Construction of a Transgenic *Beauveria bassiana* (Hypocreales: Cordycipitaceae) Strain Expressing the SPHvt/GNA Fusion Protein and Determination of its Toxicity to *Anopheles stephensi* (Diptera: Culicidae) Larvae[1]

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Abstract Mosquitoes transmit numerous diseases, including malaria and dengue fever, making mosquito control crucial for managing the spread of these and other illnesses. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) holds promise for the biological control of mosquito vectors but faces challenges such as low pathogenicity, lengthy infection periods, reduced longevity, and a dependence on specific environmental conditions (e.g., temperature and humidity) for spore germination, thus impacting its efficacy. To address this, a strain of *B. bassiana* was engineered to carry the SPHvt/GNA fusion pathogenicity protein-encoding gene. A plasmid containing the gpdA promoter, GFP gene, and SPHvt/GNA gene was constructed using molecular cloning techniques, including digestion and ligation, based on the base plasmids pBarGPE1 and pBarGFP. The engineered vector plasmid pBarGFPSPHvt/GNA was integrated into the genome of *B. bassiana* Bb252 through *Agrobacterium tumefaciens*-mediated fungal genetic transformation, resulting in *B. bassiana* strains with the SPHvt/GNA fusion pathogenicity protein-encoding gene. These transgenic strains were identified through fluorescence screening and confirmed at the genomic, transcriptional, and protein levels. A spore suspension of Bb252::BarGFPSPHvt/GNA was applied to *Anopheles stephensi* Liston (Diptera: Culicidae) larvae. The effect of pathogenicity on larval survival was assessed, and the infection process was monitored. The results indicated that the construction of a transgenic *B. bassiana* strain for potential management of mosquito larvae.

Key Words entomopathogenic fungus, vector control, snowdrop lectin, spider venom, biological control

There are approximately 3,573 species of Culicidae (Diptera) worldwide, with 419 species found in China (Fu and Chen 2018). Many of these mosquito species serve as vectors for deadly human diseases such as malaria, dengue, Japanese encephalitis, filariasis, yellow fever, banna, chikungunya, getah, and, recently, zika, which pose significant threats to human and animal health and safety (Cui et al. 2015, He and Guo 2022, Wu et al. 2022). Therefore, controlling mosquito populations is crucial in the management of vector-borne diseases.

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Mosquito control methods mainly consist of chemical and biological methods. However, the use of chemical pesticides has led to environmental pollution and the development of resistance. Thus, it is crucial to find sustainable, safe, and environmentally friendly control methods for mosquitoes. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) is an entomopathogenic fungus that offers several advantages for biological control, including a wide host range, strong pathogenicity, easy production and preservation, low residue without contamination, and safety for humans, animals, and natural enemies (Kotta-Loizou and Coutts 2017, Yuan et al. 2015). As a result, *B. bassiana* has great potential for the biological control of vector mosquitoes (Deng et al. 2019; Evans et al. 2018; Jaber et al. 2016; Kirsch and Tay 2022; Lee et al. 2019; Liu et al. 2012, 2019; Lv et al. 2021; Renuka et al. 2023). Despite the high efficiency and safety of fungal biological control, the application of fungal preparations has limitations. These include the long incubation period for infection, low persistence, and specific environmental requirements (e.g., temperature and humidity) for fungal spore germination, which can impact the effectiveness of fungal preparations in mosquito control (Fan et al. 2012; Liu et al. 2009, 2014).

With advancements in genetic engineering, the introduction of pathogenicity protein genes into entomopathogenic fungi has significantly enhanced the control efficacy against pests. For example, *Galanthus nivalis* agglutinin (GNA) is an exogenous plant hormone that has the ability to enter insect epithelial cells and the circulatory system, effectively killing pests. Additionally, it can act as a carrier protein to transport other peptides or proteins into the hemolymph of insects (Gatehouse et al. 1998, Nagadhara et al. 2004, Sukiran et al. 2023). Spider venom contains various insecticidal peptides that specifically target insect voltage-gated channels, resulting in insect paralysis and death (Bi et al. 2021, Chen et al. 2018). Hvt (ω-hexatoxin-Hv1a) is a toxin derived from the venom of the Australian funnel-web spider, *Hadronyche versuta* (Rainbow) (Araneae: Atracidae). It selectively blocks voltage-gated calcium (Ca_v) channels in invertebrates, effectively eliminating insects (Fletcher et al. 1997, Sukiran et al. 2023), without being toxic to vertebrates or bees (Nakasu et al. 2014). Some researchers have inserted the synthetic Hvt gene into the genomes of tobacco and cotton, enabling these plants to produce the Hvt toxic peptide, thereby enhancing host plant pest resistance (Khan et al. 2006).

