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ORIGINAL ARTICLE

The steroid hormone 20-hydroxyecdysone upregulated the protein phosphatase 6 for the programmed cell death in the insect midgut

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Abstract Programmed cell death (PCD) plays an important role in insect midgut remodeling during metamorphosis. Insect midgut PCD is triggered by the steroid hormone 20-hydroxyecdysone (20E) and it is mediated by a series of genes. However, the mechanism by which 20E triggers midgut PCD is still unclear. Here, we report a protein phosphatase 6 (PP6) from *Helicoverpa armigera* playing roles in midgut PCD. *PP6* was expressed in the midgut during larval growth and it is significantly increased during metamorphosis. The increase was proven to be regulated by 20E. The juvenile hormone analog methoprene has no effect on *PP6* expression. RNA interference analysis suggests that 20E upregulated the *PP6* transcript levels through the ecdysone receptor *EcRB1*. *PP6* knockdown by larval feeding or PP6 dsRNA injection resulted in the repression of the midgut PCD during the metamorphic stage. The mechanism was demonstrated to be through the suppression of genes such as *Broad (Br)*, *E74a, E75b, HR3, E93, rpr*, and *caspase*, which are involved in 20E signaling pathway or midgut PCD. These findings suggest that PP6 is involved in the 20E signal transduction pathway and participates in the PCD in midgut.

The nucleotide sequence PP6 has been submitted to the GenBank with accession number: JF704119.

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Introduction

Programmed cell death (PCD) plays an important role in animal development by removing the unnecessary cells or tissues (Jacobson et al. 1997; Vaux and Korsmeyer 1999). PCD also occurs in the insect midgut during metamorphosis (Robinow et al. 1993; Truman et al. 1994). Similar to the *Drosophila* larval salivary glands, the midgut undergoes PCD, triggered by the molting hormone 20-hydroxyecdysone (20E) (Lee et al. 2002). This hormone may induce an increase in the set of genes that participates in the 20E signaling pathway, such as the 20E receptor *EcR*, the heterodimer *USP* (Jindra et al. 1997), and the transcription factors *E75b* (Bialecki et al. 2002; Segraves and Hogness 1990), *HR3*, *Br*, and *E74a* (Langelan et al. 2000; Palli et al. 1992). Some of these genes, such as *Br* and *E74a*, also play roles in larval salivary gland and midgut PCD (Lee et al. 2000; Robinow et al. 1993; Truman et al. 1994). *Br*, *E74a*, and another transcription factor, *E93*, regulate transcription factors such as *reaper* (*rpr*), *head involution defective* (*hid*) (Jiang et al. 2000; Lee et al. 2000), and effector *caspase*, inducing midgut PCD (Daish et al. 2004). However, the 20E signaling pathway that regulates midgut PCD is not fully understood.

Protein phosphorylation and dephosphorylation play vital roles in hormonal signal transduction (Cohen 2002). Protein phosphorylation is performed by various protein kinases, whereas protein dephosphorylation is performed by protein phosphatases (PPs) (Lammers and Lavi 2007). In human, improper regulation of the phosphorylation and dephosphorylation lead to a profound influence on the cells and organism

respond to its external and internal conditions (Eichhorn et al. 2009). The abnormal action of kinases and phosphatases can lead to a series of disease including various cancers (Wang et al. 2004). However, most studies have focused on protein phosphorylation regulated by protein kinases, whereas fewer studies have investigated protein dephosphorylation by PPs. The ubiquitously expressed protein phosphatase 2A (PP2A) modifies 1% of all proteins. Some studies have confirmed the role of PP2A as a tumor suppressor. The experiments in mice showed that, okadaic acid, an inhibitor of PP2A, promotes tumor growth (Schonthal 1998).

Protein phosphatases are classified into three families based on amino acid identity and three-dimensional structure. One family is specific for phosphotyrosine residues (PTP), and the other two families are specific for phosphoserine and phosphothreonine residues (PPP), and specific for phosphoserine and phosphothreonine residues depending on Mg^{2+} (PPM). PPP family includes PP1, PP2A, and PP2B, and they are calmodulin-regulated PP while PPM family is Mg^{2+}-dependent PP includes PP2C, and pyruvate dehydrogenase phosphatase (Cohen 1997). PPs are composed of catalytic, structural, and regulatory subunits. The structural subunit connects the catalytic subunit to the different regulatory subunits for different substrates and functions (Eichhorn et al. 2009). Protein phosphatase 2Ac (PP2Ac) is the most important catalytic subunit in the PPP family (Cohen 1997). PP2Ac has been found to participate in the regulation of numerous cellular proteins, including ion channels, metabolic enzymes, kinases, and hormone receptors (Shenolikar and Nairn 1991). The protein serine/threonine phosphatase 6 (PP6), an essential phosphatase in eukaryotes that contains a PP2Ac domain and is known as Sit4 in *Saccharomyces cerevisiae*, functions in the G1–S transition during cell cycle progression (Sutton et al. 1991). However, the function of phosphatases in 20E-induced PCD has not been reported.

