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# Downregulation of a transcription factor associated with resistance to Bt toxin Vip3Aa in the invasive fall armyworm

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**Transgenic crops producing insecticidal proteins from *Bacillus thuringiensis* (Bt) have revolutionized control of some major pests. However, more than 25 cases of field-evolved practical resistance have reduced the efficacy of transgenic crops producing crystalline (Cry) Bt proteins, spurring adoption of alternatives including crops producing the Bt vegetative insecticidal protein Vip3Aa. Although practical resistance to Vip3Aa has not been reported yet, better understanding of the genetic basis of resistance to Vip3Aa is urgently needed to proactively monitor, delay, and counter pest resistance. This is especially important for fall armyworm (*Spodoptera frugiperda*), which has evolved practical resistance to Cry proteins and is one of the world's most damaging pests. Here, we report the identification of an association between downregulation of the transcription factor gene *SfMyb* and resistance to Vip3Aa in *S. frugiperda*. Results from a genome-wide association study, fine-scale mapping, and RNA-Seq identified this gene as a compelling candidate for contributing to the 206-fold resistance to Vip3Aa in a laboratory-selected strain. Experimental reduction of *SfMyb* expression in a susceptible strain using RNA interference (RNAi) or CRISPR/Cas9 gene editing decreased susceptibility to Vip3Aa, confirming that reduced expression of this gene can cause resistance to Vip3Aa. Relative to the wild-type promoter for *SfMyb*, the promoter in the resistant strain has deletions and lower activity. Data from yeast one-hybrid assays, genomics, RNA-Seq, RNAi, and proteomics identified genes that are strong candidates for mediating the effects of *SfMyb* on Vip3Aa resistance. The results reported here may facilitate progress in understanding and managing pest resistance to Vip3Aa.**

transgenic crop | *Bacillus thuringiensis* | myeloblastosis transcription factor | invasive species | *Spodoptera frugiperda*

## Significance

Crops genetically engineered to produce insect-killing proteins from the bacterium *Bacillus thuringiensis* (Bt) control some major pests and reduce use of insecticide sprays. However, evolution of pest resistance to crystalline Bt proteins has decreased these benefits, promoting adoption of transgenic crops that produce a Bt vegetative insecticidal protein called Vip3Aa. Better understanding of resistance to Vip3Aa is urgently needed to proactively combat pest resistance to this protein. Here we found that reduced expression of a gene encoding a protein that regulates transcription decreased susceptibility to Vip3Aa in the fall armyworm, one of the world's most voracious crop pests. The identification of this genetic basis of resistance may help to improve management of resistance and enhance sustainability of Bt crops.

Since 1996, millions of farmers in dozens of countries have cultivated crops genetically engineered to produce insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) (1). The total area of transgenic Bt crops planted globally from 1996 to 2019 exceeded one billion hectares (1), with benefits including pest suppression, reduced use of conventional insecticides, and better biological control by natural enemies (2–10). However, these benefits have been reduced by rapid evolution of pest resistance to Bt crops (11–14). Practical resistance that decreases the efficacy of Bt crops has been documented in at least 26 cases affecting nine crystalline (Cry) Bt proteins in populations of 11 pest species in seven countries (12).

Spurred by pest resistance to Cry toxins, farmers have increasingly adopted multi-toxin transgenic crops that produce Bt vegetative insecticidal protein Vip3Aa together with Cry proteins (13–16). Whereas Cry proteins are produced by the bacteria during sporulation and retained within cells, Vips are generated during the vegetative phase and secreted (17). Despite some structural similarity between Vip and Cry proteins, cross-resistance between them is weak or nil (17–22). Thus, Vip3Aa is essential for the efficacy of Bt crops against some devastating pests that have evolved practical resistance to Cry toxins (12, 16, 22). We are not aware of any documented cases of practical resistance to Vip3Aa. However, resistance to Vip3Aa has been selected in the laboratory in at least six lepidopteran species (22–30) and early warnings of field-evolved resistance to this toxin have been reported for two major pests, the corn earworm (*Helicoverpa zea*) and the fall armyworm (*Spodoptera frugiperda*)(12, 15, 31, 32).

Better understanding of the genetic basis of resistance to Vip3Aa is urgently needed to proactively monitor, delay, and counter pest resistance. Yet the genes involved in insect resistance to any Vip protein have not been reported previously as far as we know. Nearly all knowledge of the genes involved in Bt resistance is based on studies of Cry toxins (33–41). Resistance to Cry toxins is often caused by mutations disrupting proteins such as cadherin and ABC (ATP-binding cassette) transporters that bind Cry toxins in the midgut of susceptible larvae (33, 35, 36, 40). Like Cry toxins, Vip toxins must bind

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specifically to midgut receptors to kill insects (19, 20). However, the genes involved in resistance are likely to differ between Cry and Vip toxins because these two toxin families do not share midgut receptors (19) and resistance to Vip toxins does not necessarily entail disruption of midgut receptors (42).

Understanding the genetic basis of resistance to Vip3Aa is especially important for fall armyworm because it is one of the world's most damaging crop pests (43, 44). It has recently invaded Africa, Asia, and Australia (44), and it has evolved practical resistance in the Americas to the Cry toxins in Bt crops (12, 45). Previous work with *S. frugiperda* has identified three putative receptors for Vip3Aa and three proteins putatively involved in defense against Vip3Aa (46–50), but we are not aware of any evidence linking these proteins with resistance to Vip3Aa selected in the laboratory or field. Here, we report the identification of an association between resistance to Vip3Aa in *S. frugiperda* and downregulation of a transcription factor gene for which no role in Bt toxicity has been reported before. We also identify genes that are potential targets of the transcription factor and strong candidates for contributing to the observed resistance to Vip3Aa.

## Results

**Selection for Resistance to Vip3Aa and Survival on Corn Producing Vip3Aa.** We analyzed a Vip3Aa-resistant strain of fall armyworm called DH-R, which was derived from the DH-S strain that originated in Dehong, Yunnan Province, China, in 2019 (previously called DH19) (51–53). After 35 generations of selection with Vip3Aa in the laboratory, the concentration of Vip3Aa killing 50% of larvae ($LC_{50}$) was 206 times higher for DH-R than DH-S (4.32 and 0.021 µg Vip3Aa per $cm^2$ diet, respectively, *SI Appendix*, Table S1). On non-Bt corn, survival was similar for DH-R (62%) and DH-S (61%) (Fisher's exact test, $P = 0.92$, *SI Appendix*, Table S2). Relative to survival on non-Bt corn, survival from first instar to adult eclosion on Bt corn producing Vip3Aa was 26% for DH-R vs. 0% for DH-S (Fisher's exact test, $P < 0.0001$, *SI Appendix*, Table S2). Selection for resistance to Vip3Aa in DH-R did not cause cross-resistance to Cry1Ab, Cry1Fa, or Cry2Ab (*SI Appendix*, Tables S1 and S3).

**Inheritance of Vip3Aa Resistance.** Survival was 97% for DH-R and 0% for DH-S in bioassays with 0.4 µg Vip3Aa per $cm^2$ diet (*SI Appendix*, Table S3). At this concentration, mean survival was 12% for $F_1$ progeny from crosses between DH-R and DH-S and survival did not differ significantly between the $F_1$ progeny from reciprocal crosses (*SI Appendix*, Table S3). These results show inheritance of resistance at this concentration was autosomal and partially recessive (mean $h = 0.13$, where $h = 0$ indicates completely recessive resistance and $h = 1$ indicates completely dominant resistance).

