MAPK-activated transcription factor PxJun suppresses PxABCB1 expression and confers resistance to Bacillus thuringiensis Cry1Ac toxin in Plutella xylostella (L.)

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Running head: PxJun regulates Cry1Ac resistance in *P. xylostella*

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Abstract

Deciphering the molecular mechanisms underlying insect resistance to Cry toxins produced by *Bacillus thuringiensis* (Bt) is pivotal for the sustainable utilization of Bt biopesticides and transgenic Bt crops. Previously, we identified that MAPK-mediated reduced expression of the *PxABCB1* gene is associated with Bt Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.). However, the underlying transcriptional regulation mechanism remains enigmatic. Herein, the *PxABCB1* promoter in Cry1Ac-susceptible and Cry1Ac-resistant *P. xylostella* strains was cloned and analyzed and found to contain a putative Jun binding site (JBS). A dual-luciferase reporter assay and yeast one-hybrid assay (Y1H) demonstrated that the transcription factor PxJun repressed *PxABCB1* expression by interacting with this JBS. The expression levels of *PxJun* were increased in the midguts of all resistant strains compared to the susceptible strain. Silencing of *PxJun* expression significantly elevated *PxABCB1* expression and Cry1Ac susceptibility in the resistant NIL-R strain, and silencing of *PxMAP4K4* expression decreased *PxJun* expression and also increased *PxABCB1* expression. These results indicate that MAPK-activated PxJun suppresses *PxABCB1* expression to confer Cry1Ac resistance in *P. xylostella*, deepening our understanding of the transcriptional regulation of midgut Cry receptor genes and the molecular basis of insect resistance to Bt Cry toxins.

Importance

The transcriptional regulation mechanisms underlying reduced expression of Bt toxin receptor genes in Bt-resistant insects remain elusive. This study unveils that a

transcription factor PxJun activated by the MAPK signaling pathway represses *PxABCB1* expression and confers Cry1Ac resistance in *P. xylostella*. Our results provide new insights into the transcriptional regulation mechanisms of midgut Cry receptor genes and deepen our understanding of the molecular basis of insect resistance to Bt Cry toxins. To our knowledge, this study identified the first transcription factor that can be involved in the transcriptional regulation mechanisms of midgut Cry receptor genes in Bt-resistant insects.

***Keywords*:** *Bacillus thuringiensis*; *Plutella xylostella*; Transcription factor; Jun; ABCB1; Cry1Ac resistance

Introduction

The diverse insecticidal crystal proteins generated by *Bacillus thuringiensis* (Bt) can specifically kill various lepidopteran, coleopteran and dipteran insect pests without causing harm to non-target organisms or the environment; as such, Bt biopesticides and genetically modified Bt crops have been developed and widely used for pest control (1-3). Unfortunately, different insect pests in the field have developed high-level resistance to Bt sprays or Bt crops due to the resulting strong selection pressure, gravely threatening the sustainable application of Bt products (4, 5). Therefore, understanding the molecular mechanisms underlying insect resistance to Bt Cry toxins is crucial for the long-term utilization of these valuable Bt biotechnologies (6, 7).

As currently understood, the mode of action of Bt Cry toxins involves multiple steps that occur in the larval midgut, and the binding of Bt Cry toxins to functional receptors in the midgut is a key step in this complex toxicity process (8-10). The identified midgut receptors of Bt Cry toxins include cadherin (CAD), alkaline phosphatase (ALP), aminopeptidase N (APN) and ATP-binding cassette (ABC) transporters (i.e., ABCAs, ABCBs, ABCCs, ABCGs) (11, 12). The down-regulation of midgut Cry receptor genes to reduce toxin-receptor interactions is one of the principal drivers of Bt resistance evolution in diverse insects (13, 14). However, little is known about the transcription regulation mechanisms of these midgut Cry receptor genes.

77 ABCB1, also known as P-glycoprotein (PGP) or MDR1, is one of the
78 best-studied ABC transporters. ABCB1 can serve as a pump to efflux toxic
79 xenobiotics out of cells using ATP-driven energy, and up-regulation of the *ABCB1*
80 gene has been reported to be involved in multidrug resistance (MDR) in mammals (15)
81 and resistance to chemical and biological pesticides in insects (16-19). Moreover,
82 studies in recent years have found that ABCB1 can also serve as a functional midgut
83 receptor of Bt Cry3 toxins, and its gene mutation is associated with high-level
84 resistance to Bt Cry3 toxins in *Diabrotica virgifera virgifera* and *Chrysomela tremula*
85 (20-22). Meanwhile, we found that down-regulation of *ABCB1* is directly associated
86 with Cry1Ac resistance in *P. xylostella* (23, 24). Although research on the
87 transcriptional regulatory mechanisms of *ABCB1* expression has made great progress
88 in mammals (25, 26), little is known about this phenomenon in insects.

