MAPK-dependent hormonal signaling plasticity contributes to overcoming *Bacillus thuringiensis* toxin action in an insect host

Zhaojiang Guo1,4✉, Shi Kang1,4, Dan Sun1,4, Lijun Gong1, Junlei Zhou1, Jianying Qin1, Le Guo1, Liuhong Zhu1, Yang Bai1, Fan Ye1, Qingjun Wu1, Shaoli Wang1, Neil Crickmore2, Xuguo Zhou3 & Youjun Zhang1

The arms race between entomopathogenic bacteria and their insect hosts is an excellent model for decoding the intricate coevolutionary processes of host-pathogen interaction. Here, we demonstrate that the MAPK signaling pathway is a general switch to trans-regulate differential expression of aminopeptidase N and other midgut genes in an insect host, diamondback moth (*Plutella xylostella*), thereby countering the virulence effect of *Bacillus thuringiensis* (Bt) toxins. Moreover, the MAPK cascade is activated and fine-tuned by the crosstalk between two major insect hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH) to elicit an important physiological response (i.e. Bt resistance) without incurring the significant fitness costs often associated with pathogen resistance. Hormones are well known to orchestrate physiological trade-offs in a wide variety of organisms, and our work decodes a hitherto undescribed function of these classic hormones and suggests that hormonal signaling plasticity is a general cross-kingdom strategy to fend off pathogens.
Insects are the most speciose group of animals, with approximately 5.5 million species estimated to exist on earth. Many insects are agricultural pests regularly causing 10–20% crop losses and with on-going global warming, they pose a real threat to world food security. The gram-positive bacterium Bacillus thuringiensis (Bt) can produce protein toxins to kill different insects with high host specificity and environmental safety, which makes it the most successful biopesticide for the last few decades. Transgenic crops expressing Bt toxins (Bt crops) have become the cornerstone of bioinspired pest control technology, with >100 million hectares planted globally in 2018. Although Bt products have provided unprecedented economic, environmental, and social benefits, the rapid evolution of Bt resistance in at least nine insect species in the field has seriously eroded their potential. Unraveling the molecular mechanisms of Bt resistance has important implications for the sustainable utilization of Bt-based technology.

Bt Cry toxins exert toxicity in insect larval midguts via a multi-step process requiring protoxin activation, toxin-receptor interaction, toxin oligomerization, membrane insertion, and pore formation. Alterations of midgut receptors such as cadherin (CAD), aminopeptidase N (APN), alkaline phosphatase (ALP), and ABC transporters (e.g., ABCC2) disrupt toxin binding and are generally associated with high-level resistance to Bt Cry toxins in insects. The diamondback moth, Plutella xylostella (L.), is one of the most devastating and cosmopolitan agricultural pests. It was the first insect to develop field-evolved resistance to Bt biopesticides, and the availability of complete genome information renders it an excellent model to probe how insect hosts withstand Bt infection during host–pathogen interaction. Previously, field-evolved resistance to Bt Cry1Ac toxin in P. xylostella has been linked to both a cis-mutation in the PxABCC2 gene and MAPK-mediated differential expression of PsmALP, PxABCB1, PxABCC1-3, and PxABCG1 genes. Although we found that the MAPK signaling pathway can alter the expression of multiple midgut genes related to Cry1Ac resistance in P. xylostella, its downstream response gene repertoires and upstream activation signals remained mysterious.

APN proteins are a class of endopeptidases catalyzing the cleavage of neutral amino acids from the N-terminus of protein or polypeptide substrates. They belong to the M1 family (metallo-type) of zinc-dependent aminopeptidases that are implicated in many physiological processes of diverse organisms. In the insect midgut, besides their important role in food digestion, APNs were the functional receptors of Bt Cry toxins. Moreover, we had previously detected several differentially expressed midgut PxAPN genes in Bt Cry1Ac-resistant P. xylostella32,33. However, whether the differential expression of these PxAPN genes associated with Cry1Ac resistance was also trans-regulated by the MAPK cascade in P. xylostella was unclear.

Insect endocrinologists have studied insect hormones for more than a century, and they have discovered that two major insect hormones, juvenile hormone (JH) and 20-hydroxyecdysone (20E), act antagonistically with each other to coordinately orchestrate insect life-history traits including growth, development, and reproduction. Moreover, JH and 20E are multifunctional players that can also participate in insect immune defense to pathogenic infection, and the MAPK signaling pathway is involved in this pleiotropic hormone signaling network. Since exogenous hormone treatments can alter APN gene expression in insects, we also wanted to test whether altered levels of insect hormones can activate the MAPK cascades thereby trans-regulating the differential expression of PxAPN and potentially other midgut genes to confer Cry1Ac resistance in P. xylostella.

In this study, we further probe the mechanism of Bt Cry toxin resistance in P. xylostella, starting by looking at the potential role of APN genes, and we confirm that MAPK-mediated differential expression of APN and other midgut genes does lead to Cry1Ac resistance in P. xylostella. More importantly, we uncover that the MAPK cascade is activated and modulated by an enhanced pleiotropic hormone signaling pathway. This study provides a model for understanding how intracellular signaling networks shape the expression landscape of midgut genes causing Bt resistance/tolerance in insects within the context of balancing growth-defense tradeoffs.

Results

Genome-wide analysis of APN gene family in P. xylostella.