**Genome-Wide Association Study (GWAS) and Fine-Scale Mapping of Vip3Aa Resistance.** We conducted a GWAS with 29,248 single nucleotide polymorphisms (SNPs) and 10,266 insertions and deletions (indels) identified from 148 *S. frugiperda* larvae. These larvae were $F_2$ progeny generated by a single-pair cross between a DH-R female and a DH-S male followed by mass mating among their $F_1$ progeny. We scored the resistance phenotype as larval weight 7 d after neonates were placed on diet with 0.4 µg Vip3Aa per $cm^2$ diet. Regions on a few chromosomes showed a potential association with resistance based on either the SNPs or indels (Fig. 1). However, based on analysis of linkage disequilibrium (LD) with a threshold of 0.5 for $r^2$, only a region on chromosome 27 (Genome assembly version ZJU_Sfru_1.0, GCF_011064685.1) was associated with resistance based both on SNPs (10.01 to 10.63 Mb, Fig. 1) and indels (10.21 to 10.49 Mb, Fig. 1).

Next, we conducted fine-scale mapping with 96 $F_2$ larvae that were derived from a second set of crosses using the approach described above. Larvae that survived exposure to diet treated with 0.5 µg Vip3Aa per $cm^2$ diet were scored as resistant. The results demonstrate a highly significant association between resistance and each of the five SNP markers tested from 10.01 to 10.63 Mb on chromosome 27 ($P < 10^{-14}$ for each marker, *SI Appendix*, Table S4). The strongest association occurred for the three SNPs from 10.29 to 10.48 Mb, with $P < 10^{-31}$ for each (*SI Appendix*, Table S4).

**Resistance to Vip3Aa Associated with Reduced Expression of the *SfMyb* Gene.** Results of RNA-Seq with midguts of fourth instar larvae showed that only two of the nine genes from 10.29 to 10.48 Mb on chromosome 27 were substantially expressed: *S. frugiperda myeloblastosis* (*SfMyb*, GenBank accession no. XM_035598865.1) and *nuclear pore glycoprotein p62-like* (*SfNup62*, XM_035598955.1, Fig. 1 and *SI Appendix*, Table S5). Sequencing these two genes in 10 individuals per strain revealed no differences in the predicted amino acid sequences between DH-R and DH-S. Expression was similar between strains for *SfNup62* ($P = 0.50$) but was 2.8-fold lower in DH-R than DH-S for *SfMyb* ($P = 0.012$, *SI Appendix*, Table S5). The SfMyb protein of 746 amino acids resembles proteins in the large Myb family of transcription factors whose members have conserved DNA-binding domains, occur widely in eukaryotes, often regulate cell differentiation and proliferation, and are implicated in many types of tumors (54–56).

We conducted a backcross (BC) experiment to determine whether reduced expression of *SfMyb* is genetically linked with resistance. After a DH-R male mated with a DH-S female to generate $F_1$ progeny, the $F_1$ males mated with DH-R females to produce BC progeny. The BC larvae were fed for 7 d on either untreated diet or diet treated with 0.5 µg Vip3Aa per $cm^2$ diet. Survivors on the treated diet were deemed resistant. Analysis of 20 larvae from each group revealed the mean relative expression of *SfMyb* was 3.9-fold lower for resistant larvae (0.29, SE = 0.02) than larvae on the control diet (1.1, SE = 0.1; *t* test, $t = 7.43$, df = 21 [adjusted for unequal variances], $P < 0.0001$). These results support the hypothesis that reduced expression of *SfMyb* is genetically linked with resistance to Vip3Aa.

**Reducing *SfMyb* Expression with RNAi (RNA interference) or Gene Editing Decreases Susceptibility to Vip3Aa.** We used RNAi to determine whether experimental reduction of *SfMyb* expression would reduce susceptibility to Vip3Aa. Relative to a control injection of double-stranded RNA for green fluorescent protein (dsGFP), injection of double-stranded RNA for SfMyb (dsMyb) into third instar larvae of DH-S significantly reduced the abundance of SfMyb mRNA 24 and 48 h later (Fig. 2*A*). The relative growth rate of larvae on diet with Vip3Aa incorporated (2 µg Vip3Aa per ml diet) was significantly higher for larvae injected with dsMyb than dsGFP (Fig. 2*B*). These results indicate that reduced expression of *SfMyb* mediated by RNAi in the DH-S strain reduced larval susceptibility to Vip3Aa.

Next, we used CRISPR/Cas9 gene editing to determine whether knocking out the *SfMyb* gene by introducing a premature stop codon would reduce susceptibility to Vip3Aa. We generated a strain heterozygous for the knockout (KO) mutation in *SfMyb* [genotype: *SfMyb*-KO/wild-type (WT)] (Fig. 3). Of the 96 progeny analyzed from mating among moths within this strain, 58 were