To understand the mechanism of 20E regulation of midgut PCD, the function of PP6 in the midgut of *Helicoverpa armigera* was investigated. *PP6* was found to be upregulated by 20E during metamorphosis. The knockdown of *PP6* in the larvae resulted in the disruption of midgut PCD, and the genes involved in the 20E signaling pathway or midgut PCD, such as *Br*, *E74a*, *HR3, E93*, and *rpr*, are downregulated. These evidences indicated that PP6 played roles in insect midgut PCD by regulation 20E signaling pathway.

Experimental procedures

Insect

Helicoverpa armigera (cotton bollworms) were fed an artificial diet consisting mainly of wheat and soybean under 14:10 h light:dark cycles (Zhao et al. 2005). The cotton bollworms were obtained from the Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China.

Bioinformatics analysis and phylogenetic tree analysis

Protein translation and prediction were achieved with software from ExPASy (http://www.au.expasy.org/). Homology analysis was conducted with BLASTX (http://www.ncbi.nlm.nih.gov). Alignments were performed using CLUSTALW and GENEDOC computer software (http://www.nrbsc.org/downloads/gd322700.exe). The prediction of the mature peptide was performed with ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/).

Semi-quantitative RT-PCR

Unizol reagent (Biostar, Shanghai, China) was utilized to isolate total RNA from the different tissues from the fifth instar feeding larvae to the pupae. Five micrograms of the total RNA was reverse transcribed and subsequently used as the polymerase chain reaction (PCR) template. Before the reverse transcription PCR (RT-PCR) analysis, the PCR template under different number of cycles (24–32, took the samples every two cycles) was amplified to determine the optimal condition for the analysis. Finally, 28 cycles was selected to perform further analysis. Specific RT-PCR primers PP6F2 (5′-atgagtgacgtggataag-3′) and PP6R2 (5′-ttctaagctgtagtagccccgg-3′) were chosen for RT-PCR (263 bp). The RT-PCR program was as follows: one cycle at 94°C for 3 min; 28 cycles at 94°C for 30 s, at 53°C for 35 s, and at 72°C for 35 s; and one cycle at 72°C for 10 min. A 150-bp fragment of *β-actin* amplified with *β-actin* primers actinF (5′-cctggtattgctgaccgttgc-3′) and actinR (5′-ctgttggaaggtggagagggaa-3′), served as the quantitative control (Fabrick et al. 2003). RT-PCR products were checked on 2% agarose gels. After staining with ethidium bromide, the products were photographed under UV light. QUANTITY ONE software (Bio-Rad, Hercules, CA, USA) was used to read the intensities of the bands, and the intensity ratios of *PP6* to *β-actin* were calculated. The means of the intensity ratios from three independent experiments were used to construct the histograms (Du et al. 2007).

Hormonal regulation of PP6 gene

Methoprene (Dr. Ehrenstorfer, Augsburg, Germany) and 20E (Sigma, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) at a storage concentration of 10 mg/ml. The storage solution was diluted into 0.1 mg/ml with phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.4) and injected to the larval hemocoel. The sixth instar

6 h larvae were injected with either 20E (500 ng/larva) or methoprene (500 ng/larva). The controls were treated with equivalent amounts of DMSO at the same stage. The total RNA from the midgut of the treated larvae was isolated at various stages at 1, 3, 6, and 12 h after the injection. RT-PCR was used to analyze the differences between the controls and the experimental group. The experiment was repeated thrice using three independent samples and statistically analyzed.

Recombinant expression of PP6

A 912 bp open reading frame (ORF) encoding PP6 from *H. armigera* was inserted into the expression vector pGEX-4T-1. Then, the recombinant vector was transformed into competent *Escherichia coli* BL21 host cells. Isopropyl-*β*-D-thiogalactopyranoside (0.5 mM) was utilized to induce the host cells to produce the target protein in Luria-Bertani/ampicillin (100 μg/ml) medium. PP6 was expressed as inclusion bodies. Buffer A (50 mM Tris–HCl, pH 8.0, 5 mM EDTA) and buffer B (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 2 M urea) were used to wash the inclusion bodies twice. Then, the washed inclusion bodies were dissolved in buffer C (0.1 M Tris–HCl, pH 8.0, 10 mM dithiothreitol, 8 M urea). The proteins were then refolded in dialysis buffer (0.1 M Tris–HCl, pH 8.0, 5 mM EDTA, 5 mM cysteine) for 16 h at 4°C (Kuhelj et al. 1995). The refolded samples were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the target protein was excised from the gel. The excised samples were electrophoresed for 16 h at 4°C in the dialysis buffer and subsequently refolded in deionized water for 16 h at 4°C. The refolded samples were injected into a rabbit to produce the antibodies.

Preparation of antiserum against PP6 protein

About 200 μg of purified recombinant PP6 protein, 1 ml Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris–HCl, pH 7.5) and 1 ml complete Freund's adjuvant were mixed injected into the back of a rabbit. Three weeks later, another 200 μg PP6 protein in 1 ml TBS mixed with 1 ml incomplete Freund's adjuvant was injected into the same rabbit. After two additional weeks, the rabbit was injected with 500 μg recombinant PP6 protein without Freund's adjuvant.

dsRNA synthesis

The primers of PP6inF, 5′-taatacgactcactatagggtggga gactatgttgaccg-3′, PP6inF 5′-catttactaactgatgtgcc-3′, PP6F 5′-tgggagactatgttgaccg-3′, and PP6inR 5′-taatacgactcactata gggcatttactaactgatgtgcc-3′, were designed to amplify the gene fragment (489 bp). The PCR products were purified and used as templates to synthesize dsRNA in vitro with the MEGAscriptTM RNAi kit (Ambion, Austin, TX, USA). The purity and integrity of dsPP6 were detected by agarose gel electrophoresis. The dsPP6 was dissolved in RNase-free water.