Based on currently available transcriptome and genome databases of P. xylostella, 18 M1 aminopeptidase genes including 15 APN genes were identified in silico, and their full-length cDNA sequences were successfully cloned except for PxAAN3b (Supplementary Table 1 and Supplementary Fig. 1a). A representative lepidopteran APN protein contains six common features (Supplementary Fig. 1b), including the characteristic gluzincin aminopeptidase motif GAMEN and the zinc-binding/gluzincin motif HEX2HX18E located in the peptidase_M1 domain which are conserved in nearly all of these M1 aminopeptidases (Supplementary Table 1 and Supplementary Fig. 1c). We found that the APN1–12 gene cluster possesses highly conserved synteny in both gene order and orientation in different lepidopteran insects, indicating that it has undergone tandem gene duplication during insect genome evolution (Fig. 1a). Although the paralogous PxAAPN1–12 genes show similar features including exon number, size, and intron phase (Supplementary Fig. 1d), they share relatively low protein sequence similarity (Supplementary Fig. 1e), implying their evolutionary and functional diversity. A model-based phylogenetic analysis demonstrates that lepidopteran APN proteins cluster into 13 classes and are evolutionarily conserved in each class. Sister phylogenetic relationships were also observed between APN1 and APN3 and between APN5 and APN6, suggesting close protein structure and functional similarities within these pairs (Supplementary Table 2 and Fig. 1b).

Altered expression of four PxAAPN genes in resistant strains.

A previous study showed that cis-mutations in PxAAPN genes are not linked to Cry1Ac resistance in P. xylostella. Likewise, we did not detect mutations in PxAAPN genes in any of our resistant P. xylostella strains. Nevertheless, considering that our previous P. xylostella midgut transcriptome and RNA-Seq studies had identified differentially expressed PxAAPN genes in the resistant strains, the transcription profiles of these genes were further investigated. Unsurprisingly, given their function as digestive enzymes, the APN genes were found to be most highly expressed in the midgut and during the larval stages. Some (PxAAPN1–9) were expressed to a greater extent than the others (Supplementary Fig. 2a). These data were compared with our previous midgut transcriptome and RNA-Seq data (Supplementary Fig. 2b), which showed that the PxAAPN1–9 genes (except for PxAAPN7) were expressed in midgut tissues of third-instar Bt susceptible larvae. When the constitutive midgut transcription profiles of these genes in fourth-instar larvae from both Bt-susceptible and -resistant strains were compared, it was revealed that both PxAAPN1 and PxAAPN3a genes were substantially decreased in all of the Bt-resistant strains, whereas PxAAPN5 and PxAAPN6 genes were increased (Fig. 1c). Assessment of protein levels confirmed that PxAAPN1 and PxAAPN3a were both reduced in midgut samples from all the Bt-resistant fourth-instar larvae (Supplementary Table 3 and Fig. 1d).
PxAPN1 and PxAPN3a are functional midgut Cry1Ac receptors. In order to ascertain the role, if any, of the differentially expressed PxAPN genes in the mechanism of action of Bt toxins, the PxAPN1, PxAPN3a, PxAPN5, and PxAPN6 genes were transiently expressed in Sf9 cells. These insect-derived cells are not naturally susceptible to the Bt Cry1Ac toxin and as a result have been used to test putative toxin receptors. Specific APN activity assays showed that high levels of APN activity were detected in all the PxAPN-expressing Sf9 cells, indicating that these recombinant PxAPN proteins were biologically active (Supplementary Fig. 3a). The successful ectopic expression of these GFP-fused PxAPN proteins was further verified by Western blots (Supplementary Fig. 3b). Immunofluorescent localization detection illustrated that all four PxAPN proteins were located on the cell surface, but that only PxAPN1 and PxAPN3a proteins could bind Cry1Ac toxin (Fig. 2a). Furthermore, Cry1Ac toxin induced a time- and concentration-dependent cell cytotoxicity in cells expressing PxAPN1 or PxAPN3a but not in those expressing PxAPN5 or PxAPN6 (Fig. 2b and Supplementary Fig. 3c). These observations led to the conclusion that PxAPN1 and PxAPN3a are functional receptors of the Bt Cry1Ac toxin in P. xylostella.

PxAPN1 and PxAPN3a knockdown/out confers Cry1Ac resistance. Having established that PxAPN1 and PxAPN3a could act as receptors for Cry1Ac, we then intended to establish whether their loss could induce resistance to the toxin. RNAi-induced knockdown of PxAPN1, PxAPN3a, PxAPN5, and PxAPN6 gene expression in the Bt-susceptible strain of P. xylostella was undertaken. The transcript levels for these four PxAPN genes were significantly reduced at 24 h post-RNAi, with the lowest expression at 48 h and lasting for about 72 h in both cases. The observed silencing was specific to each targeted gene (Fig. 2c and Supplementary Fig. 4a). Subsequent bioassays conducted at 48 h post-RNAi for 72 h revealed a remarkable reduction in the susceptibility to Cry1Ac protoxin at both 1 mg/L (LC50 value) and 2 mg/L (LC90 value) in both dsPxAPN1- and dsPxAPN3a-treated
groups compared to the controls. Furthermore, the use of combinatorial RNAi to silence both PxAPN genes simultaneously by microinjection of a combination of dsPxAPN1 and dsPxAPN3a (dsMultigenes) caused incremental increases in Cry1Ac tolerance at both doses (Fig. 2d). In contrast, no susceptibility changes to Cry1Ac protoxin in DBM1Ac-S larvae injected with buffer or dsEGFP, dsPxAPN1, dsPxAPN3a or a multiple gene mixture (dsPxAPN1 and dsPxAPN3a).

CRISPR/Cas9-mediated knockout of both PxAPN1 and PxAPN3a genes was further utilized in vivo to validate their roles in Cry1Ac resistance. We used an optimized germline transformation and mutation screening strategy to successfully construct stable homozygous mutant strains of PxAPN1 knockout strains PxAPN1KO and PxAPN3aKO, and the near-isogenic resistant strain NIL-R exposed to Cry1Ac protoxin. Interstrain allelic complementation tests with diagnostic doses of Cry1Ac protoxin (10 mg/L). F1 progeny were produced by crossing one susceptible and three resistant strains in all pairwise combinations. Data are presented as mean values (c) and mean values ± SEM (b, d, f), n = 6 (b, c), n = 3 (d), and n = 5/10 (f) biologically independent samples, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant, one-way ANOVA with Holm-Sidak’s test was used in (c), (d), and (f) for comparison. Images in (a) are representative micrographs of three independent experiments that produced similar results. Source data are provided as a Source Data file.