89 Recently, our studies have shown that the insect hormone-activated
90 mitogen-activated protein kinase (MAPK) signaling pathway can act as a common
91 switch to *trans*-regulate the differential expression of multiple midgut genes,
92 including ALP, APNs (*APN1*, *APN3a*, *APN5* and *APN6*) and ABC transporters
93 (*ABCB1*, *ABCC1*, *ABCC2*, *ABCC3* and *ABCG1*), thereby conferring Bt Cry1Ac
94 resistance in *P. xylostella* without fitness costs (14, 23, 24, 27, 28). The above
95 researches have also indicated that there is a significant level of redundancy in
96 receptors such that multiple proteins within a given insect can act as functional
97 receptors and that change of individual gene results in incremental increase in
98 resistance. The involvement of the MAPK cascades indicating that there are some

MAPK-responsive downstream transcription factors (TFs) that regulate the expression of these midgut genes, including *ABCB1* (29). Moreover, some studies have also found that TFs such as FOXA and GATAe can positively regulate the expression of some Bt Cry1Ac receptor genes, including *ABCC2*, *ABCC3*, *ALP* and *CAD,* in different insect cell lines (30, 31). Indeed, we have identified a MAPK-activated transcription factor *CREB* that can increase the expression of a P450 gene *CYP6CM1* thereby leading to imidacloprid resistance in *Bemisia tabaci* (32). Nonetheless, the TFs that regulate the reduction in expression of midgut receptor genes, including *ABCB1,* in insect resistance to Bt Cry toxins remain largely unknown.

Here, our work has shown that a TF called PxJun can directly bind to the promoter region of *PxABCB1* to reduce its expression. Furthermore, midgut *PxJun* expression was significantly higher in all the Cry1Ac-resistant strains than in the Cry1Ac-susceptible strain. Silencing of *PxJun* expression up-regulated *PxABCB1* transcription and enhanced larval susceptibility to Cry1Ac toxin in the Cry1Ac-resistant NIL-R strain, and silencing of *PxMAP4K4* expression inhibited *PxJun* and increased *PxABCB1* transcription. These results indicate that PxJun activated by the MAPK signaling pathway participates in Cry1Ac resistance in *P. xylostella* by inhibiting the expression of the *PxABCB1* gene. Our results provide important insights into the transcriptional regulation of Bt toxin receptors, providing a better understanding of the evolution of insect resistance to Bt Cry toxins.

Results

PxABCB1 promoter analyses in susceptible and resistant strains

Having previously observed that differential expression of *PxABCB1* associates with resistance to the Bt Cry1Ac toxin in *P. xylostella* (23, 24), we sought to compare the promoter region of this gene between a susceptible and resistant strain in order to investigate possible reasons for this differential expression.

We amplified the 5′-flanking region of the *PxABCB1* gene from gDNA samples of the Cry1Ac-susceptible strain DBM1Ac-S and its near-isogenic Cry1Ac-resistant strain NIL-R. Analysis of the *PxABCB1* promoter sequence revealed a conserved initiator of transcription site (Inr) motif 5′-TCAGT-3′ located 127 nucleotides (nt) upstream of the start codon (ATG) of the *PxABCB1* gene. The adenine (A) of the Inr was designed as the transcriptional start site (TSS) and marked as "+1" (Fig. S1). With respect to promoter identification no typical TATA box was found near the TSS, although a putative CAAT box was predicted 139 nt upstream of the TSS (Fig. S1). Nucleotide sequence alignment showed that multiple single nucleotide polymorphism (SNP) sites existed in the *PxABCB1* promoters of the two strains and that the *PxABCB1* promoter in the NIL-R strain contained a number of deletions as well as a small insertion compared with that in the DBM1Ac-S strain (Fig. 1A and Fig. S1).

To investigate whether the differences in the 5′-flanking region sequences between DBM1Ac-S and NIL-R strains lead to differences in promoter activity, and thus cause differential expression of the *PxABCB1* gene, a transcriptional reporter

assay was employed. Three full-length and truncated promoters to reflect regions of similarity and dissimilarity between the two strains were cloned into the pGL4.10 vector to drive the expression of the luciferase reporter gene (Fig. 1). The relative luciferase activities of these *PxABCB1* promoter recombinant plasmids were detected in S2 cells at 48 h post-transfection. The results indicated that there was no significant difference in activity for any of the *PxABCB1* promoter regions (Fig. 1B), suggesting that genomic differences were not responsible for the altered expression of *PxABCB1* in the resistant strain. What was noticeable however was that the promoter activity was significantly reduced when the section between -280 and +125 was used, despite this containing the putative CAAT box promoter motif (Fig. 1B and Fig.S1).

Identification of the critical regulatory regions in the *PxABCB1* promoter

To investigate the loss of promoter activity associated with the -280/+125 region, we created a further range of constructs containing decreasing amounts of the promoter region from the DBM1Ac-S strain (Fig. 2). The data from these constructs confirmed that promoter activity was reduced once sequences upstream of -460 were removed. Interestingly though, the promoter activity was partially restored when the deletion extended as far as -72 (Fig. 2). Based on the luciferase activity data, these two important regulatory regions in *PxABCB1* promoter from -621 to -460, and from -112 to -72 likely contain critical positive and negative regulatory elements, respectively, suggesting that they could potentially control *PxABCB1* expression

163 through a combination of *cis*-regulatory elements and *trans*-acting factors (33).