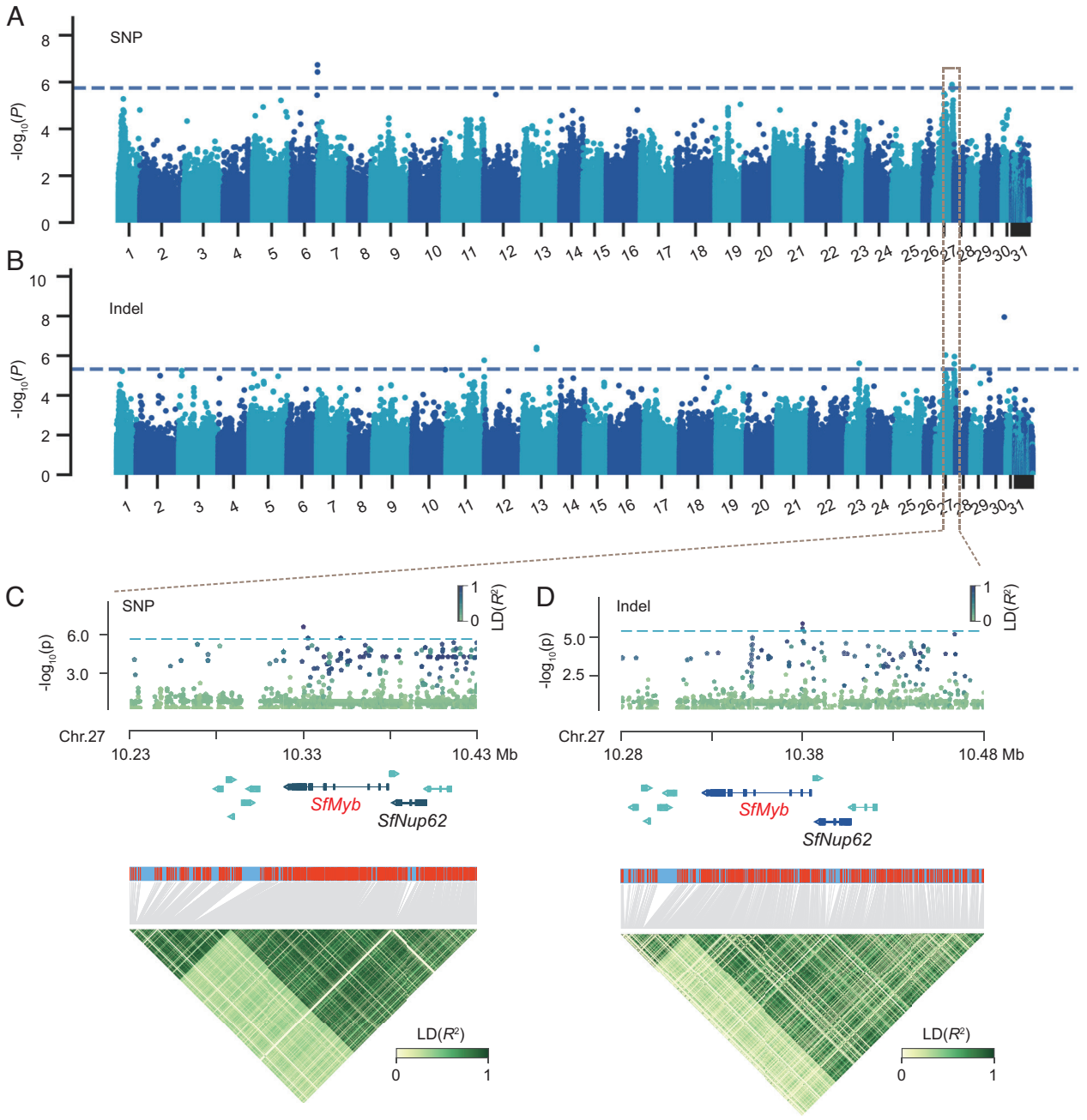


**Fig. 1.** Region on chromosome 27 associated with *S. frugiperda* resistance to Vip3Aa. Manhattan plots: (*A*) SNPs and (*B*) indels for all 31 chromosomes; (*C*) SNPs and (*D*) indels for a region on chromosome 27. Horizontal dashed lines indicate the threshold for significant association. Below the Manhattan plots for (*C*) and (*D*) are models of the nine genes in the region and LD heatmaps based on SNPs and indels, respectively. The gene models show the two expressed genes (*SfMyb* and *SfNup62*) in dark blue and the other seven genes in light blue.

heterozygous for the KO and 38 were homozygous WT, showing that individuals homozygous for the KO did not survive.

We crossed 30 female *SfMyb*-KO/WT heterozygotes with 30 males from DH-R that were homozygous for the mutant *SfMyb* allele and reared their progeny for 7 d on diet that was either untreated or treated with 0.5 µg Vip3Aa per $cm^2$ diet. On untreated diet, all larvae survived (n = 96). All survivors on untreated diet had the mutant allele from DH-R (as expected), whereas the other allele was *SfMyb*-KO for 42 larvae and the WT allele from DH-S for the remaining 54 larvae. The observed genotype ratio (42:54) does not differ significantly from the expected 1:1 genotype ratio if survival on untreated diet was independent of the *SfMyb* genotype (Fisher's exact test, $P = 0.47$). By contrast, on the treated diet, survival was 44% (85 of 192). All 85 survivors on treated diet had two mutant *SfMyb* alleles, one from DH-R and the other from *SfMyb*-KO. This differs significantly from the expected 1:1 genotype ratio if survival was independent of *SfMyb* genotype (Fisher's exact test, $P = 5 \times 10^{-16}$). These results indicate survival on Vip3Aa-treated diet was strongly associated with the lack of a WT *SfMyb* allele.

The results above imply that 0.5 µg Vip3Aa per $cm^2$ diet killed all wild type/DH-R heterozygotes, which is comparable to the partially recessive inheritance of resistance ($h = 0.13$) inferred from testing the $F_1$ progeny of DH-S and DH-R at the slightly lower concentration of 0.4 µg Vip3Aa per $cm^2$ (*SI Appendix*, Table S3). In addition, the 44% survival of larvae on treated diet noted above does not differ significantly from the 50% survival expected if the genotype ratio of survivors on untreated diet was 1:1 (*SfMyb*-KO/DH-R:

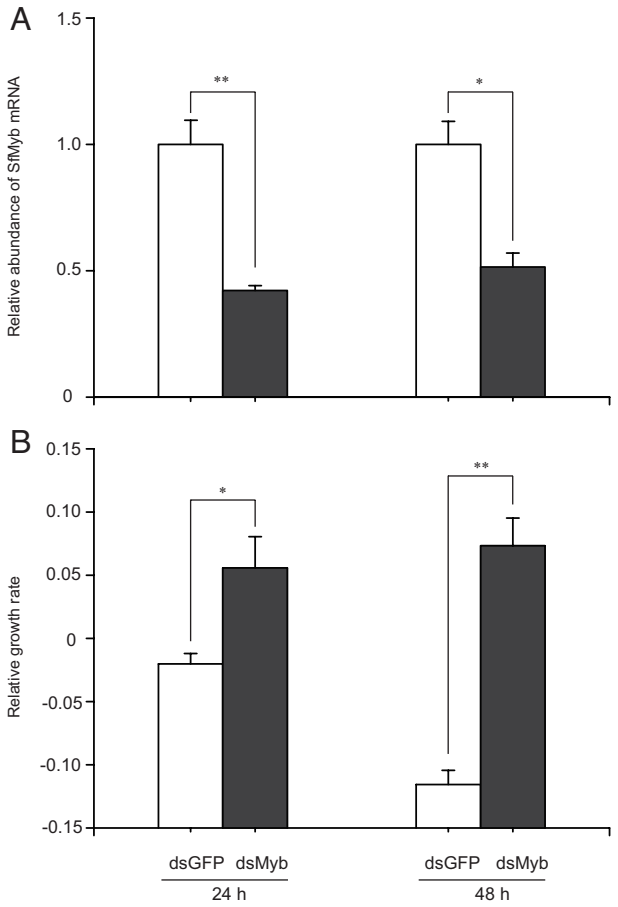


**Fig. 2.** Susceptibility to Vip3Aa reduced by RNAi suppression of *SfMyb* expression. Third instar larvae of susceptible strain DH-S were reared for 24 or 48 h on diet containing Vip3Aa after injection with dsRNA of Myb (dsMyb) or GFP (dsGFP) as a control. (*A*) Relative abundance of *SfMyb* mRNA. (*B*) Relative growth rate [(final weight – initial weight)/initial weight]. Comparisons between treatments based on *t* tests: $*P < 0.05$, $**P < 0.01$.

WT/DH-R), survival was 0% for wild type/DH-R, and survival was 100% for *SfMyb*-KO/DH-R (Fisher's exact test, $P = 0.31$). Moreover, the estimated survival of *SfMyb*-KO/DH-R larvae on treated diet is 100% if we use the observed genotype ratio of survivors on untreated diet (42 *SfMyb*-KO/DH-R to 54 wild type/DH-R). Assuming a 1:1 genotype ratio (*SfMyb*-KO/DH-R: WT/DH-R) without exposure to treated diet, the estimated survival of the *SfMyb*-KO/DH-R larvae on diet containing 0.5 µg Vip3Aa per $cm^2$ diet is 89% (85 of 96), which does not differ significantly from the observed 88 or 96% survival of DH-R larvae on diet containing 0.4 µg Vip3Aa per $cm^2$ diet in two independent bioassays (*SI Appendix*, Tables S1 and S3, Fisher's exact test, $P = 1$ and 0.22, respectively). Thus, resistance to Vip3Aa at the concentrations tested was not significantly lower for *SfMyb*-KO/DH-R than DH-R. Because the concentration of Vip3Aa was higher for *SfMyb*-KO/DH-R than DH-R (0.5 vs. 0.4 µg Vip3Aa per $cm^2$ diet, respectively), the conclusion that resistance was not lower for *SfMyb*-KO/DH-R than DH-R is conservative.

**Deletions in the *SfMyb* Promoter of DH-R.** In light of the compelling evidence reported above that reduced expression of *SfMyb* caused decreased susceptibility to Vip3Aa, we compared the *SfMyb* promoter between DH-R and DH-S. We found deletions of two and eight base pairs in DH-R relative to DH-S as well as 15 SNPs between the two strains (Fig. 4). Dual-luciferase transient expression analysis in *Sf9* cells showed the mean relative activity of the *SfMyb* promoter was 1.7-fold lower for DH-R (21.2, SE = 0.7) than DH-S (35.2, SE = 1.9, two-way ANOVA, $F_{1,12} = 54.9$, $P < 10^{-5}$, *SI Appendix*, Table S6).