RNAi in the HaEpi cell line

The *H. armigera* epidermal cell line HaEpi (Shao et al. 2008) were cultured to 80% confluence at 26°C in Grace's medium supplemented with 10% fetal bovine serum (FBS) and then changed to FBS-free medium. Then, 1 ml of FBS-free Grace's medium with 8 μg dsRNA and 10 μl Lipofectamine 2000 was diluted to 4 ml with Grace's medium and directly added to the cells. The final dsRNA concentration was 2 μg/ml in the FBS-free medium. After 12-h incubation at 26°C, the cells were rinsed and then re-fed with a 10% FBS medium containing 0.4 μM 20E. After 12 h of culturing, total RNA was isolated from the cells for RT-PCR analysis. Control cells were prepared using the same amount of Lipofectamine 2000 without 20E (Shao et al. 2008). The unrelated dsRNA dsGFP was added to the cells to as another control treatment.

PP6 interference by injection

The dsRNA was diluted to 7 μg/μl with DEPC water and 7 μl was injected into the larvae at the fifth instar 24 and 48 h, as well as at the sixth instar 24 h. The mRNA from the sixth instar 36 h larvae to the sixth instar 96 h larvae were isolated with the aforementioned method to detect the expression profiles of *PP6* and other PCD-related genes.

PP6 interference by feeding

For the bioassay of the *PP6* interference, the third instar larvae were fed an artificial diet with dsRNA. Every 20 μg dsRNA was diluted in 100 μl PBS and added to the artificial diet (1 cm^3). For the respective dsRNA control, the same concentration of dsGFP in the artificial diet was used. After continuous feeding of dsRNA, the mRNA from the sixth-36 h, sixth-60 h, and sixth-96 h larvae were isolated with the aforementioned method to detect the expression profiles of *PP6* and other development-related genes.

Results

PP6 gene had high conservation in animals

The full-length cDNA sequence of *PP6* was 1,101 bp with a 93 bp 5′ untranslated region (UTR), a 912 bp ORF, and a