164

165 **The transcription factor PxJun represses *PxABCB1* promoter activity**

166 Informatic analyses were undertaken to identify putative transcription factor (TF)
167 binding sites. Potential sites for CncC, Vvl and Ubx were predicted in the positive
168 regulatory region and the binding sites of Jun and Ubx were predicted in the core
169 negative regulatory region (Fig. 3A and Fig. S1). To explore whether these proteins
170 were involved in the transcriptional regulation of the *PxABCB1* gene, the full-length
171 coding region of each TF was cloned by PCR amplification and subcloned into the
172 expression vector pAc5.1. They were then co-transfected into S2 cells along with the
173 luciferase reporter plasmid bearing the full *PxABCB1* promoter region. The
174 co-transfection assays revealed that PxJun significantly decreased the activity of the
175 *PxABCB1* promoter compared with the control, while other TFs had little effect on
176 promoter activity (Fig. 3B), suggesting that PxJun might be involved in the negative
177 regulation of *PxABCB1* expression through interacting with the Jun binding site
178 (JBS).

179

180 **PxJun interacts with the JBS to repress *PxABCB1* promoter activity**

181 To confirm that PxJun inhibits promoter activity through the predicted JBS between
182 -112 and -72 (Fig. 3A), recombinant plasmids bearing *PxABCB1* promoters
183 containing a JBS deletion or mutation were constructed and co-transfected with the
184 PxJun plasmid into S2 cells. The luciferase activity of the *PxABCB1* promoter

containing normal JBS was significantly decreased by PxJun, whereas the activities of the JBS deleted or mutated promoters showed no change compared with the control (Fig. 4A). These results suggested that PxJun repressed *PxABCB1* promoter activity mainly through the identified JBS. Subsequently, a yeast one-hybrid (Y1H) assay was carried out to further test the interaction between PxJun and the JBS. The yeast strain containing the PxJun prey vector and normal JBS bait grew normally on SD/-Leu medium supplemented with 500 ng/mL AbA, while the yeast strain containing the PxJun prey vector and mutant JBS-M bait did not grow (Fig. 4B). This result indicated that the PxJun prey plasmid interacted with the JBS but not the JBS-M bait. Together, the above results confirmed that PxJun suppressed the promoter activity of the *PxABCB1* gene by interacting with the JBS.

Analysis of the PxJun protein and its possible interaction with PxFos

The PxJun protein contains two characteristic domains: the Jun domain at the N-terminus and the bZIP_Jun domain at the C-terminus (Fig. S2). The bZIP_Jun domain consists of a basic DNA-binding domain (DBD) for specific DNA recognition and binding, followed by a leucine zipper domain for protein dimerization (Fig. S3) (29). Both domains are conserved among different insects and mammals (Fig. S2 and Fig. S3). We performed a phylogenetic analysis of PxJun to determine the evolutionary relationships among c-Jun proteins in different species (Fig. S2). The results showed that the c-Jun proteins are evolutionarily conserved and clearly clustered into groups corresponding to each insect order; as expected, the

mammalian c-Jun proteins also grouped into one cluster (Fig. S2).

Jun-related subfamily members typically interact with themselves or other proteins, and function as dimers in their regulatory role, and Fos subfamily proteins are the most common co-factors (34, 35). Thus, we aimed to further explore whether the PxFos protein is also involved in the regulation of *PxABCB1* transcription with PxJun. A recombinant plasmid encoding PxFos with or without PxJun was co-transfected with the *PxABCB1* promoter into S2 cells to measure the activity of the luciferase reporter. Transfection of PxFos alone or in combination with PxJun had no significant effect on the activity of the *PxABCB1* promoter (Fig. 5), indicating that the PxFos protein did not participate in the regulation of the *PxABCB1* gene.

Increased expression levels of the *PxJun* gene in resistant strains

The spatio-temporal transcription profiles of the *PxJun* gene were monitored by qPCR in different tissues of fourth-instar DBM1Ac-S larvae and different developmental stages. The tissue expression profile showed that the *PxJun* gene was widely expressed in different tissues with no obvious tissue-specific expression pattern, implying that the *PxJun* gene plays important roles in a variety of tissues (Fig. S4). Meanwhile, developmental expression analysis indicated that the *PxJun* gene was also expressed in different periods with no obvious stage-specific expression pattern (Fig. S4), suggesting that the *PxJun* gene is involved in the regulation of growth, development and reproduction in *P. xylostella*.

229 To further investigate the relationship between *PxJun* and *PxABCB1*, the
230 transcript levels of *PxJun* were detected in the midgut tissues of fourth-instar larvae
231 of different *P. xylostella* strains. As indicated by the qPCR results, the expression
232 levels of the *PxJun* gene were significantly higher in all four Cry1Ac-resistant
233 strains than in the susceptible DBM1Ac-S strain (Fig. 6). The expression trend of
234 *PxJun* was negatively correlated with the *PxABCB1* expression trend in the midgut
235 tissues of different strains (detected in our previous study) (23), whereas it was
236 positively correlated with Cry1Ac resistance level, supporting the concept that *PxJun*
237 promotes Cry1Ac resistance by repressing *PxABCB1* expression.
238
239 **Silencing *PxJun* enhances *PxABCB1* expression and susceptibility to Cry1Ac**
240 **toxin**
241 To validate whether *PxJun* depresses *PxABCB1* expression in *P. xylostella*,
242 *PxJun*-specific dsRNA was synthesized and injected into third-instar NIL-R larvae.
243 The transcription levels of *PxJun* and *PxABCB1* were measured by qPCR after 48 h.
244 The expression of *PxJun* was reduced by approximately 50% in the midguts of
245 dsRNA-injected larvae compared with controls; by contrast, the transcriptional level
246 of *PxABCB1* in their midguts was significantly increased compared to that of
247 controls (Fig. 7A). In addition, toxicity bioassays with 1000 mg/L Cry1Ac protoxin
248 were carried out at 48 h post-injection. The mortality of the *PxJun*-silenced larvae
249 was dramatically increased (from 10% to 35.56%) (Fig. 7B). These data again
250 supported the role of PxJun in negatively controlling the expression of *PxABCB1 in*

