Eur. J. Entomol. **111**(4): 457–468, 2014
doi: 10.14411/eje.2014.064
ISSN 1210-5759 (print), 1802-8829 (online)

Identifications of *cytochrome c* and *Apaf-1* and their mRNA expressions under heat stress in insecticide-susceptible and -resistant *Plutella xylostella* (Lepidoptera: Plutellidae)

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Key words. Lepidoptera, Plutellidae, *Plutella xylostella*, diamondback moth, chlorpyrifos-resistant and -susceptible, c*ytochrome c*, *Apaf-1*, fitness

Abstract. To study the component and gene expressions of apoptosome in the mitochondrial-associated apoptotic pathway, *cytochrome c* and *Apaf-1* were identified in the diamondback moth (DBM), *Plutella xylostella*, collected from Fuzhou, China. The full-length cDNA of c*ytochrome c* comprised 873 bp, including a 327 bp ORF encoding a putative protein of 108 amino acids. Two full-length cDNAs of *Apaf-1*, *Apaf-1a* and *Apaf-1b*, were identified. *Apaf-1a* shared the same region of 5'-UTR (5'-untranslated region) (172 bp) and ORF (4,647 bp) of cDNA, but was 73 bp longer than *Apaf-1b* in the region of 3'-UTR of the cDNA. The ORF of *Apaf-1a* and *Apaf-1b* encoded a putative protein of 1,548 amino acids and shared 100% identity. No significant up-regulation of c*ytochrome c* mRNA expression was found in both the chlorpyrifos-resistant (R_R) and chlorpyrifos-susceptible (S_S) DBM. Compared with the control (at 25°C for 3 h), mRNA transcript levels of *Apaf-1a* and *Apaf-1b* increased with the increase of temperature and were significantly higher at 37, 42, 47 and 50°C than at 25 and 33.5°C in R_R DBM. However, the extent of up-regulation expression of *Apaf-1a* and *Apaf-1b* in S_S DBM was slight under heat stress except at 42°C. In general, significantly higher increase in the mRNA transcript level of *Apaf-1a* and *Apaf-1b* was found in R_R DBM than in S_S DBM. It has been suggested that significantly higher expression of *caspase-7* and lower biological fitness occurs in R_R DBM under heat stress, a suggestion confirmed in our previous publications, and a result which might be associated with their higher up-regulation expression of *Apaf-1a* and *Apaf-1b*.

INTRODUCTION

Apoptosis, the main form of programmed cell death, is an active biological mechanism in both pathological and physiological conditions. It is evolutionarily conserved, highly organized and genetically regulated in all multicellular organisms (Torkzadeh-Mahani et al., 2012). Apoptosis is a powerful way of deleting superfluous and unwanted cells, and plays a critical role in the balance of life and death during the development of embryo and normal tissue turnover (Qi et al., 2010). There are three main pathways of apoptosis, including a mitochondrial-associated apoptotic pathway (intrinsic), a death receptor pathway (extrinsic) and an endoplasmic reticulum signal transduction pathway (Cullen & Martin, 2009).