**Identification of Target Genes of the SfMyb Transcription Factor That Are Candidates for Contributing to Vip3Aa Resistance.** Results from yeast one-hybrid assays revealed 12 motifs of seven DNA base pairs each that bound the SfMyb transcription factor and match motifs in *Drosophila melanogaster* promoters known to bind transcription factors (*SI Appendix*, Table S7). A scan of the *S. frugiperda* genome predicted 8,744 instances of binding of the SfMyb transcription factor to promoters containing one or more of these 12 motifs (*SI Appendix*, Table S7). Accounting for promoters predicted to bind more than one of the 12 motifs narrowed the number of candidate genes to 4,875. Of these 4,875 candidate genes, RNA-Seq of larval midguts identified 27 genes that showed significant differences in expression between DH-R vs. DH-S and between DH-S larvae treated with dsMyb (which reduced susceptibility to Vip3Aa) vs. dsGFP (control) (*SI Appendix*, Table S8). We excluded 3 of these 27 genes because the proteins they encode were not detected in DH-S in a previous study (57) and the abundance of their mRNA was significantly lower in DH-R than DH-S (*SI Appendix*, Table S8).

For 20 of the remaining 24 genes, mRNA abundance differed in the same direction in comparisons between DH-R vs. DH-S and between DH-S larvae treated with dsMyb vs. dsGFP: 14 were downregulated in both comparisons and 6 were upregulated in both comparisons (*SI Appendix*, Table S8). The remaining four were downregulated in DH-R relative to DH-S but upregulated in DH-S larvae treated with dsMyb relative to dsGFP (*SI Appendix*, Table S8). The latter four genes are not strong candidates for causing resistance to Vip3Aa because of the opposite direction of the difference in mRNA abundance from the two comparisons. Of the remaining 20 candidate genes, the expected locations of the encoded proteins are the plasma membrane (7), cytoplasm (7) extracellular (3), and nucleus (3) (*SI Appendix*, Table S8).

**Evaluation of Six Genes Previously Reported to Affect Responses to Vip3Aa in *S. frugiperda*.** We evaluated the potential role in resistance to Vip3Aa of three *S. frugiperda* genes encoding proteins that putatively defend against Vip3Aa (*autophagy related gene 5*, *phenyloxidase-activating enzyme-like*, *phenyloxidase subunit 2-like*) and three encoding putative Vip3Aa receptors (*scavenger receptor-C*, *fibroblast growth factor receptor*, and *ribosomal S2*) (46–50). In the GWAS we conducted, these six genes were not associated with resistance to Vip3Aa (Fig. 1 and *SI Appendix*, Table S9). Also, the promoters for these six genes did not have any of the 12 SfMyb-binding motifs identified from the yeast one-hybrid assay. Thus, none of the six genes were candidates in the analysis described above (*SI Appendix*, Table S8). For five of these genes, the abundance of their mRNA in larval midguts did not differ significantly between DH-R and DH-S (*SI Appendix*, Table S9). Abundance of mRNA for *ribosomal S2* was 2.5-fold lower for DH-R than DH-S ($P < 0.001$, *SI Appendix*, Table S9), but it was not lower in DH-S larvae treated with dsMyb vs. dsGFP (i.e., 1.3-fold higher in DH-R, $P = 0.52$).

## Discussion

The results here from GWAS, fine-scale mapping, and RNA-Seq identify the *SfMyb* transcription factor gene as a strong candidate for contributing to the 206-fold resistance to Vip3Aa in the DH-R strain of *S. frugiperda*. Although the amino acid sequence of the protein encoded by *SfMyb* did not differ between DH-R and the susceptible strain DH-S from which DH-R was derived, expression

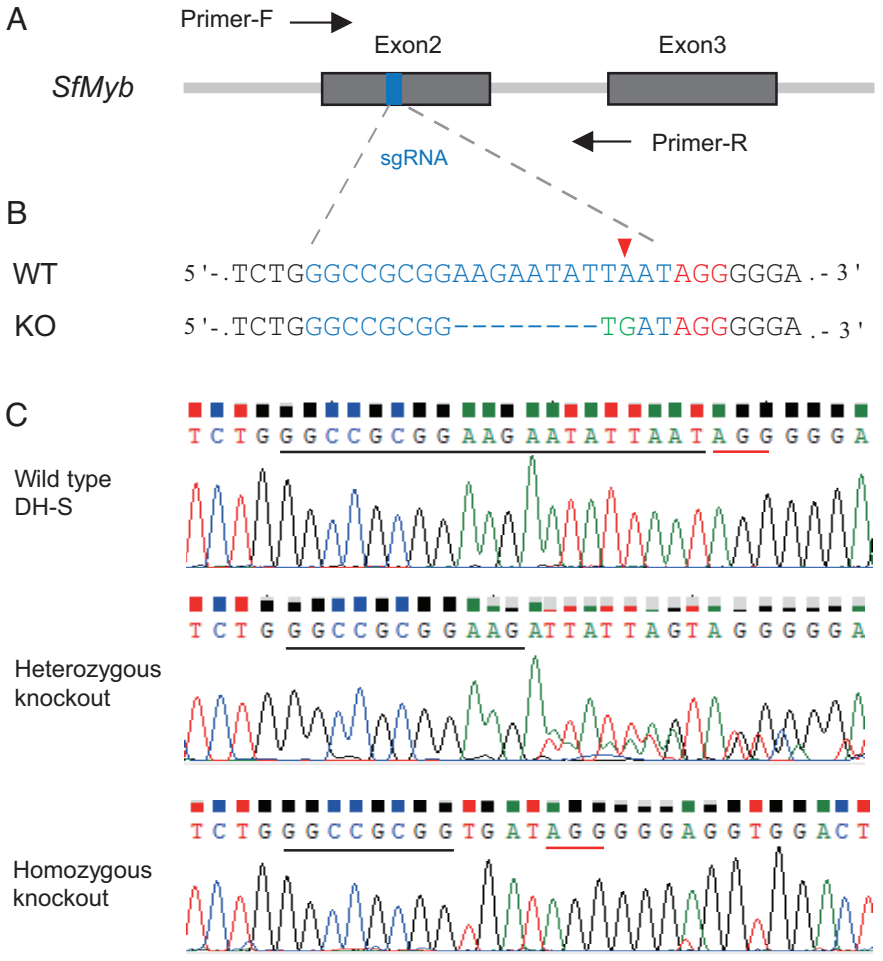


**Fig. 3.** CRISPR/Cas9 KO of *SfMyb*. (*A*) Target in exon 2 and primers-F and -R for PCR detection. (*B*) WT sequence from susceptible strain DH-S and KO sequence with deletion of 8 bp and insertion of 2 bp (TG in green) yielding a premature stop codon (TGA). Target sequences of sgRNA (blue), protospacer adjacen motif (PAM, red), Cas9 cleavage site (red triangle). (*C*) Chromatograms of direct sequencing of PCR products for determining genotype. The sgRNA target sequence is underlined in black and PAM in red.

of this gene was reduced in DH-R relative to DH-S. Also, based on our analysis of previously reported data from RNA-Seq (57), exposure of DH-S larvae to Vip3Aa significantly reduced expression of *SfMyb* (Mann-Whitney *U* test, $U = 18$, $P = 0.02$). Moreover, experimental reduction of *SfMyb* expression in DH-S using RNAi or CRISPR/Cas9 gene editing decreased susceptibility to Vip3Aa, which implies the observed reduced expression in DH-R contributes to this strain's resistance to Vip3Aa. The results from bioassays with 0.4 and 0.5 µg Vip3Aa per $cm^2$ diet imply that resistance was similar in larvae from DH-R and those with one allele from the *SfMyb* KO and the other from DH-R (*SfMyb*-KO/DH-R). In addition, the promoter for *SfMyb* had lower activity in DH-R than DH-S. It remains to be determined whether the deletion of 8 bp or other mutations we found in the *SfMyb* promoter in DH-R reduce expression of *SfMyb*.