vivo, contributing to Cry1Ac resistance in *P. xylostella*.

The MAPK signaling pathway activates *PxJun* transcription

Jun subfamily proteins are typical downstream targets of the MAPK signaling pathway and can be activated at the transcriptional and protein phosphorylation levels in mammals (34, 36, 37). Our previous studies revealed that the insect hormone-activated MAPK signaling pathway regulates the reduced expression of multiple midgut receptors including *PxABCB1* gene in resistant *P. xylostella* strains (24). Thus, we speculated that the decrease in *PxABCB1* expression might be controlled by MAPK-responsive *PxJun*. To investigate whether *PxJun* expression leading to the down-regulation of *PxABCB1* is induced by the MAPK signaling pathway, we silenced *PxMAP4K4* expression in resistant NIL-R larvae and then detected the expression levels of *PxJun* and *PxABCB1* at different periods (Fig. 8). The results reflected that after PxMAP4K4 dsRNA injection, the transcript level of PxJun decreased, while the expression level of *PxABCB1* increased (Fig. 8).Therefore, PxJun responded to MAPK signaling pathway and repressed the expression of the *PxABCB1* gene in Cry1Ac-resistant *P. xylostella*.

Discussion

Down-regulation of Bt Cry toxin receptor genes in the midgut usually results in high-level Bt resistance in insects; nevertheless, the specific transcriptional regulation mechanisms of these midgut genes remain poorly understood. Changes in gene expression can result from changes in *trans*-regulatory factors and *cis*-regulatory elements (TFBS in promoter, enhancer and silencer) (38). In this study, we revealed that the MAPK-activated TF PxJun regulates the reduced expression of the *PxABCB1* gene, thereby mediating Bt Cry1Ac resistance in *P. xylostella*.

Jun subfamily members in mammals include c-Jun, JunB and JunD, important stress-responsive TFs that are activated by the MAPK signaling pathway under various physiological and external stimuli and participate in multiple cellular processes, such as cell proliferation, differentiation, apoptosis and inflammation (34, 39). Intriguingly, whereas there are three Jun paralogs in mammals, only one Jun protein, a homolog of the mammalian c-Jun, has been identified in insects (36, 40). As in mammals, the MAPK signaling pathway in *D. melanogaster* can activate DJun in response to various stimuli to ensure normal development, immunity and homeostasis (37, 41).

Jun-related subfamily members belong to the basic leucine zipper (bZIP) family of proteins, which typically exert their functions through homodimerization or heterodimerization to form an AP-1 complex (34, 35). Jun proteins in animals participate in essential biological processes, including cell proliferation, differentiation, apoptosis and immune response (39). *PxJun* is a homolog of

mammalian *c-Jun*. We demonstrated that PxJun can specifically bind to the JBS and inhibit the promoter activity of the *PxABCB1* gene. In mammals, activated c-Jun can act as an activator or repressor to control *MDR1* (*ABCB1*) expression in different cancer cells (25, 42-45). Therefore, c-Jun appears to play a dual regulatory role in the regulation of *ABCB1* expression in different types of cells and species, probably depending on its binding partners and on the cellular environment.

In mammals, the AP-1 complexes Jun-Jun and Jun-Fos preferentially bind to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE, 5'-TGA(C/G)TCA-3'), while Jun-ATF preferentially binds to the cAMP response element (CRE, 5'-TGACGTCA-3') (35, 39). Distinct cofactors of Jun have different effects on the DNA binding affinity and function of the dimer, thus greatly expanding the spectrum of regulated genes (34). Our structural analysis of the PxJun protein showed a highly conserved bZIP_Jun domain for DNA binding and protein interaction at the C-terminus. Fos is the most common partner of Jun (34, 35). However, we demonstrated that the PxFos protein did not participate in the regulation of the *PxABCB1* gene. Thus, further study is needed to identify whether additional TFs interact with PxJun to co-regulate *PxABCB1* expression in the midgut, which will help us understand the complex functions of AP-1.

Mammalian AP-1 family members are expressed in a cell- and stage-dependent manner during development and under different stimuli, thereby mediating different transcriptional levels of specific target genes (34). In *D. melanogaster*, *DJun* expression is observed in all tissues and developmental stages (46). Similarly, we

found that the *PxJun* gene is extensively expressed in multiple tissues and developmental stages of *P. xylostella*. The temporally and spatially broad expression pattern of c-Jun implies its essential role in maintaining immunity and homeostasis, normal growth and development, and response to various stress factors (35, 39, 47).