Reduced PxAPN1 and APN3a expression is linked to resistance. Genetic linkage analysis was conducted to test the cosegregation of differential expression of PxAPN1, PxAPN3a, PxAPN5, and PxAPN6 genes with Cry1Ac resistance in the NIL-R strain. qPCR analysis showed that the transcript levels of PxAPN1 and PxAPN3a genes in both Cry1Ac-unselected F2 backcross families exhibited two distinct groups: one group displayed reduced expression of PxAPN1 and PxAPN3a genes, whereas the other group showed transcript levels resembling larvae from the susceptible parental strain or the F1 progeny (Fig. 3a, b). Moreover, the ratio between the numbers of individuals in each group was 9:9 in both backcross families for the PxAPN1 and PxAPN3a genes, which was a perfect fit to the theoretically random assortment ratio 1:1 (p = 1.0; $\chi^2$ test). In stark contrast, all of the survivors from Cry1Ac-treated F2 backcross families had reduced PXAPN1 and PXAPN3a transcript levels compared to larvae from the DBM1Ac-S strain or the F1 progeny, indicating tight linkage (cosegregation) with Cry1Ac resistance in NIL-R (p < 0.001, $\chi^2$ test). The transcript levels for PxAPN5 and PxAPN6 genes in both Cry1Ac-treated and non-treated groups remained significantly

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**Fig. 2** PxAPN1 and PxAPN3a are functional Cry1Ac receptors in *P. xylostella*. a Detection of Cry1Ac binding to Sf9 cells expressing PxAPN proteins by immunolocalization. Gray panels: differential interference contrast (DIC) microscopy views; green panels: GFP-expression fluorescent signal; red panels: Cry1Ac-binding fluorescent signal. Mixed color panels: merged images from both the green and red fluorescent channels. The scale bar is 20 μm. b Susceptibility to a series of concentrations of Cry1Ac toxin (0.1 nM to 1 μM) in untransfected Sf9 cells (control), Sf9 cells transfected with only an empty expression vector (NON) and PxAPN-expressing Sf9 cells after 24 h incubation. c RNAi-mediated silencing of PxAPN1 orPxAPN3a is expressed relative to transcript level for each gene at time 0 h, which are displayed as expression fold changes and color-coded according to the gradient. d Susceptibility to Cry1Ac protoxin in DBM1Ac-S larvae injected with buffer or dsEGFP, dsPxAPN1, dsPxAPN3a or a multiple gene mixture (dsPxAPN1 and dsPxAPN3a). e Non-linear log dose-response curves for *P. xylostella* larvae from the susceptible DBM1Ac-S strain, the CRISPR/Cas9-mediated PxAPN1 and PxAPN3a knockdown strains PxAPN1KO and PxAPN3aKO, and the near-isogenic resistant strain NIL-R exposed to Cry1Ac protoxin. f Interstrain allelic complementation tests with diagnostic doses of Cry1Ac protoxin (10 mg/L). F1 progeny were produced by crossing one susceptible and three resistant strains in all pairwise combinations. Data are presented as mean values (c) and mean values ± SEM (b, d, f), n = 6 (b, c), n = 3 (d), and n = 5/10 (f) biologically independent samples, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant, one-way ANOVA with Holm-Sidak’s test was used in (c), (d), and (f) for comparison. Images in (a) are representative micrographs of three independent experiments that produced similar results. Source data are provided as a Source Data file.
up-regulated and did not associate with Cry1Ac resistance (Fig. 3c, d).

MAPK-induced differential expression of diverse midgut genes. We previously established that an activated MAPK cascade trans-regulates differential expression of PxmALP, PxABC1, PxABC2, PxABC3, and possibly other midgut genes involved in Cry1Ac resistance in P. xylostella26-28. Hence, silencing of PxMAP4K4 expression in resistant NIL-R larvae was performed to ascertain whether this MAPK-mediated mechanism also accounts for the differential expression of PxAPN and other midgut genes to cause Cry1Ac resistance. After dsRNA injection in the Bt-resistant strain, the transcript levels of PxMAP4K4 were substantially reduced at 48 h post-RNAi (Fig. 4a). Correspondingly, the transcript levels of PxmALP, PxABC1, PxABC2, PxABC3, PxBCG1, PxAPN1, and PxAPN3a were increased, while the transcript levels of PxABC1, PxAPN5, and PxAPN6 decreased at 48 h post-injection (Fig. 4a). Bioassays conducted at 48 h post-RNAi for 72 h indicated that silencing of PxMAP4K4 expression led to significantly increased larval susceptibility to Cry1Ac protoxin compared to the controls (Fig. 4b). Therefore, PxMAP4K4 gene silencing recovered the expression of PxAPN1, PxAPN3a and other midgut proteins and restored Cry1Ac susceptibility in NIL-R larvae.

