoria, although only a small change in virulence in P. xylostella and G. mellonella was noted. These results revealed that the roles of co-expressed pr1A and BjIT in augmenting fungal virulence may vary with the target insect species. In addition, very little sporulation was observed on the cadaver surfaces of insects killed by Mr-pr1A and Mr-pr1A-BjIT, which is consistent with a previous study (Lu et al., 2008). However, insects killed by all of the ternary transgenic strains were observed to have a dense layer of conidia on the cadaver surfaces, and since gnbp3 activates the insect’s immune responses against fungal infections, the lack of gnbp3-mediated activation of prophenoloxidase in the haemolymph leads to melanization of the insect body infected with ternary transgenic strains (Matskevich et al., 2010; Wang et al., 2013).

Protease Pr1A can degrade AaIT completely when they are expressed together in B. bassiana; however, Pr1A produced by the binary transgenic Mr-pr1A-BjIT can completely degrade BjIT in a cuticle-inductive medium and incompletely in a non-inductive medium. In a previous study, M. anisopliae did not produce any proteases in insect haemolymph, revealing that Pr1A from entomopathogenic fungi is not produced in the insect haemolymph. Therefore, Pr1A produced by Mr-pr1A-BjIT could not completely degrade BjIT in the insect haemolymph, suggesting that compared with the Mr-pr1A, the improved virulence of Mr-pr1A-BjIT was attributable to the remaining BjIT in the insect haemolymph. We failed to successfully complete the Western blot analyses of GNBP3 in L. migratoria, although we used a range of polyclonal antibodies produced by immunizing rabbits with purified proteins expressed in E. coli or synthetic polypeptides. The lack of specific antibodies also prevented us from estimating the BjIT levels in infected insect haemolymph using Western blot analysis. Overexpression of pr1A in M. robertsii did result in a significant improvement in virulence in P. xylostella, T. molitor and L. migratoria, although no significant reduction in LT50 was seen in G. mellonella, probably because Mr-WT displayed the shortest LT50 in G. mellonella compared with that in other insects. The lack of a significant difference in the virulence of Mr-pr1A and wild-type strain in G. mellonella suggested that overexpression of pr1A did not increase virulence significantly in G. mellonella, and thus the virulence of Mr-pr1A-BjIT in G. mellonella was also not improved.
Our results demonstrate an approach to augmenting fungal virulence by the simultaneous genetic engineering of both virulence genes and dsRNA specific to host immune response-associated genes, as shown by the four ternary transgenic strains described here. These ternary transgenic strains are more virulent compared with the unary and binary transgenic strains against P. xylostella, T. molitor, G. mellonella and L. migratoria, and hence have great potential as fungal formulations against agricultural and forest pests.

Many studies have reported that silencing a specific target gene by RNAi leads to developmental disorders or death of the insect, and RNAi-mediated methods for pest management have recently received attention (Xie et al., 2019). However, the potential off-target effects of transgenic fungi are a concern limiting the application in pest management. In this study, we showed that the insect-specific dsRNA-expressing strains showed no significant effect on non-target hosts. This is not surprising as gnbp3 from different insects exhibits low degrees of sequence homology, and thus we suggest the use of transgenic strains engineered for overexpression of gnbp3-targeting dsRNA, as they have no effect on non-target insects. Significant numbers of transgenic microbial pest control agents have already been marketed, and a genetically engineered Metarhizium strain was approved by the U.S. EPA for use in an outdoor field trial, thus provides a good foundation for the current work (Hu and St. Leger 2002; Wozniak et al., 2012).

In conclusion, the expression of fungal dsRNA targeting gnbp3, which is essential for the insect's immune response against a fungal infection, together with endo- or exogenous virulence genes in transgenic fungal strains, will aid in the development of more efficacious mycoinsecticides against pests in agriculture and forests.

**Experimental procedures**

**Fungal strains, Agrobacteria and growth conditions**

The wild-type *M. robertsii* strain ARSEF 23 (ATCC no. MYA-3075) was grown on PDA (20% potato, 2% dextrose and 2% agar, w/v) in the dark at 28°C for 12 days. Conidia were harvested in a 0.05% Tween-80 aqueous solution, and the resulting conidial suspension was filtered through sterile absorbent cotton to remove mycelial debris. The spore concentration was determined using a hemocytometer and diluted as required. *Agrobacterium tumefaciens* strain AGL-1 was cultured on solid yeast extract beef medium (0.5% sucrose, 1% tryptone, 0.1% yeast extract, 0.05% MgSO₄.7H₂O and 2% agar, w/v) at 28 °C.

**Plasmids construction and fungal transformation**

The vector pDHt-SK-bar-PgpdA, which harbours the ammonium glutosinate resistance gene (*bar*), the *gpdA* promoter (PgpdA) and the *trpC* terminator (*Trpc*), from *Aspergillus nidulans* was used as the backbone for fungal transformation.