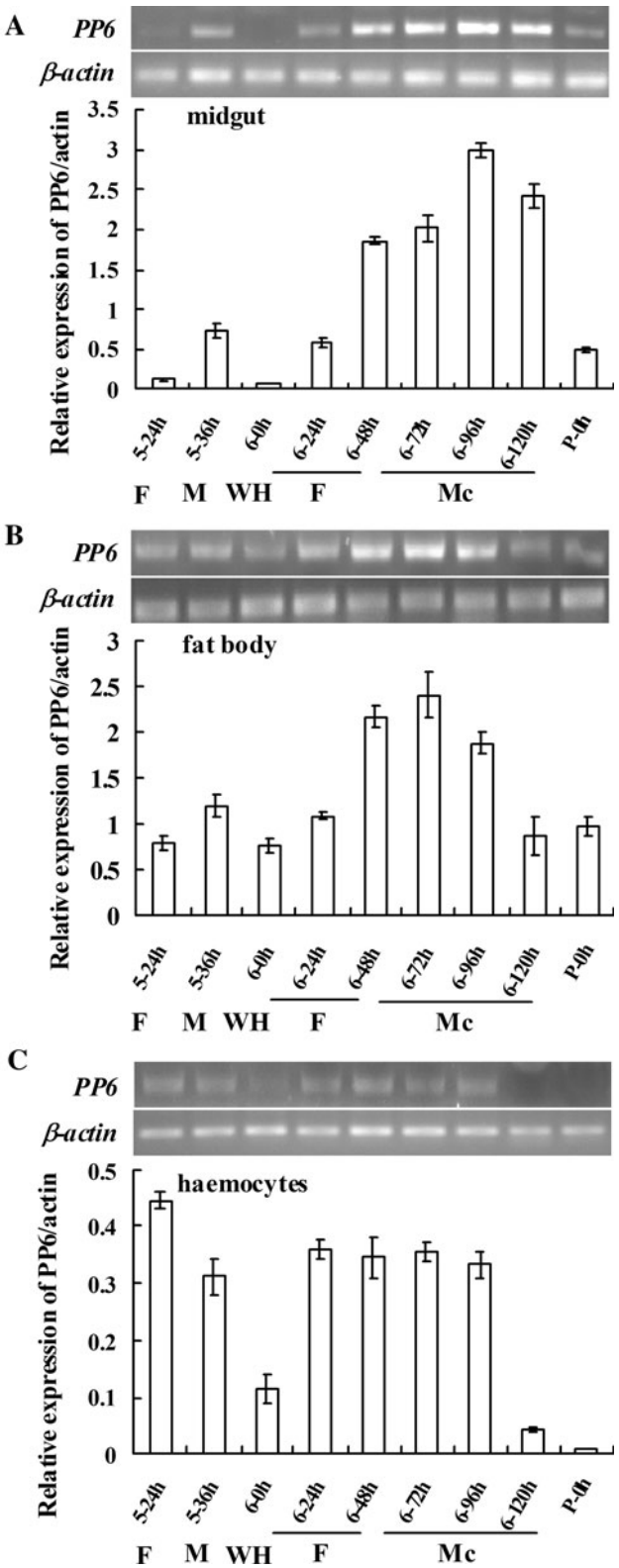

Fig. 1 Expression profiles of *PP6* in vivo by RT-PCR analysis. **a**–**c** are the expression profiles of *PP6* in the midgut, fat body, and hemocytes, respectively, at various larval developmental stages. The fifth-12 h to sixth-120 h are the larval developmental stages. *F* feeding stage, *M* molting stage (HCS), *WH* white head (within 1 h after ecdysis), *Mc* metamorphically committed stage, *P-0* h 1st day pupae. *β-Actin* was used as a quantitative control

96 bp 3′ UTR. The 3′UTR had an 18 bp polyadenylation A tail. The ORF encoded a 303 amino acid sequence with a PP2Ac domain at Leu-17 to Pro-287. The calculated molecular weight of the protein was 34.8 kDa, and the predicted isoelectric point was 5.19. There were five predicted Ser phosphorylation sites (Ser-87, Ser-165, Ser-169, Ser-206, and Ser-289), two predicted Thr phosphorylation sites (Thr-44 and Thr-299), and five predicted Tyr phosphorylation sites (Tyr-74, Tyr-80, Tyr-131, Tyr-242, and Tyr-259). No signal peptide (S-1) was found. The *Helicoverpa pp6* (*HaPP6*), showed 74.43% identity with the *PP6* from *Homo sapiens* (HsPP6, NM_002721.4), *Rattus norvegicus* (RnPP6, NM_133589.2), *Xenopus laevis* (XlPP6, NM_001095920.1), and *Danio rerio* (DnPP6 NM_201005.1) and 95.71% identity with the *PP6* from *Bombyx mori* (BmPP6, NP_001040439) and *Drosophila melanogaster* (DmPP6, NM_078506.2) (S-2).

PP6 was upregulated in the midgut and fat body during metamorphosis

To investigate the relationship between *PP6* and the metamorphosis, the expression profiles of *PP6* in various tissues during larval development were examined. RT-PCR showed that *PP6* was expressed in all the tested tissues but with different expression profiles. In the midgut, the *PP6* transcript levels obviously increased after the larvae were metamorphically committed from the sixth-48 h to prepupal stage sixth-120 h compared with the fifth-instar larvae (Fig. 1a). The transcript levels in the fat body appeared similar in pattern to that in the midgut (Fig. 1b). In the hemocytes, the target bands were weaker and had no significant variation during larval development except at sixth-0 h during larvae ecdysis (Fig. 1c). These results suggest that the higher *PP6* transcripts are closely related with metamorphosis.

20E upregulated PP6 transcription

Given that the *PP6* gene expression profiles in the midgut and fat body tissues strongly correlated with metamorphosis, *PP6* expression was speculated to correlate with 20E hormonal regulation because at this stage the juvenile hormone is greatly decreased and 20E is increased (Riddiford et al. 2003). Results show that *PP6* was evidently upregulated 1 h after 20E injection compared with the control injected with DMSO, and the effect of such upregulation lasted for 12 h after injection (Fig. 2a). In