Increased titers of insect hormones in resistant strains. Previous work had shown that exogenous hormone treatments can alter the transcript levels of two isoforms of AjAPN1 genes in Achaea janata31, so we decided to investigate whether insect hormones might be the upstream activation signals of the MAPK cascade to trans-regulate differential expression of PxAPN and other midgut proteins thereby mediating Cry1Ac resistance in P. xylostella. We initially quantified hormone titers in P. xylostella by optimized multiple reaction monitoring (MRM)-based ultra-performance liquid chromatography–tandem mass spectrometry (UPLC−MS/MS). Hormone detection showed specific single peaks for JHs, 20E and their respective analogs methoprene and 22S, 23S-homobrassinolide in the MRM mass chromatograms (Fig. 5a). As expected, the hormone titers fluctuated during different developmental stages with clear JH II peaks observed around larval molting, and the sensitivity of this initial assay only resulted in changes in 20E levels being observed during pupation (Fig. 5b). A more detailed analysis of hormone titers during the third- and fourth-instar stages showed sharp drops in 20E and small peaks in JH II during the molting stage, in contrast, the levels of both hormones did not fluctuate significantly during the feeding intermolt stage (Fig. 5c, d). When the titers of JH II and 20E in feeding third/fourth stage larvae from the resistant and susceptible strains were compared, it was found that the levels were higher in the NIL-R strain than the DBM1Ac-S strain (Fig. 5c, d). Furthermore, it was found that they were also higher in fourth-instar feeding larvae of all the resistant strains, which correlated well with their resistance ratios (Fig. 5e, f), and hinted at the potential involvement of insect hormones in Cry1Ac resistance in P. xylostella.

The MAPK cascade is modulated by insect hormonal crosstalk. To investigate a potential causal link between hormone titer and resistance, exogenous JH analog (JHA) methoprene (200 ng), 20E (2 ng), or a mixture of the two, were microinjected into newly
molted third-instar DBM1Ac-S larvae. qPCR analysis showed that the expression of PxMAP4K4 was significantly decreased at 48 h after methoprene treatment, whereas it had increased at 48 h after 20E treatment (Fig. 6a). The transcript levels of four PxAPN and other midgut genes were increased at 48 h after methoprene treatments, whereas their levels were notably decreased at 48 h after 20E treatments. However, when larvae were synchronously treated with both methoprene and 20E, while the transcript levels of PxMAP4K4, PxAPN5, PxAPN6, and PxABCC1 were increased at 48 h post-microinjection, those of PxAPN1, PxAPN3a, PxmALP, PxABC8, PxABC1, PxABC2, and PxABC3, and PxABCG1 had decreased (Fig. 6a). Intriguingly, this pattern of differential expression exactly, but inversely, mimicked that seen in the NIL-R strain following PxMAP4K4 knockdown (Fig. 4a). To investigate whether downstream elements of the MAP4K cascade (notably p38, ERK, and JNK) might be involved in the control of expression of the midgut proteins, a combination of methoprene, 20E, and a MAPK inhibitor cocktail targeting the above three kinases was microinjected into DBM1Ac-S larvae. Despite the expected increase in expression of PxMAP4K4 following this treatment, the expression levels of all of the tested midgut genes either remained the same as the control or only showed modest changes (Fig. 6a). To confirm the downstream effects of hormone treatment on the MAPK cascade, Western blots were performed to measure the levels of total and phosphorylated p38, ERK, and JNK (Fig. 6b). These data showed a significant increase in phosphorylated protein following treatment with 20E or a combination of 20E and methoprene. These increases were not observed when the MAPK inhibitor cocktail was included alongside the hormone mixture, results consistent with the qPCR data in suggesting that the hormones are exerting their effect via the MAP4K cascade. Bioassays performed at 48 h post-microinjection demonstrated that methoprene treatment resulted in significantly increased larval susceptibility to Cry1Ac protoxin compared to the controls, in contrast, 20E treatment caused significantly reduced susceptibility (Fig. 6c). Treatment with both methoprene and 20E simultaneously led to a more greatly reduced susceptibility to Cry1Ac protoxin than 20E

**Fig. 4 Effect of PxMAP4K4 gene silencing on midgut gene expression and larval Cry1Ac susceptibility.** a The effect of PxMAP4K4 gene silencing on expression of midgut genes at different periods. Gene expression level at 0 h post-RNAi was assigned a value of 1 for comparison, which are displayed as expression fold changes and color-coded according to the gradient. b Susceptibility to Cry1Ac protoxin (LC50, 1000 mg/L) in resistant NIL-R larvae post-RNAi. Data are presented as mean values ± SEM, n = 3 biologically independent samples, ***p < 0.001, ns, not significant, one-way ANOVA with Holm–Sidak’s test was used for comparison. Source data are provided as a Source Data file.

**Fig. 5 Detection of hormone titers in susceptible and resistant *P. xylostella.* a Representative MRM chromatograms of the standard solutions of JHs (JH II and JH III, 5 ng), 20E (2 ng), methoprene (0.1 ng), and 225, 23S-homobrassinolide (0.1 ng) detected by UPLC-MS/MS. b Hormone titers in whole developmental stages of the DBM1Ac-S strain. EG: eggs; L1-4: first- to fourth-instar larvae; P: pupae; A: adults. Samples were collected daily in each stage for hormone titer detection. JH II (c) and 20E (d) titers from third- to fourth-instar larvae of both DBM1Ac-S and NIL-R strains. Samples were collected every 12 h in both stages for accurate detection of hormone titer changes. e, f Box plots show JH II (e) and 20E (f) titers in all of the susceptible and resistant *P. xylostella* strains. Each orange dot represents mean hormone titer in two 1-day-old fourth-instar larvae and the black plus sign indicates mean hormone titer of all the detected individuals in each strain. Median and quartile values are provided by the central line and box boundaries, whiskers show min to max values. Data are presented as mean values ± SEM, n = 3 (b–d) and n = 10 (e, f) biologically independent samples, ’p’ < 0.05, ‘***p’ < 0.001, one-way ANOVA with Holm–Sidak’s test was used in (e) and (f) for comparison. Source data are provided as a Source Data file.
Fig. 6 Effect of hormone titers in susceptible and resistant *P. xylostella*. 

**a** Effect of methoprene and 20E treatments on gene expression levels in the injected DBM1Ac-S larval midguts. Gene expression level at 0 h was assigned a value of 1 for comparison, which are displayed as expression fold changes and color-coded according to the gradient. 