In this study, *B. bassiana* Bb252 was used as the base strain, and the SPHvt/GNA fusion gene was inserted into its genome. This resulted in the successful expression of the fusion protein in the mutant strains of *B. bassiana*, providing a novel approach for the control of vector mosquitoes.

Materials and Methods

Species origin. *Anopheles stephensi* Liston (Diptera: Culicidae) mosquitoes were obtained from the laboratory of Si-Bao Wang at the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai. The mosquitoes were kept in a controlled environment with a temperature of 28 ± 2°C, a light-dark cycle of 14 L:10 D, and a relative humidity between 70% and 80%.

Strains and plasmids. *Escherichia coli* DH5α (Enterobacteriaceae: *Escherichia*), *Agrobacterium tumefaciens* AGL-1 (Rhizobiaceae: *Agrobacterium*), and *B. bassiana*

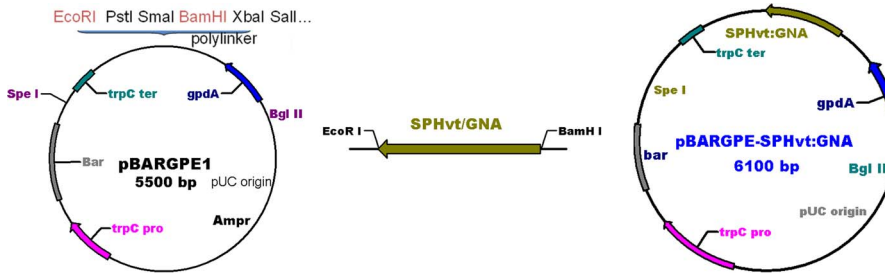

Fig. 1. Diagram of the constructed vector of pBARGPE-SPHvt:GNA.

Bb252 strains and plasmids pUC57, pBarGPE1, and pBarGFP were provided by the laboratory of Si-Bao Wang at the Chinese Academy of Sciences, Shanghai.

Principal reagents. The following reagents were used in this study: BamHI, EcoRI, SpeI, BglII, Ex TaqTM, dNTP, RNaseA, and T4 DNA ligase from TaKaRa Company (Beijing, China); protein prestaining marker from Thermo Company (Steingrund, Germany); DNA gel recovery kit, plasmid DNA rapid extraction kit, and DNA purification kit from OMEGA Bio-Tek (Norcross, GA).

Principal instrument. PCR amplification instrument (Eppendorf, Hamburg, Germany); high-speed refrigerated centrifuge (Thermo, Waltham, MA); nucleic acid electrophoresis apparatus (EPS-300, Shanghai Tianneng Technology Co., LTD, Shanghai, China); electrophoresis gel imaging system (Tanon 1600, Kunming Nari Technology Co., LTD, Kunming, China); vertical pressure steam sterilizer (HVE-50, Hirayama, Saitama, Japan); fluorescence microscopy (Olympus, Tokyo, Japan).

Acquisition and synthesis of SP-HVT-GNA sequence fragments. The amino acid sequences of the spider toxins Hvt and GNA serve as templates for this process. The codon bias of *B. bassiana* was optimized, and the sequence of flag.tag was added before the stop codon. The resulting sequence was sent to Shanghai Sunny Biotechnology Co., Ltd. (China) for synthesis and then connected to the plasmid PUC57S. Finally, the plasmid PUC57-SPHvt:GNA, which contains the BamHI and EcoRI cleavage sites on both sides, was obtained. Additionally, the optimized sequence did not contain SpeI, BglII, or NdeI cleavage sites, which can be used for subsequent vector construction.

The pBarGFP-SPHvt:GNA vector construction. The purified PUC57-SPHvt:GNA plasmid and pBarGPE-1 vector were digested by the EcoRI and BamHI enzymes, and the digested products were recovered (Fig. 1). The plasmid and the target gene fragment were then connected using T4 DNA ligase in a temperature-controlled bath at 16°C overnight. *Escherichia coli* DH5α cells were transformed with the resulting mixture, and a single colony was selected for culture. The positive clones were screened using PCR, and the recombinant plasmid was further identified using the EcoRI and BamHI enzymes.