Of the 20 candidate genes we identified as potential targets of the SfMyb transcription factor that might contribute to Vip3Aa resistance, *serine protease stubble-like (SfStubble)*, and *peste (SfPeste)* are particularly intriguing (Gene IDs 118279751 and 118265051, respectively). The proteins encoded by these genes are predicted to be transmembrane proteins (*SI Appendix*, Table S8). *SfStubble* is the only candidate for which we detected mRNA in larval midguts

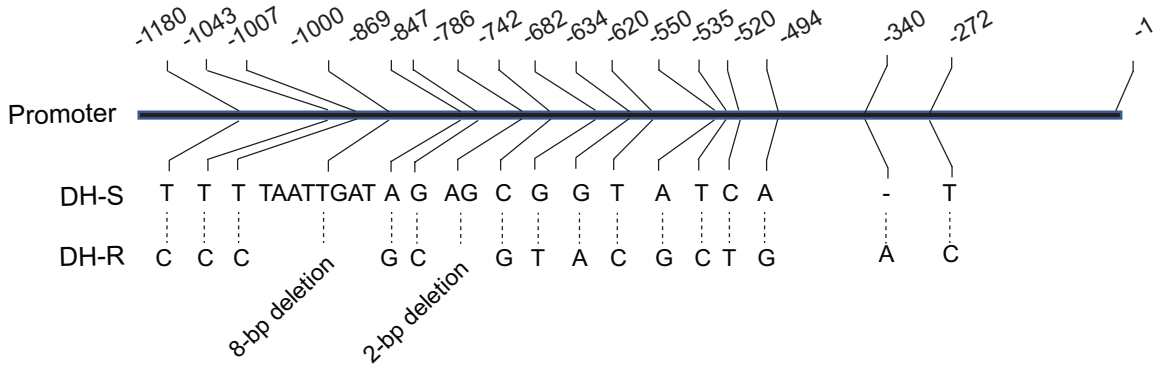


**Fig. 4.** Differences in the *SfMyb* promoter sequence between the susceptible parent strain (DH-S) and the Vip3Aa-resistant strain (DH-R) derived from DH-S.

for DH-S but not DH-R (*SI Appendix*, Table S8). Also, previous work with DH-S detected the SfStubble protein in midguts (57). Reduced conversion of Cry protoxins to activated toxins caused by downregulation of serine proteases causes resistance in *S. frugiperda* and other lepidopterans (58–60). In limited studies with Vip3Aa, slower conversion of protoxin to activated toxin was implicated in a resistant strain of *Helicoverpa armigera* (61), but conversion was not altered in a resistant strain of *H. zea* (62). It will be useful to evaluate the role of this mechanism and the potential contribution of downregulation of *SfStubble* in other Vip3Aa-resistant strains.

The abundance of *SfPeste* mRNA was 33-fold higher in DH-S than DH-R (*SI Appendix*, Table S8). The SfPeste protein is predicted to resemble members of the CD36 family of scavenger B receptors (63, 64). In *D. melanogaster*, the Peste protein facilitates infection by some bacteria (63, 64), which suggests reduced expression of this gene could decrease susceptibility to Vip3Aa. Although reduced binding of Vip3Aa in the larval midgut is not associated with resistance in some lepidopteran strains (42), this is a primary mechanism of resistance in one strain of *H. zea* (62). It will be important to determine whether reduced expression of *peste* contributes to reduced binding of Vip3Aa in *H. zea*, *S. frugiperda,* or other species.

The results here do not support a substantial role in the resistance of DH-R to Vip3Aa for six genes previously reported to affect responses to Vip3Aa in *S. frugiperda* (46–50). In previous work with *S. frugiperda*, using CRISPR/Cas9 to knock out the gene encoding the receptor protein ABCC2 caused resistance to Cry1Ab and Cry1Fa (37, 51, 52) but CRISPR/Cas9 KOs of *scavenger receptor-C* or *fibroblast growth factor receptor* did not affect susceptibility to Vip3Aa (53). Thus, the evidence from this study and the previous CRISPR/Cas9 work is not consistent with a substantial role in Vip3Aa resistance for the six previously identified genes.

In addition to the DH-R strain from China analyzed here, four other strains of *S. frugiperda* have been selected in the lab for greater than 200-fold resistance to Vip3Aa: one from Brazil and three from the United States (22, 24, 26, 27). These results attest to the widespread potential for resistance to Vip3Aa in this invasive pest. Although practical resistance to Vip3Aa has not been documented as far as we know, Amaral et al. (32) reported a 3.7-fold increase in the frequency of Vip3Aa resistance alleles in *S. frugiperda* field populations in Brazil from 0.0009 during 2013 to 2014 to 0.0033 during 2016 to 2017.

For inheritance of resistance to Vip3Aa, the dominance parameter $h$ was 0.13 indicating partially recessive resistance for DH-R in this study compared with 0 indicating completely recessive resistance in three strains of *S. frugiperda* for which this parameter was evaluated previously (24, 26, 27). The concentration of Vip3Aa (in µg Vip3Aa per $cm^2$ diet) at which dominance was evaluated was lower here (0.4) than in the three previous studies (3.6 or 10). Because the dominance of resistance to Vip3Aa increases as Bt toxin concentration decreases (26), the higher value of $h$ seen here vs. previous studies could have been caused partly or entirely by the lower concentration of Vip3Aa used here.

The association between resistance to Vip3Aa and reduced expression of transcription factor SfMyb identified here is similar to the elegantly elucidated association between resistance to Cry1Ac in *Plutella xylostella* and altered expression of transcription factors PxGATAd (downregulated) and PxJun (upregulated) (38, 39). These regulatory mechanisms differ from direct disruption of midgut receptors, which is the most common mechanism of resistance to Cry proteins (33, 35, 36, 40). Furthermore, Tiewsiri and Wang (65) demonstrated that downregulation of aminopeptidase 1 (APN1) confers resistance to Cry1Ac in the lepidopteran *Trichoplusia ni*. Although the hypothesized *trans*-regulatory factor remains to be identified in that case, the downregulation of APN1 interacts with direct disruption of the receptor ABCC2 to yield a high level of resistance to Cry1Ac (66).

As far as we know, previous work has not reported interactions between Bt toxins and Myb transcription factors. Scopus literature searches identified over 17,000 papers on Bt and over 8,000 on Myb transcription factors, but no papers addressing both topics. More work is needed to definitively demonstrate how reduced expression of *SfMyb* confers resistance to Vip3Aa. It will also be important to determine whether the target genes of *SfMyb* identified here influence resistance to Vip3Aa in other strains or field populations of *S. frugiperda* or in other insects. If reduced expression of *myb* proves to be important in the field for resistance to Vip3Aa in *S. frugiperda* or other pests, this knowledge could bolster efforts to monitor resistance and facilitate discovery of how to effectively counter resistance.

