microbial biotechnology

Open Access

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

Yulong Wang,[1] Xiangyun Xie,[1] Li Qin,[1] Deshui Yu,[1] Zhangxun Wang[1,2] and Bo Huang[1]

[1]*Anhui Provincial Key Laboratory of Microbial Pest Control, Anhui Agricultural University, Hefei, 230036, China.*

[2]*School of Plant Protection, Anhui Agricultural University, Hefei, 230036, China.*

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*αIT, the cuticle-degrading protease Pr1A, 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 *pr1A* and *Bj*αIT 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 *pr1A*, *Bj*αIT, 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.

Received 15 October, 2020; revised 20 December, 2020; accepted 29 December, 2020.
For correspondence. E-mail bhuang@ahau.edu.cn. Tel./Fax (+)86-551-65786887.
Microbial Biotechnology (2021) **14**(4), 1433–1444
doi:10.1111/1751-7915.13748

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 (Pr1A) 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 pr1A* 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 pingshaense* 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 (Georghiou, 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 promotor of an endogenous hydrophobin, exhibited high efficacy 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 acridum* strains expressing dsRNAs that target genes encoding the α and β subunits of F_1F_0-ATP synthase in *Locusta migratoria* displayed a 3.7-fold increase in virulence (LC_{50}) 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 *Dialeurodes citri* exhibited a 3.62- and a 2.91-fold increase in virulence (LC_{50}) 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. robertsii*. Genes, encoding for the cuticle-degrading protease Pr1A, which accelerates cuticle penetration, the insect-selective neurotoxin *Bj*αIT, 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*, *L. migratoria*, *Plutella xylostella* and *G. mellonella*, were created. Furthermore, unary and binary transgenic strains were constructed to express either *pr1A* or *Bj*αIT, or both of these genes. Finally, a ternary transgenic strain was generated to overexpress *pr1A*, *Bj*αIT 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 *Bj*αIT, 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-*Bja*IT and Mr-pr1A-*Bja*IT) (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-*Bja*IT-PXgnbp3, Mr-pr1A-*Bja*IT-TMgnbp3, Mr-pr1A-*Bja*IT-GMgnbp3 and Mr-pr1A-*Bja*IT-LMgnbp3) that expressed *pr1A*, *Bj*αIT 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

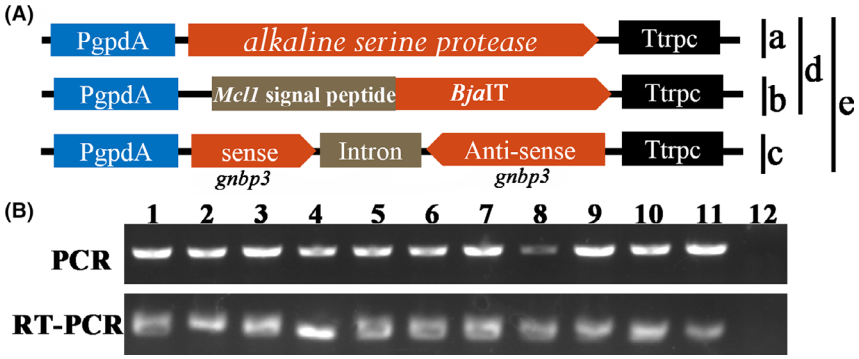


Fig. 1. Construction of different expression vectors and confirmation of different transgenic strains.A. Construction of different vectors used in this study. a, Expression vector for *pr1A*. b, Expression vector for *Bj*αIT. c, Expression vector for the double-strand RNA (dsRNA) targeting host-specific-*gnbp3*. d, Expression vector expressing both *pr1A* and *Bj*αIT. E, Expression vectors expressing *pr1A*, *Bj*αIT and dsRNA targeting host-specific *gnbp3*.B. PCR and reverse transcription PCR (RT-PCR) analysis of different transgenic strains with primers for *bar*. 1, Mr-pr1A; 2, Mr-*Bj*αIT; 3, Mr-pr1A-*Bj*αIT; 4, Mr-GMgnbp3; 5, Mr-LMgnbp3; 6, Mr-PXgnbp3; 7, Mr-TMgnbp3; 8, Mr-pr1A-*Bj*αIT-GMgnbp3; 9, Mr-pr1A-*Bj*αIT-LMgnbp3; 10, Mr-pr1A-*Bj*αIT-PXgnbp3; 11, Mr-pr1A-*Bj*αIT-TMgnbp3; 12, Mr-WT.