**b** Effect of methoprene and 20E treatments on protein expression and phosphorylation levels of three key MAPK downstream kinases p38, JNK, and ERK in the injected DBM1Ac-S larval midguts. Both the detection of protein and phosphorylation levels by Western blots (upper row) and quantitative estimation of band intensity by densitometry (graph) are presented. 

**c** Susceptibility to Cry1Ac toxin (LC50 1 mg/L) in the exogenous hormone-treated DBM1Ac-S larvae. 

**d** Effect of exogenous hormone treatment on midgut gene expression levels (d) and susceptibility to Cry1Ac toxin (LC0.3 mg/L) (e) in the *PmAP4K4*-silenced DBM1Ac-S larvae. 

**f** Box plots show the detection of Cry1Ac toxin-induced JH II (f) and 20E (g) titers in unselected or Cry1Ac-selected robust and weak DBM1Ac-S and NIL-R larvae. Each orange dot represents mean hormone titer of all the detected individuals in each strain. Median and quartile values are provided by the central line and box boundaries, whiskers show min to max values. Data are presented as mean values (a, d) and mean values ± SEM (b, c, e, g). 

**Association among hormone titer, Bt resistance, and fitness.** Based on the above observation that exposure to methoprene and 20E could directly affect resistance levels to Bt, and induce the same changes in midgut proteins as seen in the resistant strains, we proposed a model. In this model, elevated levels of 20E can induce resistance to Bt through blocking the expression of many midgut proteins, and black plus sign indicates mean hormone titer of all the detected individuals in each strain. Median and quartile values are provided by the central line and box boundaries, whiskers show min to max values. Data are presented as mean values (a, d) and mean values ± SEM (b, c, e, g), n = 3 (a–e) and n = 10 (f, g) biologically independent samples, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant, one-way ANOVA with Holm–Sidak’s test was used for comparison. Source data are provided as a Source Data file.
model, we exposed larvae to methoprene and 20E either individually or in combination and measured various life-history traits (Fig. 7a–i). These data support our model in that exposure to the individual hormones resulted in significantly reduced pupation rates, eclosion rates, adult longevity, oviposition time, female fecundity, and egg hatchability, whereas those in the hormone combination were indistinguishable from the control (Fig. 7a, c, d). Moreover, we observed that the 20E-treated third-instar larvae molted early to the fourth-instar and pupal stages with reduced appetite and food intake, while methoprene-treated third-instar larvae behaved completely opposite, i.e., molted late to the fourth-instar and pupal stages with increased appetite and food intake. In contrast, the development time and feeding behavior of third-instar larvae treated with both hormones were comparable to those in the control group. Thus, these differences might attribute to the observed variation in the resultant pupal size (Fig. 7a).

Since it is known that exposure to Bt can induce tolerance to Bt\textsuperscript{45}, and that toxin can induce the expression of PxMAP4K4 gene in the DBM1Ac-S strain\textsuperscript{26}, we further examined the effect of Bt toxin exposure on hormone levels and fitness. Both newly molted third-instar DBM1Ac-S and NIL-R larvae were exposed to LC\textsubscript{50} levels of Cry1Ac protoxin for 72 h. Surviving fourth-instar larvae showed two quite distinct groups in larval weight, some were significantly lighter than the others and were termed “weak”, whereas the others were indistinguishable from untreated larvae and were termed “robust”. For the robust larvae, it was found that

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**Fig. 7** The effect of hormone or Cry1Ac exposure on life-history traits in *P. xylostella*. a Representative pupal morphology after exogenous treatment (on day 0 of the third-instar larvae) with methoprene (upper box), 20E (middle box) or both (lower box). The scale bar is 1 mm. b–i Analysis of fitness costs of exogenous hormone treatments. j Pupal morphology after exogenous treatment of third-instar larvae with an LC\textsubscript{50} dose of Cry1Ac protoxin for DBM1Ac-S (upper box) and NIL-R (lower box). The scale bar is 1 mm. k–r Analysis of fitness costs of exogenous Cry1Ac treatments. Data are presented as mean values ± SEM, *n* = 3 biologically independent samples, *p* < 0.05, **p** < 0.01, ***p** < 0.001, ns, not significant, one-way ANOVA with Holm-Sidak’s test was used for comparison. Source data are provided as a Source Data file.
larvae from the susceptible parental DBM1Ac-S strain or the F1 exhibited two distinct groups: one group displayed increased 20E titers in both Cry1Ac-unselected F2 backcross families. Individuals in each group, 10:8 in backcross family a and 8:10 in treated and non-treated backcross groups had the same level of JH II and 20E with Cry1Ac resistance in the NIL-R strain. Linkage analysis to determine co-segregation of increased titers of 20E with Cry1Ac resistance in the NIL-R strain51. Previously, we found that a MAPK signaling pathway is unknown, in this work, we discovered an APN gene cluster, containing 12 or more APN genes in different lepidopteran insects, is estimated to have been present since the Jurassic period about 150 million years ago46. Our previous studies have shown that silencing of other potential P. xylostella Cry toxin receptor genes including PxmALP, PxABCR1, PxABCC2, PxABCC3, and PxABCG1 also results in decreased Cry1Ac susceptibility26–28.

One model for Cry toxin mechanism of action involves sequential binding of the toxin to different receptors19, whereas others suggest that multiple receptors in a susceptible host represent alternative receptors rather than members of a sequential pathway47. In a sequential model, one would predict that blocking any of the steps would lead to a similar level of resistance. In this work, we show that knocking out either APN receptor, whilst leading to high levels of resistance, does not match the level of resistance seen in the parent strain, suggesting at least some degree of receptor redundancy. The high level of resistance in the parent strain can be associated with the simultaneous down-regulation of multiple Cry toxin receptors. Whilst the loss of physiologically important proteins might be expected to impart a fitness cost to the insect, it is noteworthy that the down-regulation of proteins known to act as toxin receptors is accompanied by the up-regulation of paralogs that have no receptor function. Gene duplication and subsequent transcriptional plasticity is a major driving force for adaptive evolution of insect response to environmental stress48,49. Specifically, up-regulation of PxABN5 and PxABN6 might compensate for the down-regulation of PxABN1 and PxABN3a to diminish the fitness costs of Cry1Ac resistance and perhaps resembling the differential alteration of TnAPN1 and TnAPN6 in resistant Trichoplusia ni50.