The purified pBarGPE-SPHvt:GNA plasmid and pBarGFP vector were digested by the SpeI and BglII enzymes, and the digested products were recovered (Fig. 2). The plasmid and the target gene fragment were then connected using T4 DNA ligase in a temperature-controlled bath at 16°C overnight. *Escherichia coli* DH5α cells were transformed with the resulting mixture, and a single colony was selected

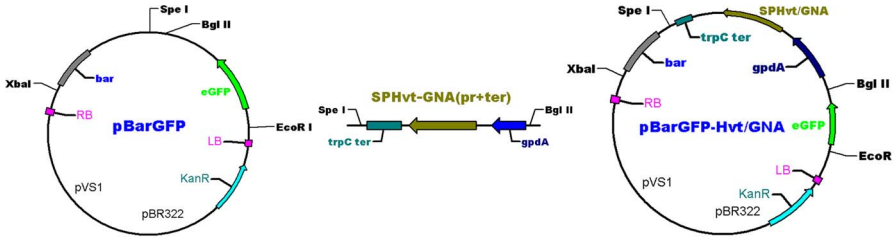

Fig. 2. Diagram of the constructed vector of pBarGFP-SPHvt:GNA.

for culture. The positive clones were screened using PCR, and the recombinant plasmid was further identified using the SpeI and BglII enzymes.

***Agrobacterium* mediates T-DNA transformation.** The constructed plasmid pBarGFP-SPHvt:GNA was subsequently transformed into *Agrobacterium* AGL-1, which was subsequently activated to an OD600 of $\approx$ 0.6 on IMAS induction medium. One hundred microliters of this bacterial solution and an equal concentration of fresh spores of the Bb252 strain (5×10^5 spores/ml) were coated on IMAS induction media (200 μmol/L AS and 400 μg/ml cefotaxime) covered with black filter paper and cultured at 27°C for 2 d.

Screening and identification of the Bb252::pBarGFPSPHvt:GNA transgenic strain. The cocultures on black filter paper were transferred to the screening medium M-100, which contained 200 μmol/L AS and 400 μg/ml cefotaxime. After incubating at 27°C for 1 d, a layer of screening medium was applied to the black filter paper, which was subsequently cultured at 27°C for 5–6 d to obtain transformants. Inverters were selected on the screening media and cultured at 27°C for 1 d. Fluorescence screening was conducted using a fluorescence microscope, and positive inverters showing green fluorescence were chosen. Strains with green fluorescence were cultured at 27°C for 5–6 d to obtain mycelia. Genomic DNA was extracted using the CTAB method, and PCR amplification was performed to screen for transgenic Bb252::pBarGFPSPHvt:GNA strains using the specific primers pBarGPEF and pBarGPER1. The PCR amplification conditions were as follows: 30 cycles of predenaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min and 30 s. Finally, extension was performed at 72°C for 7 min. Total RNA was extracted using the TRIzol method, and RT–PCR was used to verify the transcription of the HvtGNA gene in the Bb252::pBarGFPSPHvt:GNA transgenic strain. The internal reference used was GAPDH. The sequence of the upstream primer used for HvtGNA was HVTGNAF: 5′-CTACAACCCCTCCAACAAGCC-3′, and the sequence of the downstream primer used was HvtGNAR: 5′-CGGTCCGTGCCGTAAATGA-3′. The length of the amplified product fragment was 111 bp. For GAPDH, the upstream primers GAPDH-RT-F (5′-GTCATCCACGACAAGTTCGG-3′) and GAPDH-RT-R (5′-AGGACACGGCGGGAGTAG-3′) were used as internal controls. Western blotting was performed to detect the expression of the target protein in the total protein and extracellular protein fraction of the Bb252::pBarGFPSPHvt:GNA transgenic strain, which was labeled with flag.

Determination of pathogenicity. The pathogenicity of the strains was determined using a *per os* feeding method. Healthy larvae of *A. stephensi* were selected

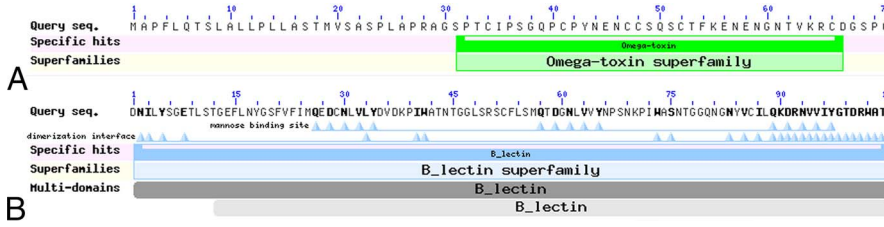

Fig. 3. Conserved domains of Hvt and GNA.

from the end of the second instar to the beginning of the third instar. In each of 12 beakers, 50 ml of dechlorinated ionized water was added along with 50 healthy mosquito larvae. The beakers were then placed in an artificial climate chamber at 27°C, 70% relative humidity (RH) and a photocycle of 12 L:12 D. The larvae were divided into 4 groups: the blank control group, control (0.01% Triton X-100) group, WT (Bb252) group, and transgenic strain (Bb252::SPHvt:GNA) group, with 3 replicates. Spore suspensions of 2.5×10^8 were prepared with 0.01% Triton X-100, and larval toxicity of each treatment was assessed. The number of dead insects was recorded daily for several consecutive days, after which the survival rate was calculated. The dead insects were placed in a Petri dish to observe the growth of *B. bassiana* mycelia and to identify the cause of death.