## Materials and Methods

**Insect Strains.** To start the susceptible strain DH-S [previously called DH19 (51)] of *S. frugiperda*, eggs and larvae were collected from more than 20 non-Bt corn fields from Dehong, Yunnan Province, China, in January 2019. Approximately 100 field-collected individuals were reared to pupae on non-Bt corn in the laboratory. In subsequent generations, DH-S was reared on wheat germ diet in the laboratory without exposure to Bt toxins or other insecticides and was susceptible to Cry1Ab and Cry1Fa (51). In February 2019, we started the Vip3Aa-resistant strain DH-R by exposing approximately 5,000 first instar larvae from DH-S to wheat germ diet treated with Vip3Aa and rearing the first ca. 500 to 1,000 larvae to reach third instar to continue the strain. Before we analyzed the genetic basis of resistance, DH-R had been selected using this approach for more than 35 generations with increasing concentrations of Vip3Aa. For maintenance and in diet bioassays, larvae were reared on wheat germ diet at 27± 2 °C and 75 ± 10% relative humidity with 14 h light:10 h dark. Adults were held under similar conditions and provided with a solution of 10 g sugar per 100 mL water.

**Bt Toxins and Diet Bioassays.** Vip3Aa protoxin was provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. We bought Cry1Ab, Cry2Ab, and Cry1F protoxins from Meiyan Agricultural Technology Co., Beijing. We used standard diet overlay bioassays (51) to test first or second instar larvae in all experiments except the RNAi experiment described below in which we used a diet incorporation bioassay (67) to test third instar larvae. Diet overlay bioassays work well with first or second instars because they feed primarily on the diet surface, whereas the diet incorporation bioassay is better for third instars because they feed primarily below the diet surface.

We tested first instar larvae using the previously described standard diet overlay bioassay to determine $LC_{50}$ values and in the GWAS (*SI Appendix*, Tables S1 and S3 and Fig. 1). Following the standard method, larvae were considered dead if they died or weighed less than 5 mg after 7 d (51). The rationale for considering larvae dead if they weigh less than 5 mg after 7 d is that such larvae rarely survive to become adults. We tested second instar larvae for fine-scale mapping and genetic linkage experiments (*SI Appendix*, Table S4 and Results). In these bioassays, live larvae that reached the third or fourth instar after 7 d were considered survivors because such larvae were likely to survive to become adults. We used second rather than first instar larvae in these experiments to increase survival and the size of survivors, which made it easier to extract DNA for subsequent analyses.

In the RNAi experiment, we tested third instar larvae using a diet incorporation bioassay (67) to measure relative growth rate (see details below). We tested third instar larvae in this experiment because they are easier to inject with double-stranded RNA (dsRNA) than first or second instar larvae.

The concentration of Vip3Aa or other toxins that killed 50% of the larvae ($LC_{50}$) and their 95% fiducial limits were calculated with probit analysis using Statistical Product and Service Solutions (SPSS) (version 25.0, IBM Corp, USA). Two $LC_{50}$ values were considered significantly different if their 95% fiducial limits did not overlap. We calculated resistance ratios as $LC_{50}$ for DH-R divided by $LC_{50}$ for DH-S for the same toxin. We used Fisher's exact test to determine whether

survival differed significantly between DH-R and DH-S on non-Bt corn and corn producing Vip3Aa.

**Plant Bioassays.** We conducted plant bioassays during November 2022 in a greenhouse at the Chinese Academy of Agricultural Sciences in Beijing. Seeds were obtained from the DBN Group (Beijing) for corn producing Vip3Aa (DBN9501), Cry1Ab (DBN9936), or Cry1Ab + Vip3Aa (DBN9936 × DBN9501), and non-Bt corn (Nonghua106). Corn was grown with 5 plants per pot in a greenhouse at about 26 °C. We transferred first instar larvae to corn when the plants were about 30 cm high and had four leaves and one shoot. We measured survival until adult eclosion. For each type of corn and each insect strain (DH-R and DH-S), we conducted 3 replicates with 72 larvae per replicate (n = 216 larvae for each combination of corn and insect strain).

**Inheritance of Vip3Aa Resistance.** To evaluate the inheritance of Vip3Aa resistance in strain DH-R, we allowed 30 virgin male moths from DH-R to mate with 30 virgin female moths of the susceptible strain DH-S. We also performed the reciprocal cross with 30 per strain. The susceptibility to Vip3Aa toxins of both strains and their $F_1$ progeny was tested at 0.4 μg Vip3Aa per $cm^2$ diet. We used Fisher's exact test to determine whether survival differed significantly between the progeny from the two reciprocal crosses. We calculated the dominance parameter *h* as: (Survival of $F_1$ – Survival of DH-S)/(Survival of DH-R – Survival of DH-S) (68).

**GWAS.**

***$F_2$ population construction.*** We conducted a single-pair cross between a DH-R female and DH-S male, then a mass cross with the $F_1$ progeny (30 females and 30 males) to obtain $F_2$ progeny.

***Phenotyping by bioassay.*** To record the phenotypes associated with the resistance traits, 200 larvae from the $F_2$ generation were reared on a diet treated with 0.4 μg Vip3Aa per $cm^2$ diet. After 7 d, we weighed each larva.

***DNA extraction.*** Genomic DNA was extracted from whole larvae using an EasyPure Genomic DNA kit (TransGen Biotech, Beijing, China) according to the manufacturer's recommendations.

***Genomic sequencing.*** A total of 148 individuals were used for sequencing. Libraries with an insert size of 250 bp were constructed, and paired-end reads were produced on the BGISEQ-500 platform at BGI-Shenzhen, China, following the manufacturer's procedures. All samples were sequenced with 20× depth.

***Read mapping and variant calling.*** Variant calling was carried out following the Genome Analysis Toolkit (GATK version 4.2.3) Best Practices (69). Raw reads of 200 bp were filtered using SOAPnuk (v1.5.6) software (70) with parameter '-n 0.1 -q 0.5 -l 12 -Q 2' and aligned to the *S. frugiperda* reference genome [ZJ version (71)] using BWA-MEM (72) with default parameters (version 0.7.17). The alignment bam files were then sorted, and PCR duplicates were marked using Picard (http://broadinstitute.github.io/picard/). We identified variants for every sample with the HaplotypeCaller module to obtain the genomic variant call format (GVCF) files. The GVCF files from the 148 individuals were consolidated into a single GVCF file, from which SNPs and small indels were identified using a joint calling approach. The SNPs and indels were further filtered using the following criteria: SNPs were filtered with "QD <2.0||MQ<40.0||FS>60.0||MQRankSum <−12.5||ReadPosRankSum <−8.0" and indels with "QD < 2.0 || FS > 200.0 || SOR >10.0 || InbreedingCoeff <−0.8 || ReadPosRankSum <−20.0"; variants with a missing rate of >10% or a minor allele frequency of <0.05 were removed, resulting in a set of 9,251,345 filtered SNPs and a set of 3,552,978 indels for further analysis. The SNPs were annotated using SnpEff (73) (version4.3).

***GWAS analysis.*** Phenotypic data consisted of the weight of each larva after 7 d on diet treated with Vip3Aa. GWAS was performed on the LD-pruned SNP (or indel) set using the linear mixed model (LMM) in the program genome-wide efficient mixed model association (GEMMA) (74) (version 0.98.1). Kinship of samples was calculated and scaled for the relatedness matrix using the setting -gk2 in GEMMA. The entire analysis was done with the kinship correction. A Wald test was used to calculate the significance (-lmm1) with the thresholds of 0.05 divided by the number of independent SNPs (or indels). LD pruning was performed with PLINK (75) (version 1.9) using a window size of 10 kb and an $r^2$ threshold of 0.5. We used the Wald test to calculate significance and set the probability threshold as 0.05 divided by the number of samples: For SNPs, $P < 1.77 \times 10^{-6}$; for indels, $4.87 \times 10^{-6}$. The threshold of $r^2$ for LD was 0.5.