The amplified *M. robertsii* pr1A cDNA (MAA_05675) was digested with *Eco*RI and *Pst*I and cloned into the backbone vector, forming pDHt-SK-bar-PgpdA-Pr1A. The sequence of the *Mcl1ss-BjzIT* was synthesized, digested with *Eco*RI and cloned into the backbone vector, forming pDHt-SK-bar-PgpdA-BjzIT. The amplified sequence of the PgpdA-BjzIT-Trpc was digested with *Xhol* and cloned into pDHt-SK-bar-PgpdA-Pr1A, resulting in the binary vector pDHt-SK-bar-PgpdA-Pr1A-BjzIT. The dsRNAs targeting the *gnbp3* of different insects were synthesized as ~350-bp sense and antisense fragments of the *M. robertsii* cutinase intron spacer, and the PgpdA promoter was used to drive the dsRNA expression as described previously (Hu and Xia 2019). The fragments were digested with *Eco*RI and *Pst*I and inserted into pDHt-SK-bar-PgpdA, forming different RNAi vectors (pDHt-SK-bar-PgpdA-x, x = PXgnbp3, TMgnbp3, GMgnbp3 or LMgnbp3). The fragment of PgpdA-BjzIT-Trpc-PgpdA-Pr1A-Trpc was amplified, digested with *XbaI*, and inserted into each RNAi vector, yielding the ternary expression plasmids pDHt-SK-bar-PgpdA-Pr1A-BjzIT-x. All vectors were transformed into *E. coli* cells and verified by DNA sequencing. Positive plasmids were transformed into the wild-type strain through *Agrobacterium*-mediated transformation. The synthesized sequences are listed in Fig. S2. Unary, binary and ternary transgenic strains were confirmed through PCR and RT-PCR analyses with primers for the *bar*. The transgenic strains were evaluated in parallel with the parental wild type in the following experiments.

**Phenotypic experiments**

For all strains, aliquots of 1 µl of 10⁶ conidia ml⁻¹ suspension were spotted on the centre of PDA plates. The radial growth (diameter) rate of each colony at 28°C was measured daily. The sporulation capacity of each strain was quantified from the PDA cultures, which were initially seeded with 100 µl of a 10⁷ conidia ml⁻¹ conidial suspension and incubated for 20 days at 28°C. The conidia on each plate were harvested in 50 ml of 0.05% Tween-80 solution by vortexing, and the concentration of the conidial suspension was measured with a hemocytometer and converted to the number of conidia per square centimetre of the colony. All experiments were performed in six replicates for all phenotypic assays.
Insect bioassays

Third-instar *P. xylostella*, fourth-instar *T. molitor*, and fifth-instar *G. mellonella* and *L. migratoria* larvae were used to assess the virulence of fungal strains. Conidia were administered by immersing the larvae into a $10^6$ conidia mL$^{-1}$ suspension. Each treatment consisted of three replicates (60 larvae per replicate). After inoculation, mortality was recorded every 12 h for 12 days. The mortality rate was estimated and compared using Kaplan–Meier analysis (SPSS software v. 23.0; IBM, New York, IL, USA; https://spss.en.softonic.com). Mummified insect cadavers were maintained for fungal outgrowth and conidiation at 28°C and high humidity for 12 days. The experiment was performed six times.

Pr1A activity assay and Western blot analysis

Total Pr1A activity in each strain was quantified following a previous protocol (Lu et al., 2008). Briefly, mycelia from unary, binary and ternary transgenic strains were collected by vacuum filtration, transferred into 250 ml flasks with 100 ml cuticle-inductive medium or SDB and incubated at 200 rpm at 28°C for up to 72 h. Samples (500 μl) were taken every 12 h to test the Pr1A enzyme activity using the substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA). The OD$_{410}$ nm values for different samples were recorded at each time point using a microplate reader (Synergy™ HTX; BioTek, USA). Six parallel replicates were performed in each treatment.

Western blot analyses were used to estimate the expression of Pr1A, *Bj*IT and GNBP3. For preparation of protein samples, unary, binary and ternary transgenic strains were grown in SDB or cuticle-inductive medium for 3 days, and the supernatant was collected for Western blotting of Pr1A and *Bj*IT. The different insects infected with unary and ternary transgenic strains were collected 72 h after topical infection, and the haemolymph was harvested for Western blot analysis of GNBP3. The total protein amount was determined using the Bradford Protein Assay kit (Beyotime, Shanghai, China) using bovine serum albumin (BSA) as the standard, and the protein was separated using 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Polyclonal antibodies against Pr1A, *Bj*IT and GNBP3 were produced by immunizing rabbits with purified proteins expressed in *E. coli*. The experiment was performed three times.

Transcriptional profiling of antimicrobial peptides (AMPs)

The expression levels of different insect antimicrobial peptides (AMPs) in infected larvae of *P. xylostella* were detected using qPCR. The samples collected from the control larvae and at 6, 12, 24, 48 and 72 h post-infection from each strain were immediately ground in liquid nitrogen. Total RNA was extracted and transcribed into cDNA for qPCR analysis, as described previously (Zhou et al., 2019). *β*-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as controls. The primers used for qPCR are listed in Table S1. Transcripts of target genes were normalized to the control gene, and the $2^{-ΔΔCt}$ method was used to calculate the relative expression level (mean ± standard deviation) of each gene. Six independent experiments were performed for each gene.

Statistical analyses

One-way analysis of variance (ANOVA) and Tukey’s HSD test were used to analyse each dataset and to compare different groups. SPSS 23.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. *P* values less than 0.05 were considered as significant.

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Conflicts of interest

None declared.

Author contributions

B.H and YL.W conceived the experiments. B.H, ZX. W and XY.X wrote and revised the manuscript. YL.W, XY.X, L.Q, ZX.W and DS.Y performed the experiments and data collection.
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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Expression of the *pr1A* in different transgenic strains. All strains were grown on potato dextrose agar (PDA; 20% potato, 2% dextrose, and 2% agar, w/v) in the dark at 28 °C for 12 days. Error bars, standard deviation from six replicate assays. *Significant difference* (*P* < 0.05).

**Fig. S2.** Sequences of the synthesized transgenic vectors

**Table S1.** PCR primers used in this study.