Observation of the infection process of larvae of *A. stephensi* by the Bb252::pBarGFPSPHvt:GNA transgenic strains. After *A. stephensi* were treated by the Bb252::pBarGFPSPHvt:GNA transgenic strains fluorescence microscopy was used to observe the fungal infection process at 36, 60, 80, and 96 h. These data were statistically analyzed using GraphPad Prism 6 software. A 2-tailed Student's *t* test with Welch correction was performed with a significance level of $P < 0.05$.

Results

Synthesis of the SPHvt/GNA fusion gene. The Hvt gene combines with the GNA gene while introducing a flag sequence. A BamHI restriction site was added upstream of the combined sequence, and an EcoRI restriction site was added downstream. These modifications were optimized based on the codon bias of *B. bassiana* and contracted to a biological company for synthesis. Subsequently, the pUC57-SPHvt/GNA positive plasmid was obtained.

BLAST analysis results of Hvt and GNA. The amino acid sequences of the spider toxin Hvt were compared and analyzed using the NCBI BLAST online analysis tool. The analysis revealed that the spider toxin Hvt belongs to the omega toxin superfamily and possesses a conserved omega toxin domain, which is an insect-specific neurotoxin (Fig. 3A). On the other hand, GNA belongs to the B-lectin superfamily, carries a conserved B-lectin domain, and is categorized as a type of lectin protein (Fig. 3B).

Signal peptide analysis of the SPHvt/GNA fusion gene. The SignalP 4.1 server, an online analysis tool available on ExPASy, was used to analyze the proteins encoded by the SPHvt/GNA genes. The analysis identified amino acids 1–22

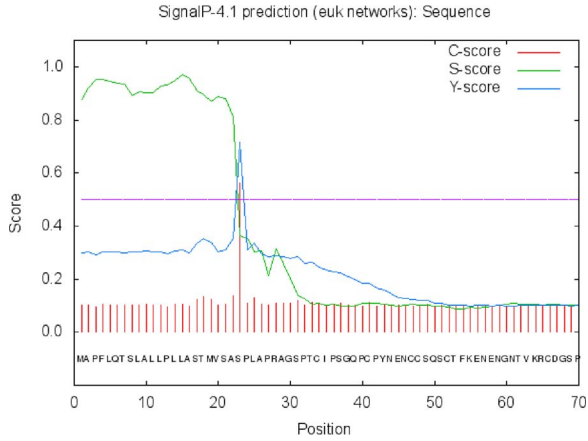

Fig. 4. Signal peptide site analysis of the fusion protein SPHvt/GNA (Note: max.C 23 0.560 max.Y 23 0.715 mean S 1-22 0.914 D 1-22 0.823 0.450; cleavage site between pos. 22 and 23: VSA-SP D = 0.823, D-cutoff = 0.450).

as signal peptides, with the cleavage site located between amino acids 22 and 23. The mature peptide is initiated at amino acid 23 (Fig. 4). In addition, analysis via the ProtParam tool of the ExPASy website revealed that the molecular weight of SPHvt/GNA was 19996.3 Da, the isoelectric point was 4.43, and the molecular formula was C869H1338N232O284S13. The predicted molecular weight of the mature peptide was 16988.7 Da, the isoelectric point was 4.33, and the calculated instability index (II) was 38.93, indicating that the protein was stable in solution.

Identification of recombinant plasmid transformants. The synthetic fusion gene SPHvt/GNA was inserted into the pBarGPE1 plasmid using the BamHI and EcoRI restriction sites in the polyclonal sites of the pBarGPE1 plasmid, resulting in the pBarGPE-SPHvt/GNA recombinant plasmid. The recombinant plasmid was verified through sequencing to ensure its accuracy and the absence of frameshift mutations and base mutations.