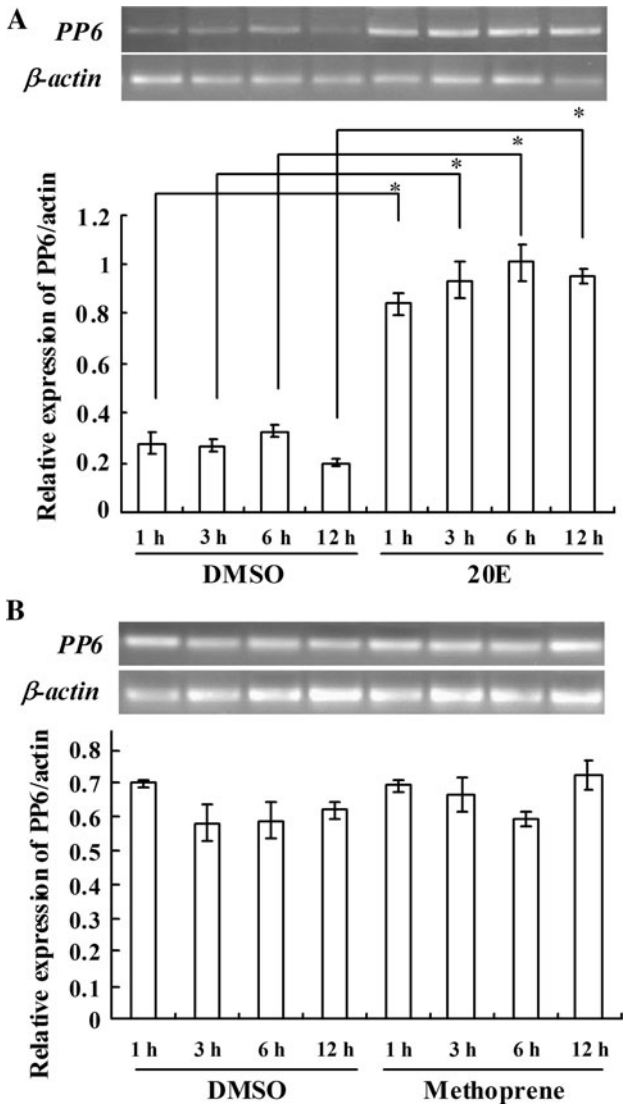

Fig. 2 Hormonal effects on *PP6* expression in the midgut. **a** Sixth instar 6 h larvae were injected with DMSO or 20E; **b** sixth instar 6 h larvae were injected with DMSO or methoprene. 1, 3, 6, and 12 are durations (h) after injection. *Asterisk* indicated significant differences with $P < 0.05$, analyzed by Student's *t* test. *β-Actin* was used as a quantitative control

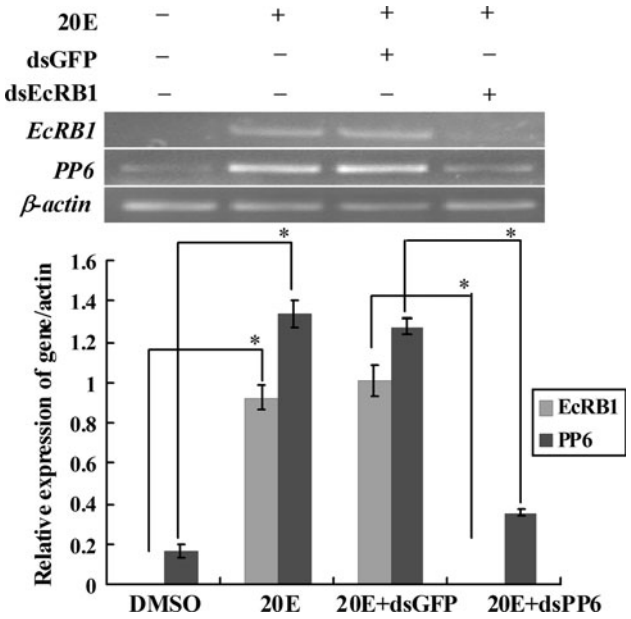

Fig. 3 20E upregulated *PP6* through its receptor EcRB1, analyzed by RT-PCR. *Asterisk* indicates significant differences with $P < 0.05$, analyzed by Student's *t* test. *β-Actin* was used as a quantitative control

contrast, juvenile hormone analogy methoprene had no effect on this gene compared with the control (Fig. 2b). These results suggest that *PP6* was upregulated by 20E in vivo, but not by juvenile hormone.

20E upregulated PP6 through its receptor EcRB1

To confirm *PP6* upregulation by 20E, the 20E receptor *EcRB1* on the *Helicoverpa* epidermal cell line HaEpi was knocked down. After *EcRB1* knockdown, the *PP6* gene was not upregulated by 20E (Fig. 3), suggesting 20E upregulated *PP6* through its receptor EcRB1.

PP6 is involved in midgut PCD

The effects after *PP6* knockdown by RNAi via dsPP6 injection into the larval hemocoel were examined to understand the function of PP6 in the larval midgut during metamorphosis. Immunohistochemistry analysis showed that PP6 was detected mainly in the larval midgut at the wandering stage during metamorphosis (sixth-96 h) after dsGFP injection as a negative control (Fig. 4a, green fluorescence). However, PP6 was not detected in the larval midgut at the wandering stage after dsPP6 injection (Fig. 4d), indicating that PP6 was knocked down. Structural comparison of the midgut after *PP6* knockdown showed that typical PCD occurred in the larval midgut under the control of dsGFP injection and that the larval midgut condenses and detaches from the newly formed imaginal midgut (Fig. 4b, c). In contrast, the larval midgut did not undergo PCD, which still kept the larval structure, and no imaginal midgut was formed after *PP6* knockdown (Fig. 4e, f). In addition, metamorphosis of these larvae was delayed for about 24 h. This evidence suggests that PP6 is involved in larval midgut PCD.

PP6 knockdown suppressed some genes involved in the 20E signaling pathway

The role of *PP6* in the 20E signaling pathway was examined in the *Helicoverpa* epidermal cell line HaEpi to