Jun subfamily proteins in mammals are activated both at the transcriptional level and via post-translational modification by the MAPK signaling pathway in response to many physiological and environmental stimuli, affecting the transactivation potential, DNA binding capacity and stability of AP-1 components (34, 36, 37). Studies in human cells found that c-Jun can be activated by the MAPK signaling pathway, leading to elevated transcript and phosphorylation levels and thus regulating the expression of the *MDR1* (*ABCB1*) gene (43, 44, 48, 49). Silencing of *PxMAP4K4* expression down-regulated *PxJun* and up-regulated *PxABCB1*, demonstrating that the expression of the *PxABCB1* gene is negatively regulated by the MAPK-responsive PxJun in *P. xylostella*. Persistent alteration of AP-1 activity and/or *ABCB1* expression can induce the oncogenic transformation of cells and tumor formation, as well as the development of chemo-resistance in mammals (39). In fact, the expression trend of *PxJun* in midgut tissues of different *P. xylostella* strains is similar to that of *PxMAP4K4* and opposite to that of *PxABCB1* (23, 24, 27). Thus, a model explaining the transcriptional regulation mechanisms of reduced expression of the *PxABCB1* gene is shown in Fig. 9. In the Bt-resistant *P. xylostella* larvae, the activated MAPK signaling pathway can induce *PxJun* transcription thereby leading to reduction of *PxABCB1* gene expression and causing Cry1Ac

resistance. Therefore, the MAPK/c-Jun signaling cascade might be a common mechanism of transcriptional regulation of the *ABCB1* gene. Since the MAPK pathway has been shown to regulate the expression of a number of Cry1Ac receptors within *P. xylostella*, it is possible that PxJun is directly involved in the downregulation of other receptors and therefore that the increase in mortality observed in Fig. 7B may not be solely attributed to *PxABCB1* overexpression. Further research is needed to evaluate whether the MAPK signaling pathway activates PxJun at both the transcript and phosphorylation levels, and whether PxJun also participates in the regulation of other receptors.

In conclusion, we found that PxJun negatively regulates *PxABCB1* expression by interacting with the JBS in the *PxABCB1* promoter, and the activated MAPK cascade enhances *PxJun* expression and thus represses *PxABCB1* expression to result in Cry1Ac resistance in *P. xylostella*. This study provides new insights into the transcriptional regulation mechanisms of midgut receptor genes and lays the foundation for comprehensively understanding the complex molecular mechanisms of insect resistance to Bt Cry toxins.

Material and methods

Insect strains and cell line

The five *P. xylostella* strains used in this study, including one Bt-susceptible and four Bt-resistant strains, were described previously (27, 50, 51). In brief, the DBM1Ac-S strain has been maintained in the laboratory without exposure to any Bt products/toxins or any other insecticides. The four Bt-resistant strains, DBM1Ac-R, NIL-R, SZ-R and SH-R exhibit different levels of resistance to Bt Cry1Ac protoxin or Bt *var. kurstaki* (Btk) formulation. Their resistance ratios are approximately 3500, 4000, 450, and 1900 folds that of the susceptible DBM1Ac-S strain, respectively. The larvae of all strains were reared on Jing Feng No. 1 cabbage (*Brassica oleracea* var. *capitata*) at 25 °C, 65% relative humidity (RH) and a 16:8 (light:dark) photoperiod. Adults were supplied with a 10% honey/water solution.

The *Drosophila melanogaster* S2 cell line was transfected for the dual-luciferase reporter assay. S2 cells were cultured in HyClone SFX-insect medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin-streptomycin (Gibco, Rockville, MD, USA) at 27 °C.

Toxin preparation and toxicity bioassay

The Cry1Ac protoxin preparation and subsequent leaf-dip bioassay were performed as described previously (52). Briefly, the Cry1Ac protoxin was isolated and purified from Btk strain HD-73, and its protein concentration was quantified and stored in 50 mM Na_2CO_3 (pH 9.6) for subsequent toxicity bioassays. A three-day leaf-dip

bioassay was conducted using third-instar *P. xylostella* larvae. Ten individuals in each group were used, and the bioassays were repeated four times for each Cry1Ac concentration. The larval mortality of the control did not exceed 5%.

gDNA extraction, promoter cloning and TFBS prediction

Genomic DNA (gDNA) was isolated from fourth-instar *P. xylostella* larvae using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. A pair of specific PCR primers to amplify the *PxABCB1* promoter was designed based on the 5′-flanking sequence of the *PxABCB1* gene in the *P. xylostella* genome dataset of LepBase (http://ensembl.lepbase.org/Plutella_xylostella_pacbiov1/Info/Index). PrimeSTAR Max DNA Polymerase (TaKaRa, Dalian, China) was used according to the manufacturer's protocol for all PCR amplifications in this study. The PCR products were subsequently purified and ligated into pEASY-T1 vectors (TransGen, Beijing, China) for sequencing. Transcription factor binding sites (TFBSs) in the *PxABCB1* promoter region were *in silico* predicted via the JASPAR database (http://jaspar.genereg.net) and PROMO virtual laboratory (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Sample preparation, RNA isolation and cDNA synthesis

Samples at different developmental stages (eggs, first- to fourth-instar larvae, prepupae, pupae, 1-day-old virgin male and female adults) were collected from the

susceptible DBM1Ac-S strain, and five tissues (head, midgut, Malpighian tubules, integument and testis) were dissected from fourth-instar *P. xylostella* larvae in ice-cold insect Ringer's solution (130 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$).