To construct the recombinant vector pBarGFP-SPHvt/GNA, the recombinant plasmid pBarGPE-SPHvt/GNA was cleaved using the SpeI and BglII enzymes. The smaller fragment (approximately 2,700 bp) was then recovered through gel electrophoresis, and the SPHvt/GNA (pr+ter) fragment containing the gpdA promoter and trpC terminator was obtained. Subsequently, this fragment was ligated with the pBarGFP plasmid, which had also been digested by SpeI and BglII. This resulted in the successful generation of the pBarGFP-SPHvt/GNA recombinant vector.

PCR amplification was conducted using the specific primers pBaGPEF and pBaGPER as templates. The resulting PCR product was then analyzed using 1.0% agarose gel electrophoresis, which revealed the presence of a band at approximately 900 bp. The size of this target fragment matched the expected size (Fig. 5), confirming that it was connected to the pBarGFP vector. The recombinant plasmid pBarGFP-SPHvt/GNA was subjected to double enzyme digestion analysis

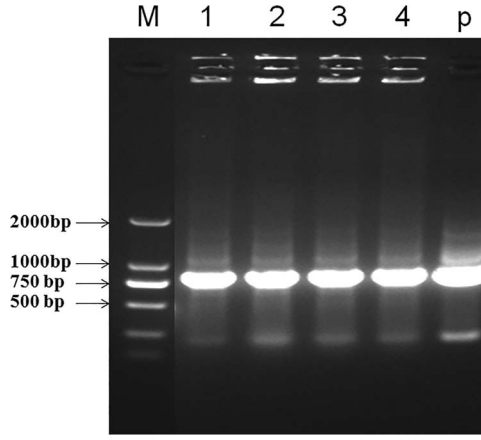

Fig. 5. Identification of the positive clones by PCR (Note: M: DNA Marker DL2000; 1,2,3,4: PCR product of convert colony as the template; 2: PCR product of pBarGFP-SPHvt/GNA the template).

using SpeI and BglII, as well as 1.0% agarose gel electrophoresis analysis. The results indicated the existence of a band at approximately 2,700 bp, consistent with the size of the target gene. Additionally, at approximately 10,000 bp, a band of the same size as that of pBarGFP was observed after double digestion (Fig. 6). This further validated the attachment of the target fragment SPHvt/GNA to the pBarGFP vector.

Strain-fluorescence screening. The transformation of the target fragment into *B. bassiana* Bb252 was facilitated by *Agrobacterium tumefaciens* AGL-1, as depicted in Fig. 7. As the vector contained the GFP gene, it was incorporated into the genome of Bb252 alongside the target fragment. Consequently, the positive transgenic strains emitted green fluorescence. Strains exhibiting fluorescence were selected and initially identified as the positive transgenic strain Bb252:: BarGFPSPHvt/GNA (Fig. 8).

DNA level verification. The genomic DNA of the GFP strain and the wild-type Bb252 strain were used as templates for PCR amplification using the specific primers pBaGPEF and pBaGPER. The binding locations of the primers are shown by gray arrows in Fig. 7. The amplification products were detected using 1.0% agar-gel electrophoresis. The results indicated that the PCR product obtained using the genomic DNA of the positive transgenic Bb252::BarGFPSPHvt/GNA strain as the template showed a specific band of approximately 900 bp, which matched the expected size of the target fragment. However, when PCR amplification was performed using the genomic DNA of the wild-type Bb252 strain as a template, no band was observed (Fig. 9), suggesting that the pathogenicity protein-encoding gene fragment had been integrated into the Bb252 genome.

Transcriptional-level verification. The total RNA of the GFP transformants and the wild-type Bb252 strain was reverse-transcribed into cDNA. cDNA was then used as the template for RT–PCR amplification with the specific primers HVTGNAF and HvtGNAR. The results of 1.0% agarose gel electrophoresis revealed that the SPHvt/

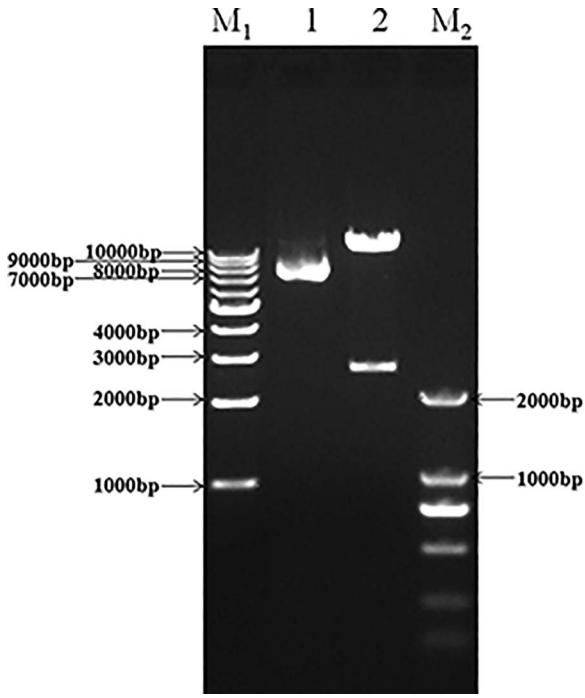

Fig. 6. Identification of the recombinant plasmid pBarGFP-SPHvt/GNA by enzyme digestion (Note: M1: DNA marker 1 kb DNA ladder; M2: DNA marker DL2000; 1: pBarGFP-SPHvt/GNA; 2: products of pBarGFP-SPHvt/GNA digested by SpeI and BglII).