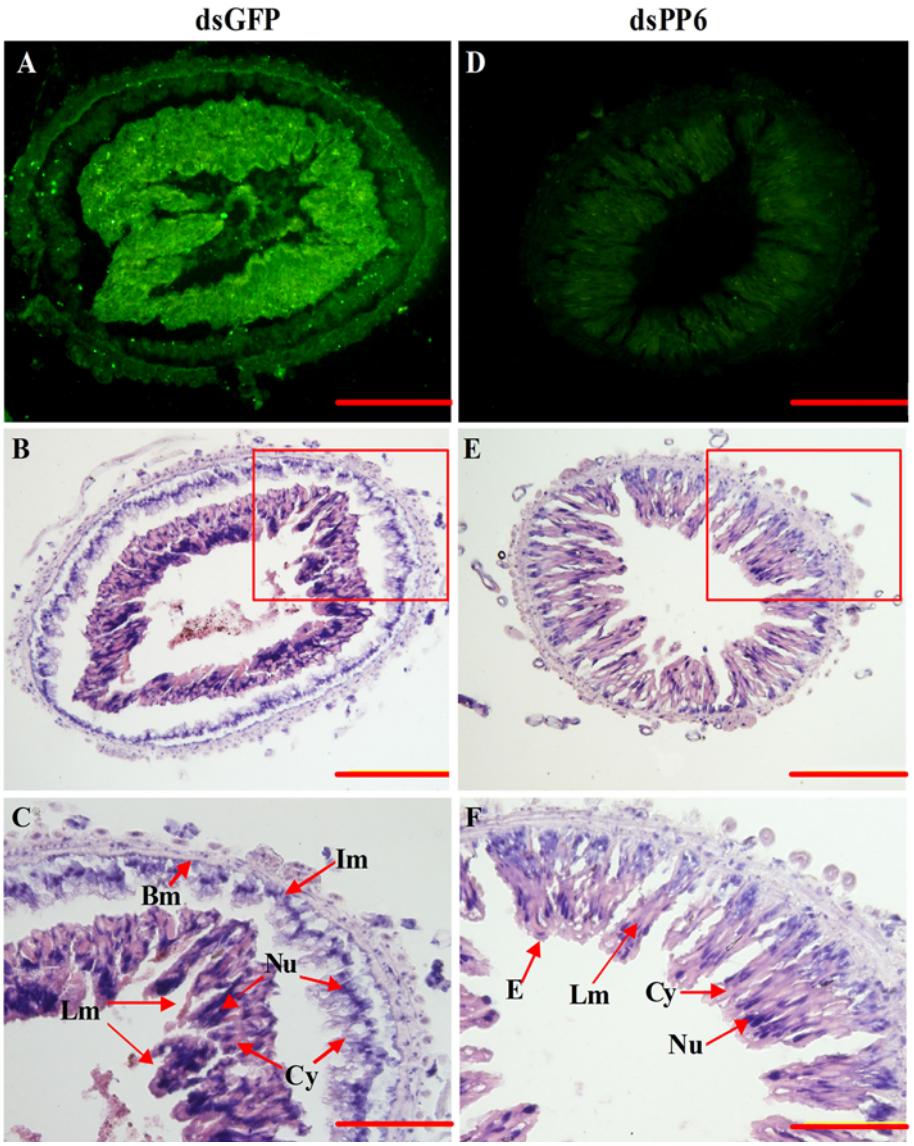

Fig. 4 PP6 is involved in midgut PCD. **a**, **d** Fluorescence immunochemistry shows the effects of dsPP6 on the midgut in the metamorphically committed larvae. PP6 stained with anti-PP6 and Alexa 488; *Scale bar* is 200 μm. **b–e** hematoxylin and eosin staining to show the midgut at sixth-96 h after knockdown of *PP6* by injection of *dsGFP* or *dsPP6*. **c–f** magnified squares in **b** and **e**. *Im* imaginal midgut, *Lm* larval midgut, *Nu* nucleus, *Cy* cytoplasm, *Bm* basement membrane, *E* enterocytes. *Scale bars* are 200 μm (in **b**, **e**), 50 μm (in **c**, **f**)

demonstrate the mechanism of *PP6* participation in the midgut PCD. The results show that knockdown of *PP6*, *EcRB1*, and *USP1* had little effect, whereas *E75b* and HHR3 were relatively decreased (Fig. 5a).

This phenomenon could also be observed in the larval experiments. After *PP6* knockdown by feeding ds*PP6*, *E75b and HHR3* expression were suppressed when *EcRB1* and *USP1* were not in effect (Fig. 5b). These results suggest that PP6 is involved in the induction of some genes such as *E75b* and *HHR3* downstream of *EcRB1* and *USP1* through the 20E signal transduction pathway.

PP6 knockdown suppressed some genes involved in PCD

Considering that the biological function of *PP6* is related to the midgut PCD, as shown by larval RNA interference analysis, some genes known to be involved in PCD were investigated. In the HaEpi cells, when *PP6* was knocked down, *Br, E74a*, and *caspase* were suppressed. However, *E93* and *rpr* did not change (Fig. 6a). Similarly, when *PP6* was knocked down in the larval interference experiments, *Br*, *E74a*, *E93*, *rpr*, and *caspase* were suppressed from the sixth-36 h to the 96 h (Fig. 6b), indicating that *PP6* was necessary for the transcription of the genes involved in midgut PCD.