Total RNA from different samples was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The first-strand cDNA used for subsequent gene cloning was synthesized using the PrimeScript II First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol.

TF cloning and sequence analysis

The initial open reading frames (ORFs) of *Jun*, *Fos*, *cap 'n' isoform C* (*CncC*) and *ultrabithorax* (*Ubx*) genes in *P. xylostella* were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/) under accession numbers XM_011559543, XM_011549928, XM_011570739 and KP245729, respectively. In addition, the ORF of the *ventral veins lacking* (*Vvl*) gene in *P. xylostella* was obtained from the LepBase (http://ensembl.lepbase.org/, Gene ID: g11291). All the ORFs of these genes were further corrected *in silico* with the assistance of our previous *P. xylostella* midgut transcriptome database (53). Using specific primers for PCR amplification (Table S1), the full-length coding sequences (CDSs) of PxJun (GenBank accession no. MW446637) and four other genes were cloned, and each obtained CDS was translated into amino acid sequences using the ExPASy translate tool (https://web.expasy.org/translate/). The presence of conserved domains in the

PxJun protein was analyzed using the Conserved Domain Database (CDD) at NCBI (https://www.ncbi.nlm.nih.gov/cdd/). Multiple sequence alignment was conducted using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and was further formatted using GeneDoc 2.7 software (http://genedoc.software.informer.com/2.7/).

Phylogenetic analysis

The Jun protein sequences used for alignment and phylogenetic analysis in insects and representative mammals were obtained from the GenBank database. A phylogenetic tree was built using MEGA 7.0 software (https://www.megasoftware.net/) with the neighbor-joining (NJ) method, "p-distance" model and 1000 bootstrap replicates.

Dual-luciferase assay

The 5′-flanking sequence of the *PxABCB1* gene was truncated into a series of fragments of different sizes. All the fragments were subcloned into linearized pGL4.10 vector (double digests with *Bgl*II and *Kpn*I) using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). The In-Fusion primers used for promoter amplification are listed in Table S1. In addition, full-length promoters with JBS mutation/deletion were obtained by gene synthesis (TsingKe, Beijing, China). The ORFs of TFs were ligated into linearized pAc5.1/V5-His B expression vector (hereinafter referred to as "pAc5.1", double digested with *Kpn*I and *Xho*I) using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). The In-Fusion

primers used for TF amplification are listed in Table S2. The pGL4.73 vector (Promega, Madison, WI, USA) containing a *Renilla* luciferase gene was used as an internal control.

Vector transfection was performed using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). S2 cells were seeded at a density of 5×10^5 cells per well in 400 µL medium without antibiotics in a 24-well plate 6 h before transfection. To detect promoter activity, the pGL4.10-promoter reporter plasmid (600 ng) was co-transfected with pGL4.73 (200 ng), and the empty pGL4.10 vector was used as the control vector. To determine the effect of TF on promoter activity, the pAc5.1-TF expression plasmid (600 ng) and pGL4.10-promoter reporter plasmid (200 ng) were co-transfected with pGL4.73 (100 ng), and the empty pAc5.1 plasmid was used as the control vector. For each transfection reaction, the plasmids and transfection reagent were diluted in 100 µL medium without antibiotics, incubated at room temperature for 15 min and then added to each well. After 48 h of transient transfection, luciferase activity was measured on a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA) by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The luciferase activity was calculated by normalizing the firefly luciferase level to the *Renilla* luciferase level. The relative luciferase activity (fold) was calculated by setting the activity of the control to an arbitrary value of 1. Three biological replicates and four technical replicates were conducted for each transfection experiment. The statistical significance of

differences between different groups were analyzed by one-way ANOVA with Duncan's test ($p < 0.05$).

Yeast one-hybrid (Y1H) assay

A yeast one-hybrid (Y1H) assay was performed using the Matchmaker Gold Yeast One-Hybrid System (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Three tandem copies of the predicted JBS (5′-AGAAAGAAATGAGAGATACG-3′) or mutant JBS (5′-AGAGGAGGGCAGAGAGCACG-3′) were ligated to linearized pAbAi vector (double digested with *Xho*I and *Hind*III) to construct bait plasmids. The bait strains were generated by integrating the pBait-AbAi vector (linearized with *Bst*BI) into the Y1HGold yeast genome and then selected on SD/-Ura medium. After 3-5 days at 30 °C, colonies were picked and analyzed by colony PCR and sequenced to further identify bait sequence inserts. For each confirmed bait strain, the minimal inhibitory concentration of aureobasidin A (AbA) to suppress basal expression of the bait construct was determined (less than 1000 ng/mL), and this AbA concentration was used to screen the prey-bait interaction. *PxJun* cDNA was subcloned into linearized pGADT7 vector (double digested with *Eco*RI and *Xho*I) to construct a prey plasmid, which was then transformed into the bait strains and selected on SD/-Leu medium with the minimal AbA inhibitory concentration. After 3-5 days at 30 °C, colonies were picked, analyzed by colony PCR and sequenced to confirm the prey protein inserts. The confirmed positive interaction colony was rescreened on SD/-Leu