GNA gene was transcribed in the suspected positive transgenic strain Bb252::BarGFPSPHvt/GNA, while the wild-type strain Bb252 did not contain the SPHvt/GNA gene; therefore, the gene could not be detected in the transcriptome. This further supported the transcription of the SPHvt/GNA gene in the Bb252::BarGFPSPHvt/GNA transgenic strain, as shown in Fig. 10.

Protein level verification. The total protein and extracellular protein of the wild-type Bb252 strain and the positive transgenic Bb252 strain were extracted for

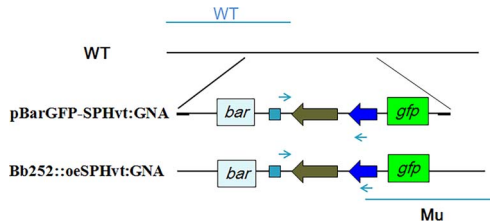

Fig. 7. The principle diagram of Agrobacterium-mediated fungal genetic transformation.

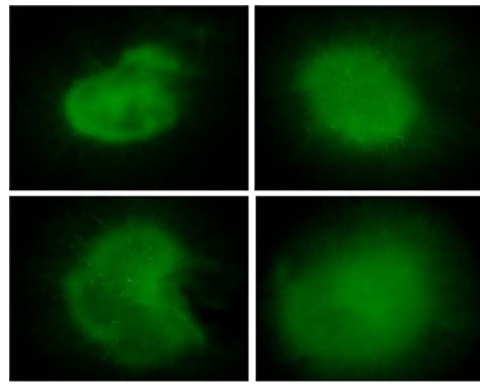

Fig. 8. The fluorescent strains chosen (4×).

Western blot analysis. The results showed that both the total protein and extracellular protein of the transgenic strain reacted with the anti-flag.tag antibody and exhibited specific binding protein bands that matched the predicted size (Fig. 11). This indicated that the Bb252::BarGFPSPHvt/GNA transgenic strain expressed the SPHvt/GNA protein. Furthermore, after the strain expressed the pathogenicity protein, the protein underwent proteolytic enzyme action to remove the signal peptide and became a mature peptide that was then secreted into the extracellular environment.

Pathogenicity of transgenic strains against *A. stephensi* larvae. We used a feeding method to assess the pathogenicity of second-instar larvae of *A. stephensi* to the wild-type strain Bb252 and the transgenic strain Bb252::BarGFPSPHvt/GNA. The survival rates at different treatment times are presented in Fig. 12. The

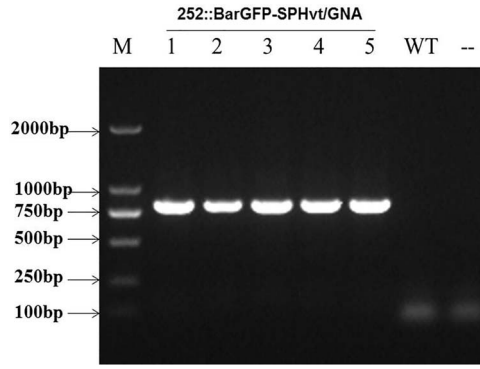

Fig. 9. Identification of the positive transgenic strains at the genomic DNA level (Note: M: DNA marker DL2000; 1,2,3,4,5: PCR product of transgenic strains with genomic DNA as the template; WT: PCR product of Bb252 genomic DNA as the template; –: PCR product of H_2O as the template).

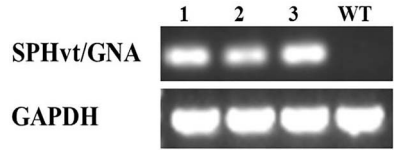

Fig. 10. Identification of the positive transgenic strains at the transcriptional level (Note: 1, 2, 3: the positive transgenic strains via RT–PCR; WT: wild-type Bb252 via RT–PCR).

survival rate of *A. stephensi* larvae gradually decreased over time after treatment with the spore suspension. Furthermore, the death rate of both strains was greater between the 3rd and 6th days. Compared with the wild-type strain, the transgenic strain caused significantly faster larval mortality ($P < 0.001$).