It is noteworthy that the total midgut APN enzyme activities were similar in our susceptible and resistant strains26 and we did not detect any fitness costs in our near-isogenic Cry1Ac-resistant NIL-R strain51. Previously, we found that a MAPK signaling pathway can trans-regulate the differential expression of PxmALP, PxABCC1–3, and possibly PxABCB1 and PxABCG1 genes thereby resulting in Cry1Ac resistance in four different P. xylostella strains26–28. We have now identified that this pathway can also trans-regulate the differential expression of four PxABN genes to mediate Cry1Ac resistance in the same four resistant P. xylostella strains.

Although the mechanism underlying the altered MAPK signaling pathway is unknown, in this work, we discovered an intriguing causal link to the insect hormones 20E and JH. Levels of both insect hormones were significantly increased in all of our resistant strains of P. xylostella, but more relevantly when treated F2 backcross families had increased 20E titers, indicating tight linkage (cosegregation) with Cry1Ac resistance in NIL-R ($p < 0.001$, $\chi^2$ test).

**Discussion**

The proteinaceous Cry toxins produced by Bt represent highly potent virulence factors which when presented to an insect at a high dose, such as in Bt crops, overwhelm the insect’s immune system leading to death5. Through modification of the toxin binding protein (receptor) on the surface of its midgut epithelium, an insect can effectively block the action of the toxin leading to high levels of resistance19. The first Cry toxin receptor identified in an insect was an APN protein in Manduca sexta20,29. In this work, we show that two isoforms of this enzyme can act as functional receptors in P. xylostella and that reduction in their expression leads to loss of susceptibility towards Cry1Ac. We have also conducted the genome-wide characterization of the APN gene family and unified their nomenclature and classification in insects. Interestingly, we discovered a syntenic APN gene cluster, containing 12 or more APN genes in different lepidopteran insects, is estimated to have been present since the Jurassic period about 150 million years ago46. Our previous studies have shown that silencing of other potential P. xylostella Cry toxin receptor genes including PxmALP, PxABCR1, PxABCC2, PxABCC3, and PxABCG1 also results in decreased Cry1Ac susceptibility26–28.

Linkage of increased 20E titers with Cry1Ac resistance. The data presented above suggest a causal association between increased titers of insect hormones and receptor-mediated resistance to Bt Cry toxin, a link which involves the MAPK signaling cascade. To further strengthen this association, we used genetic linkage analysis to determine co-segregation of increased titers of JH II and 20E with Cry1Ac resistance in the NIL-R strain. UPLC–MS/MS detection showed that JH II titers in both Cry1Ac-treated and non-treated backcross groups had the same level of up-regulation as the resistant parental strain, indicating a lack of association with Cry1Ac resistance (Fig. 8a). In stark contrast, 20E titers in both Cry1Ac-selected F2 backcross families exhibited two distinct groups: one group displayed increased 20E titers, whereas the other group showed 20E levels resembling larvae from the susceptible parental DBM1Ac-S strain or the F1 progeny (Fig. 8b). Moreover, the ratio between the numbers of individuals in each group, 10:8 in backcross family a and 8:10 in backcross family b, followed the 1:1 random assortment ratio ($p > 0.10$; $\chi^2$ test). Crucially, all of the survivors from the Cry1Ac-

![Fig. 8 Linkage of Cry1Ac resistance phenotype to increased 20E titers in the NIL-R strain.](https://example.com/fig8.png)

**Fig. 8** Linkage of Cry1Ac resistance phenotype to increased 20E titers in the NIL-R strain. JH II (a) and 20E (b) titers for the F1, Cry1Ac-selected and non-selected backcross families are relative to hormone titers in the susceptible DBM1Ac-S strain. Both results using larvae of the subfamily generated from backcrossing a male from F1 and a female from the NIL-R strain (backcross family a) and using larvae of the subfamily generated from backcrossing a male from F1 and a female from the NIL-R strain (backcross family b) are shown. Source data are provided as a Source Data file.
susceptible insects were exposed to both hormones: differences in gene expression of PxMAP4K4, PxAPN1, 3a, 5, 6, PxABC1, PxABC1-2, 3, and PxABCG1 via manipulating unidentified transcription factors (TFs)\(^{70}\), which finally results in high-level Bt resistance without growth penalty in P. xylostella.

As mentioned above, an imbalance in the ratio of the two insect hormones can shift the insect’s physiology towards either growth/reproduction or defense with a corresponding cost on the other process, and our data in which fitness costs were associated with individual hormone exposure support that. We are proposing a fine-tuning of this process in which a balanced increase in the two hormones affects the insect in such a way that it loses susceptibility to the Cry toxin whilst maintaining gut homeostasis through the expression of paralogous genes. We demonstrated that a balanced rise in both hormones is induced by sub-lethal exposure to Bt toxin and also found, that via some unknown genetic changes, a constitutive and balanced rise has occurred in the resistant strains. A similar case was documented in *Drosophila viridis*, in which increased titers of 20E and JH were identified as an induced adaptive response to the unfavorable environmental heat stress by decreasing egg production and oviposition. Moreover, in a heat stress mutant strain of *D. viridis*, elevated JH and 20E levels caused by an as yet uncharacterized gene mutation in chromosome 6 resulted in the same reduced fertility phenotype as observed for the wild type strain under heat stress\(^{39,60}\). In *P. xylostella*, we hypothesize that a balanced rise in the levels of the two hormones pushes the insect towards a growth-defense tradeoff instead of a full-scale defense response to successfully evolve a Bt resistance phenotype, and we present a model of this proposed system in Fig. 9. Although Bt Cry toxin has been shown to induce various aspects of an immune response\(^{45}\), our previous comparisons of transcript levels between susceptible and resistant *P. xylostella* strains did not find any examples of immune-related genes such as peptidoglycan recognition proteins (PGRPs), antimicrobial peptides (AMPs), or phenoloxidases (POs) in the list of genes that had significantly increased expression in the resistant strain\(^{32,33,61}\).