482 medium with AbA to establish individual interaction strains. The positive control
483 was generated by co-transforming the pGADT7-p53 and pAbAi-p53 plasmids into
484 the Y1HGold strain. A negative control was created by co-transforming the empty
485 pGADT7 with the normal pAbAi-JBS plasmids into the Y1HGold strain.
486
487 **qPCR analysis**
488 As mentioned before (14, 23), the transcript levels of the *PxJun* and *PxABCB1* genes
489 were quantified by real-time quantitative PCR (qPCR) using the specific primers
490 listed in Table S3. The qPCR experiment was run on the QuantStudio 3 Real-Time
491 PCR System (Applied Biosystems, USA) using FastFire qPCR PreMix (SYBR
492 Green) (Tiangen, Beijing, China) according to the manufacturer's instructions. Three
493 biological replicates and four technical replicates were performed for each
494 experiment. The relative expression levels of target genes were determined using the
495 $2^{-\Delta\Delta CT}$ method and normalized to the internal control ribosomal protein *L32* (*RPL32*)
496 gene (GenBank accession no. AB180441). The statistical significance of differences
497 between groups were analyzed by one-way ANOVA with Duncan's test ($p < 0.05$).
498
499 **RNA interference**
500 RNA interference (RNAi) of *PxMAP4K4* and *PxJun* was carried out to investigate
501 the regulatory relationships among the *PxMAP4K4*, *PxJun* and *PxABCB1* genes and
502 to explore whether the *PxJun* gene is involved in Cry1Ac resistance in *P. xylostella*.
503 The cDNA fragments of *PxMAP4K4*, *PxJun* or *EGFP* used as templates for

504 subsequent dsRNA synthesis were amplified by gene-specific dsRNA primers
505 containing a T7 promoter on the 5′ end (Table S3). The gene-specific primer set used
506 to produce dsRNA of *PxJun* was designed for the 5′-terminal gene-specific region
507 and not for the 3′-terminal conserved bZIP_Jun region to avoid potential off-target
508 effects, and we could not detect any specific hits to other Jun genes by BLASTN
509 search of GenBank (https://www.ncbi.nlm.nih.gov/) or the *P. xylostella* genome
510 database (LepBase:
511 http://ensembl.lepbase.org/Plutella_xylostella_pacbiov1/Info/Index), further
512 confirming the specificity of the selected dsRNA fragment. *PxMAP4K4*, *PxJun* or
513 *EGFP* dsRNA was synthesized using the T7 RiboMAX Express RNAi System
514 (Promega, Madison, WI, USA) as indicated by the manual.

515 The protocol for RNAi experiments in the early third-instar larvae of the
516 resistant NIL-R strain was conducted by dsRNA microinjection as mentioned earlier
517 (54). Thirty larvae were microinjected with buffer, dsEGFP (300 ng), dsPxJun (300
518 ng) or dsPxMAP4K4 (300 ng) for each treatment, and three biological repeats were
519 performed. The injected larvae were reared and subjected to qPCR analysis at
520 different times. In addition, toxicity bioassays were carried out at 48 h post-injection.
521 The larvae were fed cabbage containing 1000 mg/L Cry1Ac protoxin (LC_{10} value for
522 NIL-R larvae) for 72 h to calculate larval mortality as described previously (14, 52).
523 The significance of the differences between the treatment and control groups were
524 determined by one-way ANOVA with Duncan's test ($p < 0.05$).
525

Data availability

The full-length cDNA sequence of the cloned *PxJun* gene in this study has been deposited in the GenBank database under accession number MW446637.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Transcriptional activity of the *PxABCB1* promoter in susceptible and resistant *P. xylostella*. (A) A diagram of sequence alignment of the 5′-flanking regions of *PxABCB1* in the susceptible DBM1Ac-S and resistant NIL-R strains. The right-angled arrow denotes the TSS. The numbers with arrows specify the 5′ and 3′ positions of the corresponding nucleotide. The green/white rectangles indicate DNA fragment insertion/deletion (Ins/Del). (B) Detection of *PxABCB1* promoter activities in susceptible and resistant *P. xylostella*. All the fragment constructs are named with "P" as the starting letter, followed by a pair of parentheses that contain two numerals, separated by a dash (/), to specify the 5′ and 3′ positions of the corresponding promoter fragment. Relative luciferase (*Luc*) activities were detected at 48 h post-transfection in S2 cells. The relative luciferase activity (fold) of different promoter recombinants was calculated based on the value of the pGL4.10 control vector. The values shown are the means and the corresponding standard error (SEM) for three independent experiments. The significance of differences was determined by one-way ANOVA with Duncan's test ($p < 0.05$).