**Fine-Scale Mapping of Resistance.** For fine-scale mapping of resistance, we generated a second $F_2$ segregation generation. As described above, we started with a mass cross between a single female from DH-R and a single male from the DH-S strain. The $F_2$ segregating population was obtained by crossing $F_1$ population. The progeny of $F_2$ population were put on diet treated with 0.5 μg Vip3Aa per $cm^2$ diet. Ninety-six survivors were selected and named $F_2$-R. Genomic DNA was extracted individually using a Multisource Genomic DNA Miniprep Kit (Axygen, New York, USA) according to the manufacturer's recommendations.

Based on the SNPs calculated from the genomic resequencing data and the reference genome data, we designed primers specific for the exons of functional genes spanning 10.01 to 10.63 Mb on chromosome 27. PCR amplification and sequencing were performed for parent moths. SNPs that were homozygous in DH-S and different homozygous SNPs in DH-R were selected as informative markers. To evaluate genetic linkage with resistance for each marker, we used the chi-square test to check for significant deviation between the observed and expected genotype frequencies (*rr*:*rs*:*ss* = 1:2:1).

**RNA-Seq of Larval Midguts.** From midguts of fourth instar larvae of DH-R and DH-S reared on untreated diet, we extracted total RNA using the TRIzol reagent kit (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. After extraction, the quality of RNA was assessed using an Agilent 2100 Bioanalyzer and checked using RNase-free agarose gel electrophoresis. For each strain, we performed three replicates with 24 midguts per replicate.

Sequencing libraries were generated using the NEBNex1 Ultra RNA Library Prep Kit (NEB, Ipswich, MA, USA). The libraries were sequenced on the HiSeq 2500 sequencing platform at Gene Denovo Biotechnology Co. (Guangzhou, China).

The raw reads were cleaned by moving adapters, low-quality reads, and reads containing poly-N. The rRNAs were removed by filtering with Bowtie (76). The clean reads were mapped to the reference genome version ZJU_Sfru_1.0 GCF_011064685.1 by HISAT (77) (version 2.2.4). Gene expression levels of genes were estimated using RNA-seq by expectation-maximization (RSEM) (78) and normalized using FPKM (fragments per kilobase of transcript per million mapped reads). Differentially expressed genes were identified as (|log2 (fold-change)| ≥1.5 with $q < 0.05$) using the DESeq2 (79) (version 3.16).

For each of the nine genes from 10.29 to 10.48 Mb on chromosome 27 (*SI Appendix*, Table S5), we calculated the relative mRNA abundance for DH-R and DH-S using FPKM. We used the Wald test with the false discovery rate method to adjust the *P* value for multiple tests (79). We also used the same approach to compare mRNA abundance between DH-R and DH-S for six genes previously reported to affect responses to Vip3Aa in *S. frugiperda* (*SI Appendix*, Table S7).

**Genetic Linkage Analysis of Reduced Expression of *SfMyb*.** To generate a BC family to determine whether reduce *SfMyb* expression is genetically linked with resistance, a DH-R male was crossed with a DH-S female to generate $F_1$ progeny. Then, $F_1$ male moths were crossed with DH-R female moths to create a BC family. The progeny from the BC family were either fed artificial diet or artificial diet amended with a diagnostic dose (0.5 μg/$cm^2$) of Vip3Aa toxin. For 20 survivors on treated diet and 20 survivors on untreated diet, the midgut was removed and RNA was extracted from it as described above.

First-strand cDNA was synthesized using 1 μg of total RNA and TransCript One-step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Primers were designed using Primer Premier 5 software, and qRT-PCR was performed on a StepOne Plus system with TransStart Tip Green qPCR SuperMix (TransGen Biotech) as described before (80). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the β-actin gene.

**RNAi to Reduce Expression of *SfMyb* Gene in Susceptible Strain DH-S.** We used RNAi to determine whether reduced expression of *SfMyb* decreases *S. frugiperda* susceptibility to Vip3Aa. dsRNA of *SfMyb* was prepared in vitro using the T7 High Yield RNA Transcription Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Primers for the dsMyb templates were designed (Primer-F: TAATACGACTCACTATAGGGGCCTACGAAGATGTCCAG; Primer-R: TAATACGACTCACTATAGGGGGAGTTCCAATGGTTCTTTA; underlined T7 promoter sequences).

After quality and concentration of the RNA were assessed, we injected approximately 2.5 μg dsRNA into third instar larvae of DH-S using 3.5 Drummond needles and a Nanoinject III (Drummond Scientific, USA). dsGFP was injected as a control. Each treatment group consisted of 18 larvae with three replicates (total of 54 larvae per treatment). The injected larvae were reared individually in wells of a 24-well plate. After 24 h to recover from injection, the larvae were tested in diet incorporation bioassays with 2 μg Vip3Aa per mL diet. We used qPCR to measure the abundance of *SfMyb* mRNA 24 and 48 h after the injections. The larvae were

weighed three times: immediately before transfer to the diet containing Vip3Aa diet (M1), 24 h later (M2), and 48 h later (M3). We calculated relative growth rate after 24 h as (M2 – M1)/M1 and after 48 h as (M3 – M1)/M1. We used *t* tests to to compare the effects of dsGFP vs. dsMyb on abundance of *SfMyb* mRNA and relative growth at 24 and 48 h.

**CRISPR/Cas9 KO of *SfMyb* Gene in Susceptible Strain DH-S.**

***Design and synthesis of sgRNA.*** The sgRNA against the *SfMyb* gene was designed using the sgRNAcas9 design tool (81). The sgRNA target sequence (5′-GGCCGCGGAA GAATATTAATAGG-3′, PAM sequence is underlined) was selected in exon 2 of the *SfMyb* gene. The selected sgRNA target sequence was checked in a search of the *S. frugiperda* genome (https://bipaa.genouest.org) and GenBank database using sgRNAcas9 design tool, and no potential off-target sites were found. We used the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) to synthesize the sgRNA, the crRNA/tracrRNA constant region (80 nt) was contained in the kit. Then, the full-length gRNA DNA template was generated using PCR, with a reaction mixture containing 12.5 μL of Phusion High-Fidelity PCR Master Mix, 1 μL Tracr Fragment + T7 Primer Mix, 1 μL 0.3 μmol/L Target F/R oligonucleotide mix, 10.5 μL nuclease-free water. The thermal cycling program was 98 °C 10 s, 32 cycles of (98 °C 10 s, 60 °C 30 s, and 72 °C 15 s), and 72 °C 10 min. The in vitro transcription was also performed with the kit mentioned above according to the manufacturer's instructions.

***Embryo collection and microinjection.*** Freshly laid eggs (within 2 h after oviposition) from DH-S were washed using 1% (v/v) sodium hypochlorite solution and rinsed with distilled water. The eggs were placed on a microscope slide and fixed with double-sided adhesive tape. About 1 nL of a mixture of sgRNA (300 ng/μL) and Cas9 protein (50 ng/μL) was injected into individual eggs using Nanoject III (Drummond, Broomall, PA, USA). Cas9 protein (GeneArt Platinum Cas9 Nuclease) was purchased from Thermo Fisher Scientific (Shanghai, China).