A previous study using Bt-resistant *Helicoverpa armigera* larvae found that there was an increased titer of JH in the resistant strain\(^{62}\). Although that study indicated that this hormone was directly associated with fitness costs in the resistant strain, it did not look at levels of 20E nor establish any causal link between JH titer and Bt-resistance. Detailed work on Bt tolerance in *Ephestia kuhniella* found that non-genetically inherited tolerance could be induced by exposure to sub-lethal concentrations of Bt and vertically transmitted through maternal effect\(^{45}\). This work had noted that while the initial elevated immune response in this insect carried significant developmental penalties, continued exposure to Bt led to a reduced developmental penalty while tolerance was maintained\(^{63}\). Our results are consistent with a model in which tolerance and resistance to Bt are linked and both associated with hormonal regulation. The mechanistic basis of the interplay between hormone levels, MAPK signaling pathway and Bt resistance remains unclear. While there is evidence for altered protein phosphorylation levels of p38, JNK, and ERK in the MAPK signaling cascade triggering the differential expression of the midgut genes, it is less clear how the hormones feed into this process. Our data here indicate that changes in JH and 20E concentrations can affect MAP4K4, p38, JNK, and ERK, which suggests that they are acting upstream of the MAPK signaling cascade. However, in the resistant strains, the causative mutation

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**Fig. 9 Model for insect hormones-mediated growth-defense tradeoffs orchestrating Cry1Ac resistance in *P. xylostella***. The molecular basis of JH and 20E signaling pathways is highly conserved in different insects: they have antagonistic actions to control life-history traits including growth, development, reproduction, and immunity. Upon exposure of *P. xylostella* to Bt Cry1Ac toxin, in the midgut epithelial cells, we can speculate that JH/20E can bind to the respective heterodimeric receptor complex composed of Methoprene-tolerant (Met) and its partner Taiman (Tai) or edcsyne receptor (EcR) and Ultraspireacle (USP) to initiate the transcription of JH/20E-responsive genes. Meanwhile, increased levels of JH and 20E can activate the MAPK signaling cascades to trans-regulate differential expression of multiple midgut genes including four PxAPNs (PxAPN1, PxAPN3a, PxAPN5, and PxAPN6), PxmALP, PxABC1, PxABCC1-3, and PxABCG1 via manipulating unidentified transcription factors (TFs)\(^{70}\), which finally results in high-level Bt resistance without growth penalty in *P. xylostella*. **
maps to the MAP4K4 locus and most likely directly affects the expression of this gene. The fact that JH and 20E levels are also increased in these strains might suggest that their levels are tightly coordinated with MAP4K4 levels in some way. Although the MAPK signaling pathway is strongly linked to down-regulation of the Bt toxin receptors, it is quite possible that the two hormones are affecting other aspects of the insect’s response via other routes. Initial exposure to Bt can trigger an immediate defense response, that may come with fitness costs, but then a fine-tuning between 20E and JH can balance that tolerance while reducing those costs. Where that balance is not maintained, e.g., in some of our larvae exposed to Bt toxins, severe fitness costs prevail. Differences between the weak and robust larvae may reflect different levels of intoxication and a more extreme, and costly, response to deal with excessive exposure. Continued exposure can result in genetic mutation that leads to constitutive expression of the defense response now manifested as resistance. In other cases of acquired resistance, the phenotype can be as a result of less subtle genetic mutation that leads to constitutive expression of the defense response. Continued exposure can result in genetic mutation that leads to constitutive expression of the defense response now manifested as resistance. Genes selected for this study were Mediator (Med), acetylated histone H3-K18, and acetylated histone H3-K9. The Mediator (Med) gene is a transcriptional regulator that controls the expression of many genes involved in development, differentiation, and cell cycle regulation. The acetylated histones H3-K18 and H3-K9 are markere...
Substrate (Thermo Fisher Scientific), and the images were captured by the Taron-5200 Chemiluminescent Imaging System (Tanon). Densitometric analysis of the protein expression was performed by ImageJ software (U.S. National Institutes of Health; version: v1.51j). The relative intensities were calculated based on densitometric ratios between target proteins and β-actin with the band intensities in the DBM1Ac-S BBMV samples considered as 1.

**Heterologous expression.** The recombinant PxAPN-GFP fusion proteins were in vitro transcribed and translated using a T7 expression kit (Promega) in Sf9 cells. The cells were harvested, lysed and quantified, and specific APN activity assays and Western blots were performed. Enzymatic activity was detected using Leu-p-NA (Sigma) as substrate, with one enzymatic unit (U) being defined as the amount of the enzyme that would catalyze the production of the chromogenic product from 1 μmol specific substrate per minute and per mg of total Sf9 cell proteins at 37°C. Western blots were performed as mentioned above with mouse monoclonal anti-GFP antibody (1:1000, Abcam) using β-actin as an internal control. For immunolocalization of Cry1Ac toxin binding to Sf9 cells expressing PxAPN proteins, the transfected Sf9 cells were incubated with Cry1Ac toxin and fixed in ice-cold 4% paraformaldehyde. After blocking with 1% BSA, the cells were probed with rabbit polyclonal anti-Cry1Ac primary antibody (1:1000) and goat anti-rabbit secondary antibody conjugated with Alexa Fluor 555 (1:1000, Abcam). The treated cells were examined under a LSM 700 confocal laser scanning microscope (Carl Zeiss). For cytotoxicity assays, WST-8 in the Cell Counting Kit-8 (CCK-8) (Dojindo) was used to perform a CCK-8 assay following the manufacturer’s recommendations. The absorbance was measured at 450 nm after 24 h incubation with Cry1Ac toxin, and the relative cell viability was calculated in relation to untreated Sf9 cells, which were defined as 100%. In addition, the morphological changes of Cry1Ac-treated Sf9 cells were also observed under a Zeiss LSM 700 microscope.