Figure 2. Relative luciferase activity analysis of the fragments between -765 and +125. Progressive 5′ deletion constructs from -765 to +125 were transfected into S2 cells, and luciferase activity was detected. The relative luciferase activity (fold) of different constructs was calculated based on the value of the pGL4.10 vector. The values shown are the means and the corresponding standard error (SEM). One-way

730 ANOVA followed by Duncan's test was used for comparison ($p < 0.05$, $n = 3$).

731

732 **Figure 3.** Effects of potential transcription factors (TFs) on the activity of the
733 *PxABCB1* promoter. (A) Putative transcription factor binding sites (TFBSs) in
734 critical positive and negative regulatory regions (see Fig. S1 for detailed DNA
735 motifs). Different colored ellipses represent different TFBSs. (B) Effects of different
736 predicted TFs on *PxABCB1* promoter activity. Every cloned TF was subcloned into
737 the pAc5.1 expression vector to generate a recombinant vector, which was then
738 co-transfected with P(-1122/+125) to determine the luciferase activity. The empty
739 pAc5.1 without PxJun was used as a control. The relative luciferase activity (fold)
740 was calculated based on the value of the empty pAc5.1 vector. The values shown are
741 the means and the corresponding standard error (SEM). One-way ANOVA followed
742 by Duncan's test was used for statistical analysis ($p < 0.05$, $n = 3$).

743

744 **Figure 4.** PxJun represses *PxABCB1* promoter activity through the Jun binding site
745 (JBS). (A) Effect of PxJun on *PxABCB1* promoter activity with either a deleted or a
746 mutated form of the JBS via the dual-luciferase reporter assay. The JBS motif
747 (AAGAAATGAGAGAT, -107 to -94) in P(-1122/+125) was deleted or mutated to
748 GGAGGGCAGAGAGC. PxJun was co-transfected with P(-1122/+125) containing
749 normal JBS (red ellipse), deleted JBS (black ellipse) or mutated JBS (green ellipse).
750 The empty pAc5.1 without PxJun was used as a control. Three biological replicates
751 were performed for all experiments. The values shown are the means and the
752 corresponding standard error (SEM). One-way ANOVA followed by Duncan's test

753 was used for statistical analysis ($p < 0.05$). (B) Verification of direct binding of
754 PxJun to the JBS by the yeast one-hybrid assay (Y1H). Three tandem repeats
755 containing wild-type or mutated JBS sequences were fused to the pABAi vector,
756 which was subsequently integrated into the Y1HGold yeast strain to generate the
757 "bait strain". A critical aureobasidin A (AbA) concentration of 500 ng/mL was
758 detected to completely repress the growth of the bait strains on SD/-Ura media.
759 PxJun was fused to the pGATD7 vector and then transferred into the "bait strain",
760 which was grown on SD/-Leu selective media with or without AbA. EV, empty
761 vector; positive control, using pGADT7-p53 + pABAi-p53.
762
763 **Figure 5.** Effects of PxJun and/or PxFos on the activity of the *PxABCB1* promoter.
764 PxJun and/or PxFos were co-transfected with P(-1122/+125), and the luciferase
765 activity was measured. The empty pAc5.1 without TF was used as a control. The
766 relative luciferase activity (fold) was calculated based on the value of the empty
767 pAc5.1 vector. The values shown are the means and the corresponding standard error
768 (SEM) for three independent experiments. One-way ANOVA followed by Duncan's
769 test was used for statistical analysis ($p < 0.05$, $n = 3$).
770
771 **Figure 6.** The relative expression levels of the *PxJun* gene in the midgut tissues of
772 fourth-instar larvae in five *P. xylostella* strains as detected by qPCR. The *RPL32*
773 gene was used as an internal control. The relative expression level (fold) is presented
774 as the ratio to the value of the lowest expression level, observed in the DBM1Ac-S

775 strain. The average relative expression level and SEM of three independent
776 replicates are presented. Different letters used to mark bars denote significant
777 differences ($p < 0.05$, Duncan's test, $n = 3$).
778
779 **Figure 7**. Effect of *PxJun* gene silencing on *PxABCB1* expression and Cry1Ac
780 resistance in resistant NIL-R larvae. (A) Relative expression of *PxJun* and *PxABCB1*
781 at 48 h post-injection with buffer, dsEGFP or dsPxJun. The expression levels of
782 *PxJun* or *PxABCB1* in the control larvae injected with buffer were set as 1. (B)
783 Silencing of *PxJun* expression decreased the resistance of NIL-R larvae to Cry1Ac
784 protoxin. At 48 h after microinjection with buffer, dsEGFP or dsPxJun, the larvae
785 were fed diets with or without Cry1Ac protoxin (1000 mg/L). The percentage of
786 larval mortality was then counted at 72 h post-treatment. Data are presented as the
787 mean values ± SEM for three biologically independent experiments. Different letters
788 in each group indicate statistically significant differences between treatments ($P <$
789 0.05; Duncan's test; $n = 3$).
790
791 **Figure 8**. Effect of *PxMAP4K4* gene silencing on the expression levels of *PxJun* and
792 *PxABCB1* in the midgut tissues at different periods. Data are presented as the mean
793 values ± SEM for three biologically independent experiments. Asterisks (*) indicate
794 significant differences among periods for each gene ($P < 0.05$; Duncan's test; $n = 3$).
795
796 **Figure 9**. A proposed model for the transcriptional regulation of reduced *PxABCB1*
797 expression by the MAPK-activated TF PxJun. The activated MAPK signaling

pathway increases the expression of *PxJun*, which in turn represses the transcript level of Bt Cry1Ac receptor gene *PxABCB1* and enhances larval resistance to Bt Cry1Ac toxin in *P. xylostella*.