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 (LT_{50}) 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 LT_{50} in *P. xylostella*, *T. molitor* and *L. migratoria*, but no significant change ($P = 0.1214$) in *G. mellonella* (Fig. 3). The LT_{50} in *P. xylostella*, *T. molitor*, *G. mellonella* and *L. migratoria* after Mr-*Bj*αIT 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 LT_{50} (18.6–25.3%) than those induced by Mr-pr1A (12.5–17.1%) or Mr-*Bj*αIT (15.4%–22.0%), relative to the LT_{50} 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-*Bj*αIT was more virulent in the tested insects. The LT_{50} 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 LT_{50} 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-*Bj*αIT 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

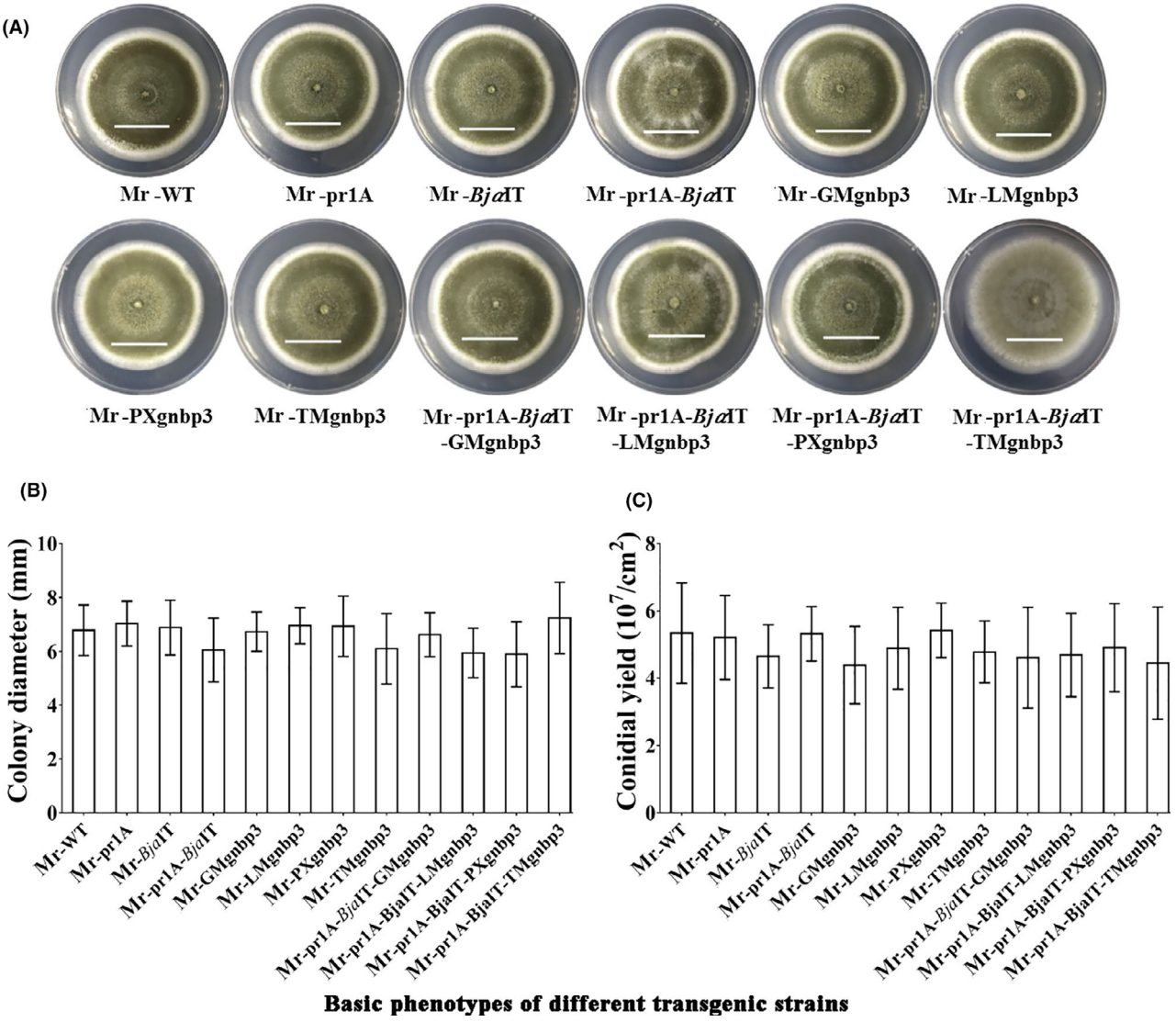
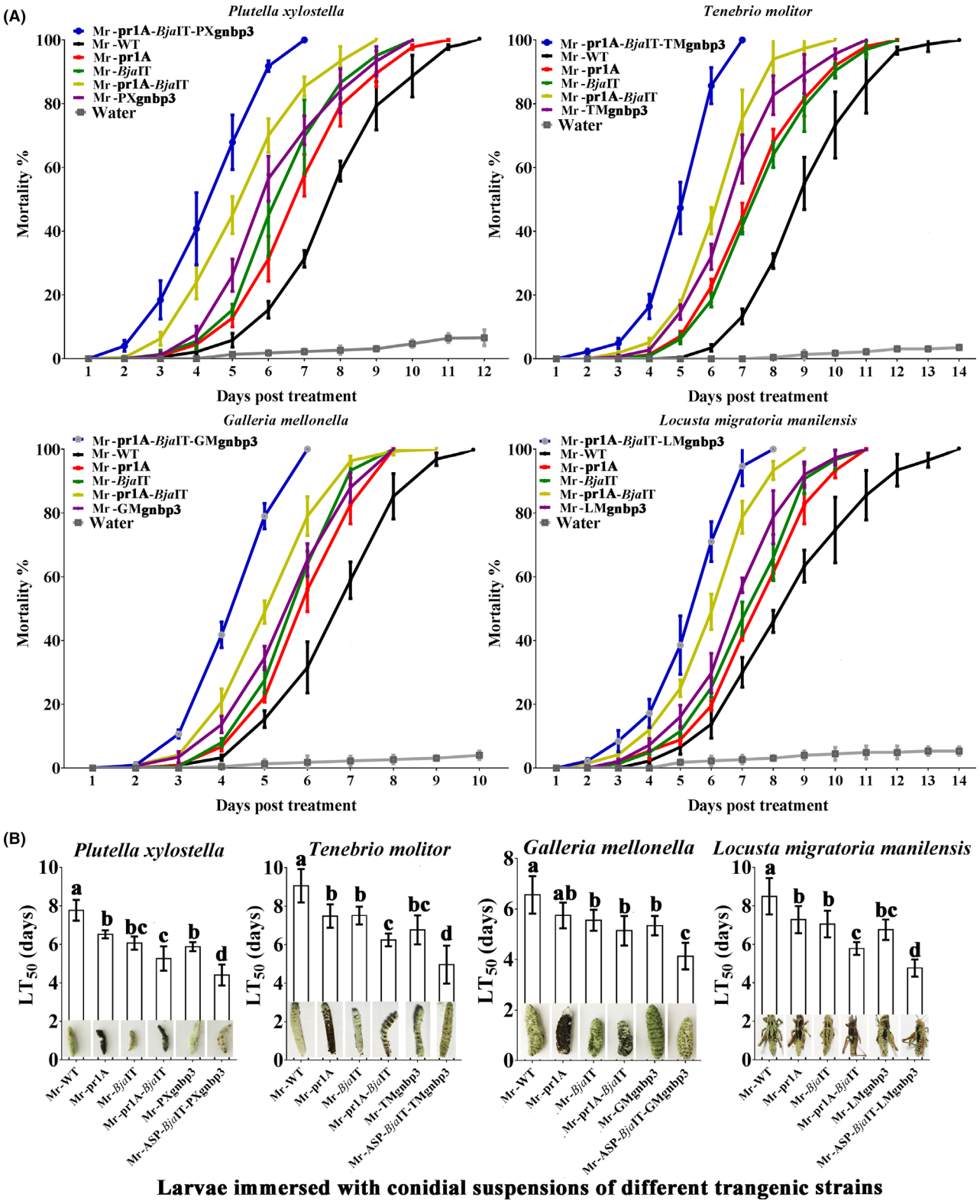