Discussion

Programmed cell death is known to occur in the insect midgut during metamorphosis, and the steroid hormone 20E regulates this process. However, the mechanism of PCD regulation by 20E is still unclear. In the present study, the phosphatase gene *PP6* was demonstrated to be

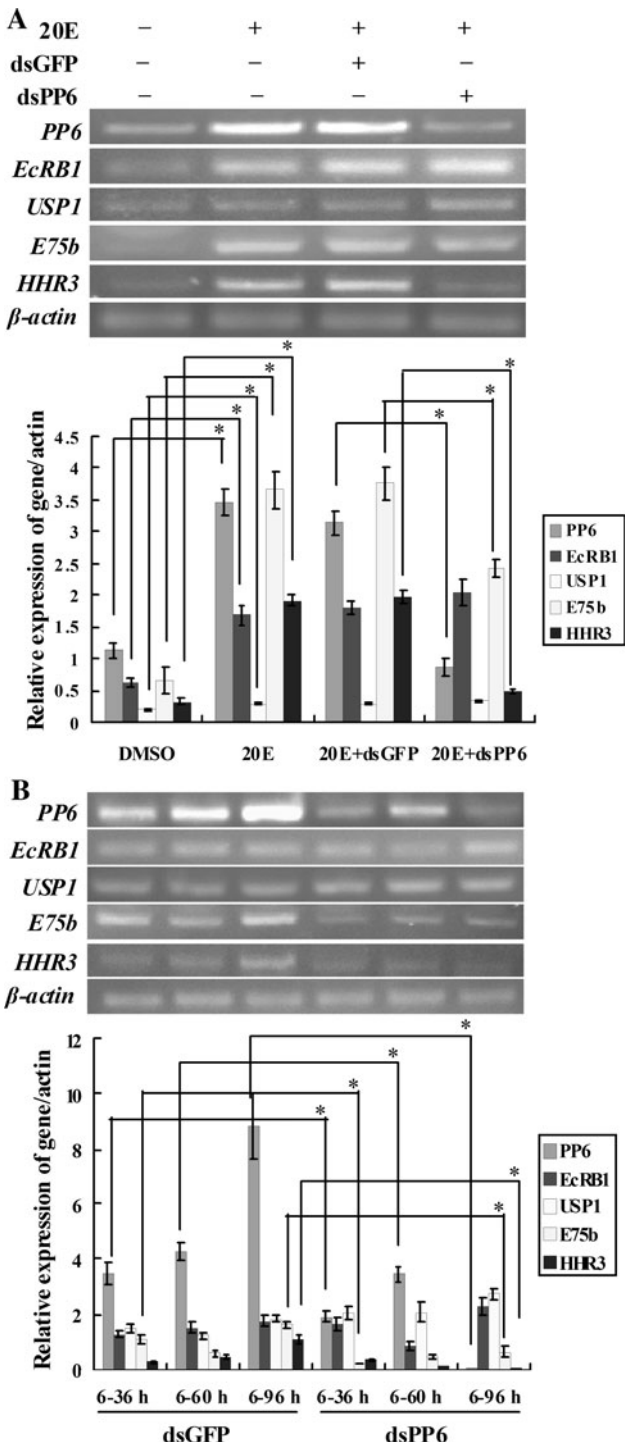

Fig. 5 Role of PP6 in the 20E signal transduction pathway. **a** *PP6* knockdown to examine the expression of genes involved in the 20E signaling pathway in HaEpi cells. **b** *PP6* knockdown to examine the expression of genes involved in the 20E signaling pathway in the larval midgut. *Asterisk* indicates significant differences with $P < 0.05$, analyzed by Student's *t* test. *β-Actin* was used as a quantitative control