Infection of *A. stephensi* larvae by transgenic strains. Under a fluorescence microscope, larvae of *A. stephensi* infected with transgenic bacteria showed fungal spores in the midgut and hindgut at 36 h after treatment. The number of spores in the larval intestine increased after 60 h of infection. At 80 h postinfection, mycelia were observed on the surface of the mosquito larvae. After 96 h of infection, the mosquito surface was covered with mycelium, and the mosquito larva died (Fig. 13). The larvae in the transgenic strain-infected group exhibited slower growth than did those in the wild-type strain-infected group, and their larval period was prolonged, or they even failed to pupate on time.

Discussion

Fungal insecticides play a crucial role in mosquito control. However, fungi isolated directly from nature and strains obtained through conventional breeding have several drawbacks, such as low pathogenicity and susceptibility to environmental influences, which affect their insecticidal effectiveness and application. To improve the pathogenicity of biocontrol fungi, strain improvement is an effective method. *Agrobacterium tumefaciens*-mediated fungal genetic transformation offers advantages such as simplicity, high conversion efficiency, genetic stability, and wide applicability. Thus, it holds great potential for the construction of fungal gene mutant libraries (Hu et al. 2021).

GNA functions as a carrier protein that transports toxic proteins to the hemolymph of insects, causing toxic effects. Building on this, numerous researchers have combined GNA with toxin proteins, such as SFI1/GNA (Down et al. 2006),

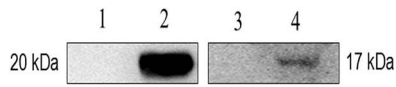

Fig. 11. Identification of the positive transgenic strains at the protein level (Note: 1: Total protein of Bb252; 2: Total protein of transgenic strains; 3 Extracellular proteins of Bb252; 4: Extracellular proteins of transgenic strains).

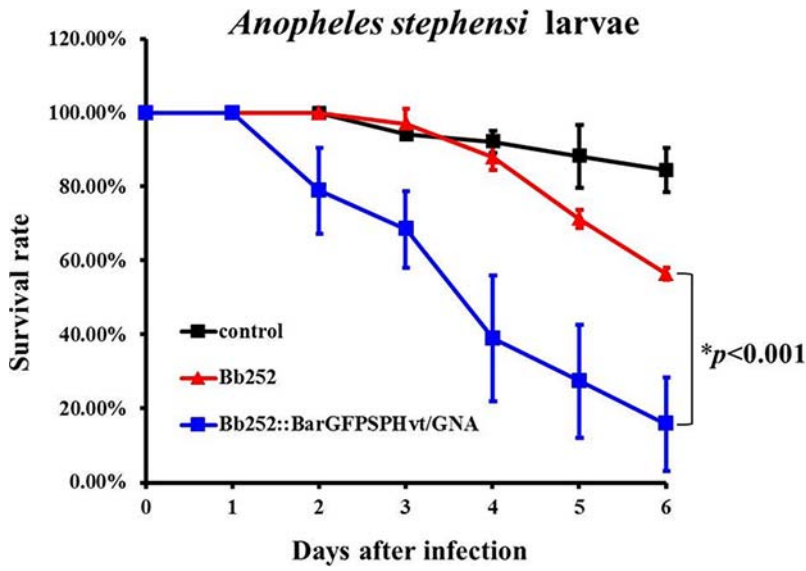

Fig. 12. Survival of *Anopheles stephensi* larvae following feeding with suspensions of 5×10^6 conidia/mL Bb252 or Bb252::BarGFPSPHvt/GNA.

ButaIT/GNA (Fitches et al. 2010), and PI1a/GNA (Yang et al. 2014). These fusion proteins have demonstrated significantly greater toxicity than their individual effects on insects. In this study, we synthesized the SPHvt/GNA fusion gene, which consists of GNA and the spider toxin Hvt, while taking into account the codon bias of *B. bassiana*. Using an *Agrobacterium*-mediated fungal genetic system, we introduced the SPHvt/GNA fusion gene into the genome of *B. bassiana* Bb252. Driven by the specific promoter gpdA of *B. bassiana*, the fusion gene was successfully expressed in the transgenic strain, allowing for the release of the pathogenicity protein SPHvt/GNA into the extracellular system. The pathogenicity of the Bb252::BarGFPSPHvt/GNA transgenic strain against *A. stephensi* larvae was significantly greater than that of the wild-type strain.