Fig. 2. Growth rates and conidiation capacities of different transgenic strains of *M. robertsii*.A, B. Images (scale: 3 cm) and diameters of fungal colonies initiated with the spotting method and incubated on PDA for 20 days at 28°C.C. Conidial yields of PDA cultures initiated by spreading 100 μl of a conidial suspension (10^7 conidia ml^{-1}) and incubating for 20 days at 28°C. Error bars, standard deviation from six replicate assays.

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.



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 *Bj*αIT was detected in Western blot analysis of *Bj*αIT-overexpressing strains grown on SDB medium. The binary transgenic strain (Mr-pr1A-*Bj*αIT) and all ternary transgenic strains showed a decreased expression of *Bj*αIT compared with that in Mr-pr1A. However, *Bja*IT was detected in Mr-*Bj*αIT 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 *Bj*αIT 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 antimicr.obial 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

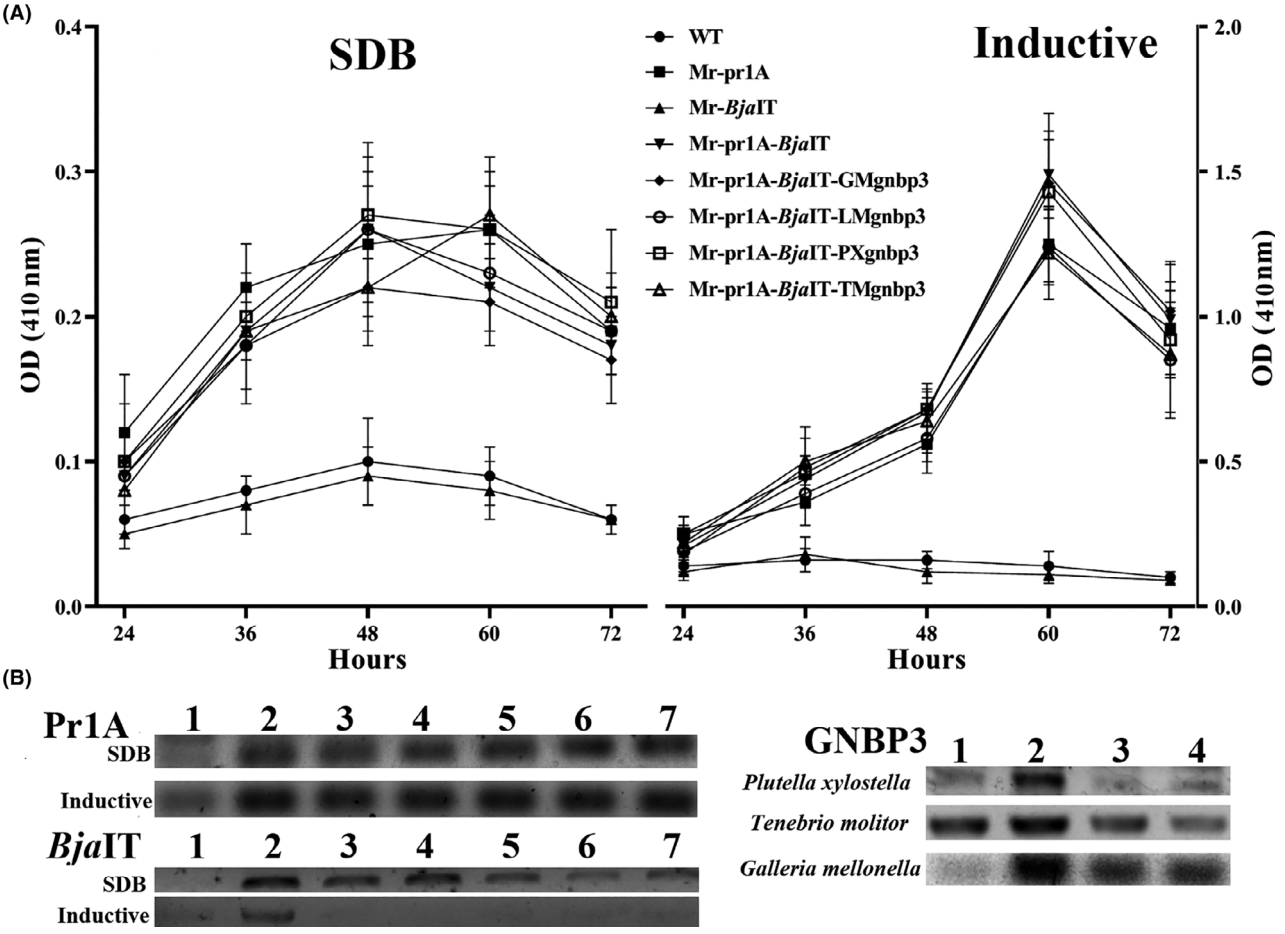


Fig. 4. Pr1A enzyme activity and Western blot analyses of protein expression in the different transgenics.A. Pr1A enzyme activity of the different transgenic strains grown in Sabouraud dextrose broth (SDB) or cuticle-inductive medium. Error bars, standard deviation from six replicate assays.B. Western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