***gDNA isolation and mutagenesis detection.*** Genomic DNA from exuviates, larvae, and moths were extracted using the Multisource Genomic DNA Miniprep Kit (Axygen, New York, USA) according to the manufacturer's recommendations. Detecting primers were designed flanking the CRISPR target sites (Primer-F: TAGCGGCTATGATTCAGAGACG; Primer-R: CTCTTTCGTCCAAGGGC CT). The PCR conditions were 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s; and 72 °C for 10 min. PCR products were directly sequenced and double sequencing peaks starting from the PAM sequences, which indicated a mutation event, were identified. PCR products were then cloned into the T3 Vector and sequenced by Sangon Biotech (Shanghai, China).

**Comparison of SfMyb Promoter Activity between DH-R and DH-S.** To compare the promoter activity of the candidate gene *SfMyb* between strains DH-R and DH-S, the promoter fragments of *SfMyb* were separately amplified from the two strains using primers designed based on the genomic data. The PCR products were digested with restriction enzyme and inserted into plasmid pGL3-Basic (Promega). The start codon (ATG) was mutated to TTG. A modified *Renilla* luciferase reporter gene pRL-OPIE2 was used as a control to normalize gene activity as described before (82). Then, $1.5 \times 10^5$ cells of the *Sf9* cells were cultured in 48-well tissue culture plate wells for 12 h at 28 °C. The cells were transfected by adding 200 μL FBS-free medium containing the transfection reagent FuGENE HD (1 μL) and plasmid (250 ng). Luciferase activity was analyzed 36 h post transfection using a Lumat luminometer LB 9501/16 (Berthold Systems, Aliquippa, PA) according to the manufacturer's instructions (Dual luciferase, Promega, WI, USA). Three independent trials were done. We used 2-way ANOVA to test for the effects of trial, strain (DH-R vs. DH-S), and their interaction.

**Yeast One-Hybrid Assay.**

***Construction of the prey library with insertion of random seven-nucleotide DNA sequences.*** We conducted a yeast one-hybrid assay in ProNet Biotech Co., Ltd using the Matchmaker One-Hybrid System. To construct a random DNA insertion prey library in the plasmid pHIS2, two single-strand DNA sequences were synthesized by Shanghai Sangon Biotechnology (China), P1: TGTAAAACGA CGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTCCCNNNNNNNGGGAGCTC ACGCGTTCGCGAATCGATCCGCGGTCTAGAAATTCCTGGCATTATCACATAATG and P2: CATTATGTGATAATGCCAGG. The seven underlined N's represent the random DNA sequences of seven nucleotides each (called motifs) that we tested for binding to the SfMyb transcription factor. DNA synthesis was carried out using P1, P2, 10 × PCR buffer, and Taq with sterile distilled water to a total volume of 20 μl. The PCR reaction conditions were 94 °C for 3 min then 68 °C for 3 min. The pHIS2 vector was Sma I digested and purified by agarose gel electrophoresis. The linearized pHIS2 (0.5 μg per μL) together with 2.0 μg of PCR reaction solution and 600 μL of competent Y187 yeast cells were gently mixed by vortexing and 1.5 mL polyethylene glycol/lithium acetate solution was added. The transformation process was done according to the user manual of Yeastmaker Yeast Transformation System 2 (Clontech). Next, the transformed cells were grown on SD/-His/-Leu/-Trp medium supplied with 30 mM 3-AT at 30 °C. After the colonies grew for 14 h, the plates were washed with 10 mL PDA buffer. We used the buffer containing the material washed from the plates to establish the prey library.

***Construction of the bait vector.*** To construct the bait vector, the coding sequence of SfMyb was synthesized by Genescript Corporation (Nanjing, China) and cloned into vector pGADT7-Rec2-ERF2 using the yeast recombination method. The recombinant pGADT7-Rec2-ERF2 gene was isolated from yeast cells using an Easy Yeast Plasmid Isolation Kit (Clontech).

***Screening the prey library.*** The recombinant vector pGADT7-Rec2-ERF2 was transformed into the yeast strain harboring the random DNA insertion prey library using Yeastmaker Yeast Transformation System 2 (Clontech). The positive clones were initially selected on the SD/-His/-Leu/-Trp medium with 30 mM 3-AT and further selected on the concentration series of 3-AT selection media (0, 10, 20, 30, 40, 50, 75, and 100 mM) to identify the clones that had high binding affinity. The primary cultures of yeast transformants were transferred to fresh medium and grown to about 0.6 at OD600 nm before spotting. The positive transformants were further confirmed by spotting yeast cells onto SD/-His/-Leu/-Trp medium supplied with 75 mM 3-AT. The transformants grown at SD/-Leu/-Trp were used as growth-positive controls.

**Analysis of the Insertion Sequences of Positive Clones.** The pHIS2 plasmids were rescued from the positive clones and sequenced. This yielded 30 seven-nucleotide insertion sequences that bound the SfMyb transcription factor in the yeast one-hybrid assay (*SI Appendix*, Table S7). Analysis of these 30 motifs using CIS-BP database (http://cisbp.ccbr.utoronto.ca/TFTools.php) identified 12 motifs that are known to bind to promoters of *D. melanogaster*.

**Identification of Target Genes of the SfMyb Transcription Factor That Are Candidates for Conferring Resistance to Vip3Aa.** We scanned the *S. frugiperda* genome version ZJU_Sfru_1.1 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_011064685.2/) to determine which promoters (2 kb upstream of the transcription start site) contain each of the 12 identified motifs. We considered the genes with one or more of these motifs potential target genes of the SfMyb transcription factor. We used four criteria to screen for the potential target genes most likely to be associated with resistance:

1) Significant difference between DH-R and DH-S in the abundance of corresponding mRNA in larval midguts based on RNA-Seq
2) Significant difference between DH-S larvae treated with dsMyb vs. dsGFP (control) in the abundance of mRNA in larval midguts based on RNA-Seq
3) The differences in 1) and 2) are in the same direction: downregulation in DH-R vs. DH-S and in DH-S larvae treated with dsMyb vs. dsGFP, or upregulation in both comparisons; and
4) Previously reported results (57) indicate the corresponding protein occurs in DH-S larval midguts (Unique peptide score greater than or equal to one).

In both 1) and 2), we used adjusted probability values that account for multiple comparisons (83).

**Comparing Responses to RNAi of DH-S Larvae Treated with dsMyb vs. dsGFP.** As described above, we synthesized dsRNA (dsMyb or dsGFP as the control) using the T7 High Yield RNA Transcription Kit and injected approximately 2.5 μg dsRNA into each larva (*RNAi to Reduce Expression of SfMyb Gene in Susceptible Strain DH-S*). We conducted three replicates with 12 larvae injected with dsRNA in each treatment and subsequently analyzed with RNA-Seq (total of 36 larvae injected with dsMyb and 36 with dsGFP). As described above, 2 d after injection, total RNA was extracted using the TRIzol reagent kit, libraries were constructed, and RNA-Seq data were analyzed (*RNA-Seq of Larval Midguts*). Confirming the RNAi was effective, the mean relative abundance of *SfMyb* mRNA was half as much in the dsMyb treatment (0.55) compared to the controls treated with dsGFP (1.1) (*t* test, $t = 7.6$, df = 4, $P = 0.0016$).

**Predicted Subcellular Localization of Proteins Encoded by Candidate Genes.** We used WoLF PSORT (https://wolfpsort.hgc.jp/) to predict where proteins encoded by candidate genes would occur among the following six sites: cytoplasm, endoplasmic reticulum, extracellular, mitochondria, nucleus, and plasma membrane.

**Data, Materials, and Software Availability.** The authors declare that the data supporting the findings of this study are available within the paper and *SI Appendix*. The sequence reported in this paper has been deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) database (accession no. PRJNA893082) (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA893082/) (84).

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