**RNA interference.** RNAi-induced gene silencing and subsequent functional assays were performed in vitro using P. xylostella Sf9 cells. Briefly, specific dsRNA target sequences were designed in exon 12 of PxAcp5 and exon 13 of PxAcp3α (Supplementary Table 6). In vitro transcription was conducted to generate sgRNAs with the MEGAscript Transcription Kit (Ambion) following the manufacturer’s instruction. The mixtures of sgRNA (150 ng/μl) and Cas9 (300 ng/μl) were microinjected into blastoderm-stage P. xylostella eggs using FemtoJet 4i and InjectMan 4 Microinjection System (Eppendorf). Nondestructive genotyping of CRISPR/Cas9-induced indel mutations around the sgRNA target site was performed using PCR amplification with gDNA samples prepared from exuviates of individual fourth-instar larvae as templates, followed by direct sequencing and TA cloning. The germline transmission and mutation screening strategy was developed and optimized to construct stable homozygous mutant strains of both the PxAcp5 and PxAcp3α genes designated APNKO and APN3KO, respectively (Supplementary Table 6). Leaf-dip bioassays were conducted to determine whether knock-out of PxAcp5 and PxAcp3α genes affects susceptibility to Bt Cry1Ac protein and to test their functionality. Further experiments were performed to confirm the activator function of Bt Cry1Ac resistance phenotype in both knock-out strains.

**Hormone assays.** Hormone treatments were performed using newly molted third-instar P. xylostella larvae. 20E and methoprene were dissolved in ethanol (20 mg/ml) and diluted with ice-cold insect Ringer’s solution (130 mM NaCl, 0.5 mM KCl, 0.1 mM CaCl2) before microinjection. The MAPK inhibitors SB203580 (specific p38 inhibitor, Merck Millipore), SP600125 (specific JNK inhibitor, Merck Millipore) and PD0325901 (specific MEK/ERK inhibitor, TargetMol) were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich). Specific dsRNA targeting PxAcpMAP4K4 (dsPxAcpMAP4K) was prepared as described in the RNAI section. The treatment time of hormone-induced effects on gene expression and the quantities of hormones in the haemolymph were optimized in preliminary microinjection experiments (Supplementary Fig. 8). Subsequently, newly molted third-instar DBM1Ac-S larvae were starved for 6 h and anesthetized for 30 min on ice, 70 ml solutions containing 20E (2 ng), methoprene (200 ng), mixture of 20E (2 ng) and methoprene (200 ng) or the hormone mixture with 100 μM MAPK inhibitor cocktail and dsRNA combined with hormone treatments were respectively microinjected into some of these larvae using the Nanoliter 2000 microinjection system, whereas the remaining control larvae were injected with the equal volumes of buffer (diluted ethanol and DMSO), with the final concentration of ethanol not exceeding 0.05%. More than 30 larvae were microinjected for each treatment, and these injected larvae were allowed to recover for about 3 h at room temperature and were then returned to normal rearing conditions, the final survivors from each control and treatment groups were used for the subsequent midgut dissection, qPCR analysis, and Western blots as described above. Meanwhile, leaf-dip bioassays were performed for 72 h using larvae at 48 h after hormone, MAPK inhibitors or dsRNA injection and Cry1Ac intoxication using a total of about 15 larvae (0.3 mg/L) value for non-injected DBM1Ac-S larvae. Bioassays were performed with 20 larvae in each group, and each bioassay replicated three times. Mortality in
control treatments was below 5% and bioassay data processing was as described above.

Toxin induction assays. Toxin induction of insect hormones was conducted using newly molted third-instar DBM1Ac-S and NIL-R larvae26. The DBM1Ac-S or NIL-R larvae were selected with 1 or 3500 mg/L Cry1Ac protoxin (respective LC50 values for each strain) for 72 h as for the leaf-dip bioassay mentioned above, unselected larvae from both strains were used as control groups. After 72 h, sample preparation from both robust and weak survivors to detect hormone titers was undertaken as described above. Three independent assays were performed, and one-way ANOVA with Holm–Sidak’s test (overall significance level = 0.05) was used to determine the significant difference between control and treatment groups.

Fitness cost analysis. The effects of exogenous hormone and Cry1Ac protoxin treatments on subsequent developmental stages of surviving larvae to determine potential fitness costs were analyzed by comparing biological parameters including pupation percentage, pupal weight, pupation duration, eclosion percentage, adult longevity, oviposition duration, female fecundity, and egg hatchability. Larvae injected with buffer and unselected with toxin were used as negative controls in the longevity, oviposition duration, female fecundity, and egg hatchability. Larvae pupation percentage, pupal weight, pupation duration, eclosion percentage, adult female insect reproduction.

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Author contributions
Z.G., X.Z., N.C., and Y.Z. designed the research. Z.G., S.K., D.S., L. Gong, J.Z., J.Q., L. Guo, L.Z., Y.B., F.Y., Q.W., and S.W. performed the experiments. Z.G. analyzed the data. Z.G., N.C., X.Z., and Y.Z. wrote and revised the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Z.G. or Y.Z.

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