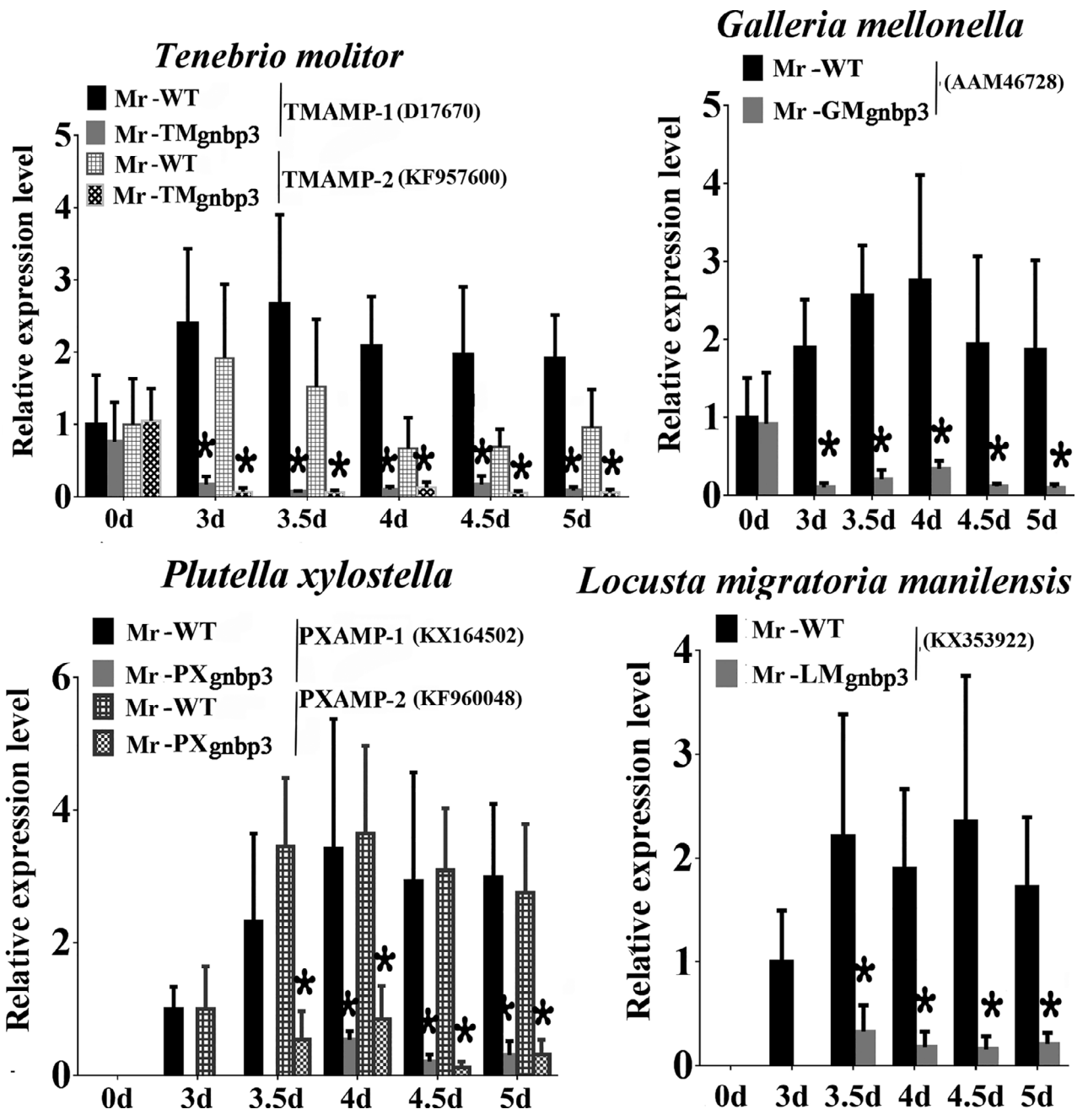


Fig. 5. Expression levels of *gnbp3* and antimicrobial peptide-coding genes (AMPs) in different insect species infected by dsRNA-inclusive transgenic strains versus the wild-type (Mr-WT) strain at 72–120 h post-infection. Error bars, standard deviation from six replicate assays. *Significant difference ($P < 0.05$).

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

Degrading insect cuticles, colonizing the host haemocoel and evading the insect's immune responses are essential steps for lethal actions of fungal pathogens against

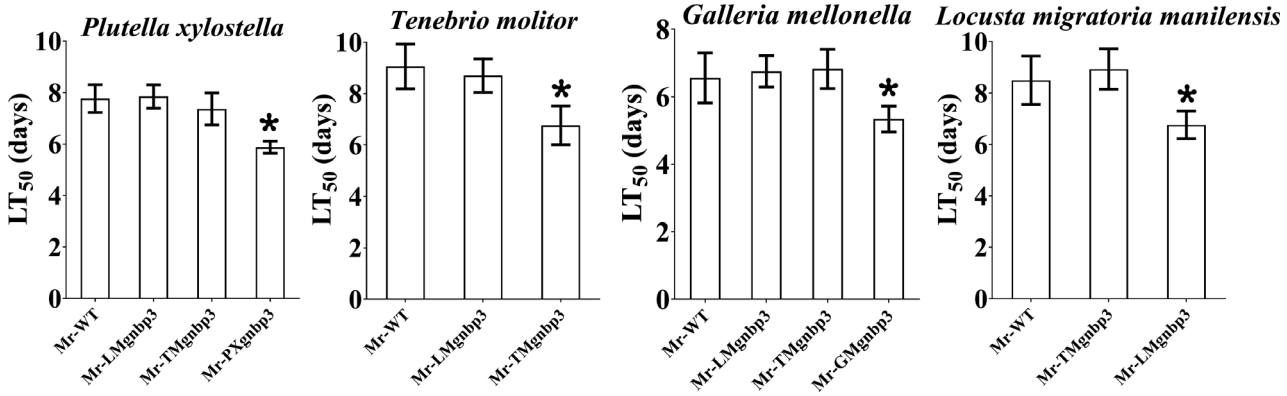


Fig. 6. LT_{50} values of Mr-LMgnbp3 and Mr-TMgnbp3 in four insect species. Error bars, standard deviation from six replicate assays. *Significant difference ($P < 0.05$).

insect pests. In this study, unary transgenic strains overexpressing *Bj*αIT 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 LT_{50} of *M. acridum* expressing dsRNA that target genes encoding the F_1F_0-ATPase subunit was reduced by 13–14% (Hu and Xia 2019). This reduction is clearly lower than the reduction in LT_{50} 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 F_1F_0-ATPase subunit gene-specific dsRNA in fungal cells.

Binary transgenics that have been engineered for the overexpression of scorpion *Aa*IT 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-*Bj*αIT, the binary transgenic Mr-pr1A-*Bj*αIT 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 *Bj*αIT 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-*Bj*αIT, 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-*Bj*αIT can completely degrade *Bj*αIT 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-*Bj*αIT could not completely degrade *Bj*αIT in the insect haemolymph, suggesting that compared with the Mr-pr1A, the improved virulence of Mr-pr1A-*Bj*αIT was attributable to the remaining *Bj*αIT 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 *Bj*αIT 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 LT_{50} was seen in *G. mellonella*, probably because Mr-WT displayed the shortest LT_{50} 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 and the virulence of Mr-pr1A-*Bj*αIT 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% $MgSO_4.7H_2O$ and 2% agar, w/v) at 28 °C.