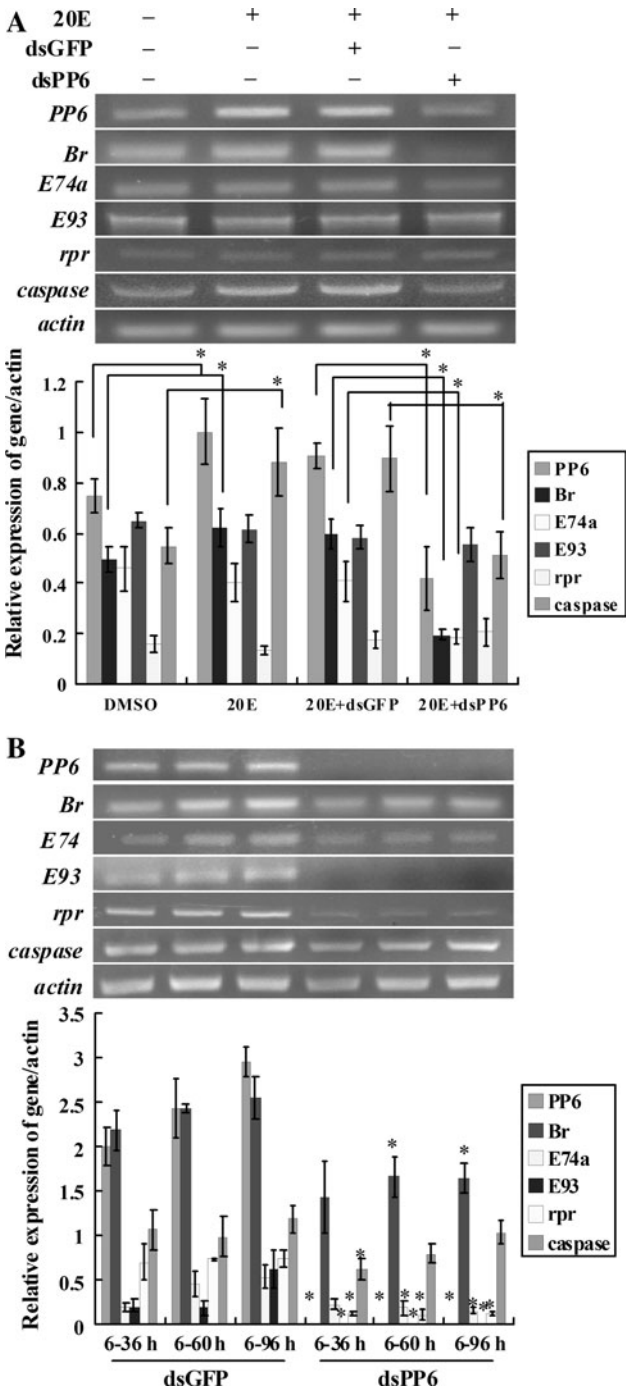

Fig. 6 Role of *PP6* in PCD. **a** *PP6* knockdown to examine the expression of genes involved in the PCD in HaEpi cells. **b** *PP6* knockdown to examine the expression of genes involved in PCD on the sixth instar larval midgut. *Asterisk* indicates significant differences with $P < 0.05$ (significant differences shows the genes in dsPP6 group with the genes in dsGFP group), analyzed by Student's *t* test. *β-Actin* was used as a quantitative control

upregulated during metamorphosis by 20E. *PP6* is involved in the larval midgut PCD during metamorphosis. This gene participates in the gene transcription in the 20E signaling pathway and in midgut PCD.

PP6 was necessary for the gene transcription in 20E signaling transduction

In the 20E signaling pathway, 20E first bound with its receptor EcR and the heterodimer USP to initiate the early gene transcriptions such as *E75b* and *HR3* transcriptions (Hiruma and Riddiford 2004) and then followed by some relative effector genes such as proteinases (Wei et al. 2007). 20E triggered *PP6*, which increased during metamorphosis and was necessary for the transcription of some gene such as *E75b* and *HHR3* in the 20E signaling pathway. This also indicated that 20E signaling pathway needs to regulate some protein dephosphorylation for some gene transcription. PP6 may be involved in protein dephosphorylation induced by 20E but the target proteins needs further study.

PP6 is necessary for the gene transcription during midgut PCD

Midgut PCD is regulated by a set of 20E-regulated genes. *BR* and *E74a* are upregulated by 20E and are the early genes involved in the midgut PCD during metamorphosis (Lee et al. 2000). These genes are essential for late cell death regulators *rpr*, *hid*, and *caspase* (Lee et al. 2000). The data show that PP6 can regulate midgut PCD by regulating the transcription of *BR*, *E74a*, *E93*, and *caspase*. The expression of some genes in the HaEpi cells did not change might because of the differences between the HaEpi cell line and the midgut.

There are three types of PCD in the developing vertebrate embryos (Schweichel and Merker 1973). The first is called apoptosis, which can be observed in the nuclei and cytoplasm of the dying cells, which degrade their contents (Kerr et al. 1972). The second is known as autophagy, which is usually observed in a group of similar cells or an entire tissue, wherein autophagic vacuoles are found in the cytoplasm (Kerr et al. 1972; Lee et al. 2002). The third one is called the non-lysosomal cell death, wherein the cell membrane becomes swollen and then the whole cell degenerates without lysosomal activity (Schweichel and Merker 1973). Autophagic cell death is the major form of cell death in the midgut (Denton et al. 2009). *E74a*, *Br*, and *E93* have been proven to be involved in the autophagic cell death of the midgut during metamorphosis (Lee et al. 2000), which may suggest that the PCD that occurs in *Helicoverpa* is autophagic cell death.

PP6 participated in the PCD in the midgut during metamorphosis

During larval–pupal transition, the midgut undergoes remodeling, which has been investigated in several insects such as *D. melanogaster*, *Aedes aegypti*, and *Heliothis virescens* (Cakouros et al. 2004; Nishiura et al. 2005). Midgut remodeling includes a series of physiological events. The most critical are the larval midgut PCD and the formation of a new imaginal midgut by intestinal stem cells (Parthasarathy and Palli 2007; Rabossi et al. 2004). Midgut PCD causes the larval midgut to condense to the yellow body, thus leading to decomposition to provide recycled material for the formation of the new midgut. The regenerative cells proliferate and differentiate into an imaginal midgut (Hakim et al. 2009). Although 20E is known to induce midgut PCD, the mechanism by which 20E triggers midgut PCD is not fully understood. The present experiments show that 20E upregulates PP6, and that PP6 participates in midgut PCD by regulating the transcription of some genes involved in 20E signal transduction or midgut PCD.

Previous studies have not yet determined the initiating events of midgut remodeling, larval midgut PCD, or the intestinal stem cell proliferation. The present data supports that the larval midgut PCD is the initiating event in midgut remodeling because in normal midgut remodeling, PP6 is expressed in the larval midgut where it regulates midgut PCD, causing the intestinal stem cells to proliferate and form the imaginal midgut. However, after PP6 knockdown, the larval midgut could not perform PCD, and the intestinal stem cell could not proliferate to form the imaginal midgut. Thus, PP6 plays an important role in the midgut remodeling during metamorphosis by participating in 20E signaling transduction and PCD.

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Conflict of interest The authors declare that they have no conflict of interest.

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