The process by which *B. bassiana* infects insects involves several stages, including adhesion, germination, penetration, and vegetative growth (Wang et al. 2005). Insecticidal fungi degrade the insect epidermis by releasing enzymes, and the germinating tubes apply mechanical pressure to the insect epidermis, thereby disrupting it and invading the insect. Once inside, insecticidal fungi secrete toxins or induce a series of physiological, biochemical, and pathological changes in the insect body, leading to the death of the host. In this study, *A. stephensi* larvae were chosen as the test subjects for the pathogenicity test, and the results indicated that the transgenic strain effectively killed them. Through observation of the infection process in *A. stephensi* larvae by the transgenic strain, traces of fungal spores entering the midgut of *A. stephensi* larvae were found, and mycelia covered the entire surface of the larvae during the later stages of infection. This suggests that after the Bb252::BarGFPSPHvt/GNA transgenic strain infects mosquito

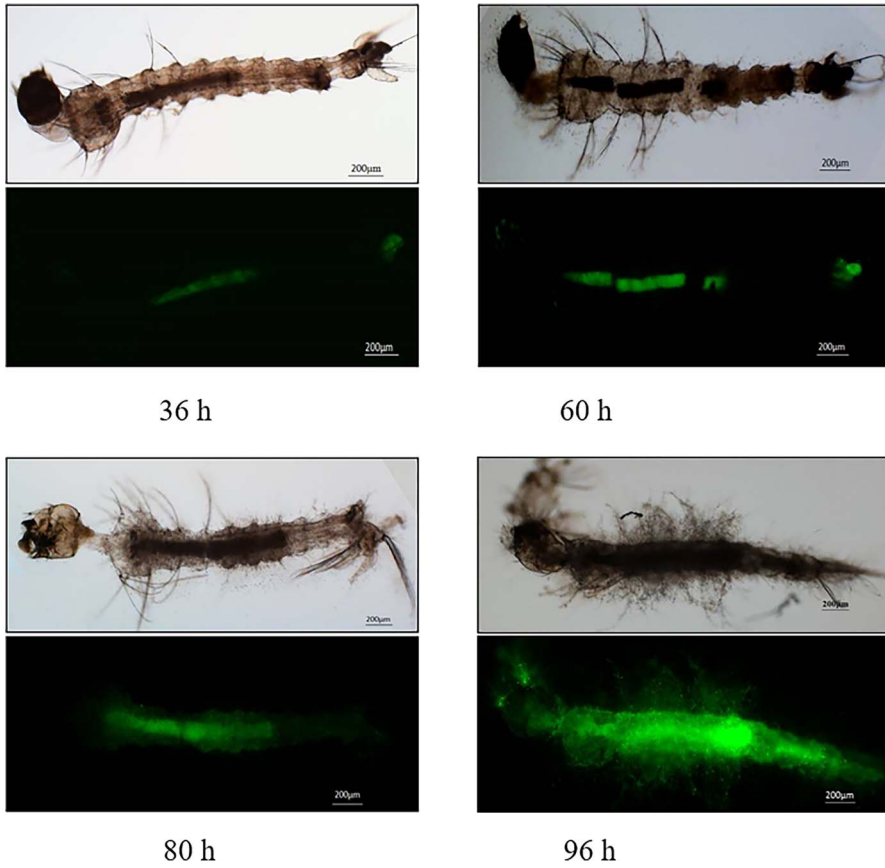

Fig. 13. Mosquito larvae post infection.

larvae, under the influence of the SPHvt/GNA pathogenicity protein, spores penetrate midgut epithelial cells, germinate into mycelia, and continuously deplete nutrients in mosquitoes, ultimately resulting in their death.

In this study, spore suspensions of 2.5×10^8 were prepared with 0.01% Triton X-100, and larval toxicity of each treatment was assessed. It was confirmed that the construction of a transgenic *B. bassiana* strain for potential management of mosquito larvae. Probit analysis of an appropriate range of concentrations is in order to determine the concentration-mortality responses as well as the lethal time, which appears to be important in these comparisons. Therefore, it is necessary to bolster with concentration-mortality response as well as lethal time responses in future work to further determine the effect of transgenic *B. bassiana* strain on mosquito larvae.

In summary, this study successfully generated a *B. bassiana* strain carrying the SPHvt/GNA fusion gene through *Agrobacterium tumefaciens*-mediated fungal genetic transformation. This significantly enhanced the strain's pathogenicity against *A. stephensi* larvae and accelerated the eradication of mosquitoes in these

preliminary bioassays. These findings highlight the promising application prospects of fungal genetic transformation in the development of new insecticides that are both safe and effective.

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