Plasmids construction and fungal transformation

The vector pDHt-SK-bar-PgpdA, which harbours the ammonium glufosinate resistance gene (*bar*), the *gpdA* promoter (PgpdA) and the *trpC* terminator (Ttrpc), 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-Bj*αIT was synthesized, digested with *Eco*RI and cloned into the backbone vector, forming pDHt-SK-bar-PgpdA-*Bj*αIT. The amplified sequence of the PgpdA-*Bj*αIT-Ttrpc was digested with *Xho*I and cloned into pDHt-SK-bar-PgpdA-Pr1A, resulting in the binary vector pDHt-SK-bar-PgpdA-Pr1A-*Bj*αIT. 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-*Bj*αIT-Ttrpc-PgpdA-Pr1A-Ttrpc was amplified, digested with *Xba*I, and inserted into each RNAi vector, yielding the ternary expression plasmids pDHt-SK-bar-PgpdA-Pr1A-*Bj*αIT-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 10^5 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 10^7 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 10^5 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*αIT 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*αIT. 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*αIT 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* (KX164502, KF960048), *T. molitor* (D17670, KF957600), *G. mellonella* (AAM46728) and *L. migratoria* (KX353922) 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 $2^{-\Delta\Delta Ct}$ 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.

Acknowledgments

This work was supported by National Key R&D Program of China (2017YFD0200400), and the National Science Foundation of China (31772226, 31471821 and 31272096), and Scientific Research Foundation of Anhui Agricultural University (Grant No. yj2019-09) and China Postdoctoral Science Foundation (Grant No. 2020M671838).

Funding Information

This work was supported by National Key R&D Program of China (2017YFD0200400), and the National Science Foundation of China (31772226, 31471821 and 31272096), and Scientific Research Foundation of Anhui Agricultural University (Grant No. yj2019-09) and China Postdoctoral Science Foundation (Grant No. 2020M671838).

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.

References

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., *et al*. (2007) Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* **25:** 1322–1326.

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., *et al*. (2012) Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *PLoS One* **7:** e47534.

Chen, A.H., Wang, Y.L., Shao, Y., Zhou, Q.M., Chen, S.L., Wu, Y.H., *et al*. (2018) Genes involved in *Beauveria bassiana* infection to *Galleria mellonella*. *Arch Microbiol* **200:** 541–552.

Fan, Y.H., Fang, W.G., Guo, S.J., Pei, X.Q., Zhang, Y.J., Xiao, Y.H., *et al*. (2007) Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase. *Appl Environ Microbiol* **73:** 295–302.

Fang, W.G., Vega-Rodríguez, J., Ghosh, A.K., Jacobs-Lorena, M., Kang, A., and St Leger, R.J. (2011) Development of transgenic fungi that kill human malaria parasites in mosquitoes. *Science* **331:** 1074–1077.

Georghiou, G.P. (1994) Principles of insecticide resistance management. *Phytoprotection* **75:** 51–59. https://doi.org/10.7202/706071ar.

Gottar, M., Gobert, V., Matskevich, A.A., Reichhart, J.M., Wang, C.S., Butt, T.M., *et al*. (2006) Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *Cell* **127:** 1425–1437.

Hu, G., and St. Leger, R.J. (2002) Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl Environ Microbiol* **68:** 6383–6387.

Hu, J., and Xia, Y.X. (2019) Increased virulence in the locust-specific fungal pathogen *Metarhizium acridum* expressing dsRNAs targeting the host F1 F0 -ATPase subunit genes. *Pest Manag Sci* **75:** 180–186.

Hughes, A.L. (2012) Evolution of the βGRP/GNBP/beta-1,3-glucanase family of insects. *Immunogenetics* **64:** 549–558.

Liu, Y.J., Liu, J., Ying, S.H., Liu, S.S., and Feng, M.G. (2013) A fungal insecticide engineered for fast *per os* killing of caterpillars has high field efficacy and safety in full-season control of cabbage insect pests. *Appl Environ Microbiol* **79:** 6452–6458.

Lovett, B., Bilgo, E., Millogo, S.A., Ouattarra, A.K., Sare, I., Gnambani, E.J., *et al*. (2019) Transgenic *Metarhizium* rapidly kills mosquitoes in a malaria-endemic region of Burkina Faso. *Science* **364:** 894–897.

Lu, D.D., Pava-Ripoll, M., Li, Z.Z., and Wang, C.S. (2008) Insecticidal evaluation of *Beauveria bassiana* engineered to express a scorpion neurotoxin and a cuticle degrading protease. *Appl Microbiol Biotechnol* **81:** 515–522.

Maina, U., Galadima, I., Gambo, F., and Zakaria, D. (2018) A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *J Entomol Zool Stud* **6:** 27–32.

Matskevich, A.A., Quintin, J., and Ferrandon, D. (2010) The *Drosophila* PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function. *Eur J Immunol* **40:** 1244–1254.

Ochiai, M., and Ashida, M. (2000) A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, *Bombyx mori*. *J Biol Chem* **275:** 4995–5002.

Qin, Y., Ying, S.H., Chen, Y., Shen, Z.C., and Feng, M.G. (2010) Integration of insecticidal protein Vip3Aa1 into *Beauveria bassiana* enhances fungal virulence to *Spodoptera litura* larvae by cuticle and *per os* infection. *Appl Environ Microbiol* **76:** 4611–4618.

Roberts, D.W., and St Leger, R.J. (2004) *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Adv Appl Microbiol* **54:** 1–70.

St Leger, R.J., Joshi, L., Bidochka, M.J., and Roberts, D.W. (1996) Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc Natl Acad Sci USA* **93:** 6349–6354.

Wang, C.S., Fan, M.G., Li, Z.Z., and Butt, T.M. (2004) Molecular monitoring and evaluation of the application of the insect-pathogenic fungus *Beauveria bassiana* in southeast China. *J Appl Microbiol* **96:** 861–870.

Wang, C.S., and St Leger, R.J. (2007) A scorpion neurotoxin increases the potency of a fungal insecticide. *Nat Biotechnol* **25:** 1455–1456.

Wang, C.S., and Wang, S.B. (2017) Insect pathogenic fungi: genomics, molecular interactions, and genetic improvements. *Annu Rev Entomol* **62:** 73–90.

Wang, Y.D., Yang, P.C., Cui, F., and Kang, L. (2013) Altered immunity in crowded locust reduced fungal (*Metarhizium anisopliae*) pathogenesis. *PLOS Pathog* **9:** e1003102.

Wang, Y.B., Zhang, H., Li, H.C., and Miao, X.X. (2011) Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PLoS One* **6:** e18644.

Wozniak, C.A., McClung, G., Gagliardi, J., Segal, M., and Matthews, K. (2012) *Regulation of agricultural biotechnology: The United States and Canada: Regulation of Genetically Engineered Microorganisms Under FIFRA, FFDCA and TSCA*. Netherlands, Dordrecht: Springer, pp. 57–94.

Xie, J., Li, S., Zhang, W., and Xia, Y. (2019) RNAi-knockdown of the *Locusta migratoria* nuclear export factor protein results in insect mortality and alterations in gut microbiome. *Pest Manag Sci* **75:** 1383–1390.

Yu, S.J., Pan, Q., Luo, R., Wang, C.L., Cheng, L.Y., Yang, J.S., *et al*. (2019) Expression of exogenous dsRNA by *Lecanicillium attenuatum* enhances its virulence to *Dialeurodes citri*. *Pest Manag Sci* **75:** 1014–1023.

Zhang, L., Ying, H.S., and Feng, M.G. (2014) Assessment of oral virulence against *Spodoptera litura*, acquired by a previously non-pathogenic *Metarhizium anisopliae* isolate, following integration of a midgut-specific insecticidal toxin. *Biol Control* **79:** 8–15.

Zhou, Q.M., Shao, Y., Chen, A.H., Li, W.Z., Wang, J.X., and Wang, Y.L. (2019) *In vivo* transcriptomic analysis of *Beauveria bassiana* reveals differences in infection strategies in *Galleria mellonella* and *Plutella xylostella*. *Pest Manag Sci* **75:** 1443–1452.

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.