The mitochondrial-associated apoptotic pathway involves the apoptotic signal transduction of apoptosome (Cytochrome c-Apaf-1-Procaspase-9) and the apoptotic effector or "executioner" proteins involved in the caspase cascade (Liu et al., 2012a). Different kinds of cellular stresses such as heat shock, oxidative stresses and DNA-damaging reagents result in Cytochrome c releasing from mitochondria into the cytosol (Torkzadeh-Mahani et al., 2012). The binding of Cytochrome c to Apaf-1 (apoptotic protease-activating factor-1) induces conformational changes in Apaf-1 which unmask its nucleotide binding sites to dATP/ATP (Riedl & Salvesen, 2007). In the presence of dATP/ATP, a further conformational change in Apaf-1 results in exposure of its caspase recruitment domain (Arnoult et al., 2002). With the subsequent binding of Procaspase-9, the apoptosome is formed (Marek, 2013). The apoptosome facilitates the autoproteolytic activation of Procaspase-9, and active Caspase-9 is formed. The active Caspase-9, as an initiator caspase (transducers at the upstream of caspase cascade), then cleaves and activates the effector caspases (executioners at the downstream of caspase cascade) such as Caspase-3 and Caspase-7, which, involved as they are in the final enactment phase of apoptosis, cleave various cellular proteins and eventually lead to cell death (Qi et al., 2010). In the nematode *Caenorhabditis elegens*, three genes (*ced-3*, *ced-4* and *ced-9*) that control programmed cell death have been identified (Ellis & Horvitz, 1986). In contrast, in the fruit fly *Drosophila*, a unified nomenclature of Apaf-1 homologs has not been established, and the region has been variously labeled as Dark (Rodriguez et al., 1999), ARK (Zimmermann et al., 2002; Mills et al., 2006), HAC-1 (Zhou et al., 1999), or Dapaf-1 (Kanuka et al., 1999). During apoptosis, Dark may interact with the initiator caspase Dronc (the *C. elegens* CED-3 and mammalian Caspase-9 homologue) through CARD-CARD interactions (Dorstyn et al., 1999). The binding of Dronc to the

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Dark facilitates the Dronc cleavage at DrICE (an effector caspase) (Kumar, 2007; Dorstyn & Kumar, 2008), which in turn cleaves many cellular proteins thereby leading to the chain of steps ultimately involved in cell death.

However, whether Cytochrome c is involved in insect cell apoptosis has proven highly controversial. The results obtained in *Drosophila* showed that Cytochrome c might not actually be involved in apoptosis. In *Drosophila* SL2 cells, Cytochrome c was not found to be released from mitochondria into cytosol during apoptosis induced by UV, cycloheximide or actinomycin D (Zimmermann et al., 2002). Down-regulation or silencing of *cytochrome c* by RNA interference was found not to protect *Drosophila* SL2 cells from stress-induced apoptosis (Zimmermann et al., 2002). Furthermore, over-expression of Cytochrome c or addition of recombinant Cytochrome c to the extracts from *Drosophila* BG2 cells failed to increase Caspase activation (Dorstyn et al., 2004). Structural analysis of the Dark gene region complex demonstrated that Cytochrome c did not participate in the assembly of this complex in *Drosophila* (Yu et al., 2006). These studies indicated that Cytochrome c is not indispensable for the apoptosis program in this insect. In contrast from the situation in *Drosophila*, involvement of Cytochrome c in cell apoptosis of lepidopteran insects was demonstrated by accumulating evidence. For instance, the release of Cytochrome c from mitochondria into cytosol has been observed in many apoptotic lepidopteran cells, such as in Sf-9 cells of the moths *Spodoptera frugiperda* (Sahdev et al., 2003), SL-1 cells of *Spodoptera litura* (Liu et al., 2007, 2012b) and IPLB-LdFB cells of *Lymantria dispar* (Malagoli et al., 2005).

Antioxidants could prevent UV-induced apoptosis by inhibiting the release of Cytochrome c and Caspase activation in Sf-9 cells (Mohan et al., 2003). Addition of Cytochrome c and dATP to labial gland extracts from the moth *Manduca sexta* may induce an apparent apoptosis (Facey & Lockshin, 2010). The role of Apaf-1 in cell apoptosis has also been studied. Silencing of the Apaf-1 homolog ARK by RNA interference inhibited stress-induced apoptosis in *Drosophila* SL2 cells (Zimmermann et al., 2002). Caspase activity was lower in *D. melanogaster ark* mutants that were deficient for ARK function during the radiation-induced apoptosis (Mills et al., 2006). Over-expression of Apaf-1 in human embryonic kidney 293 cells was found to result in rapid Caspase-3 activation (Zou et al., 1997). Bcl-X_L (an antiapoptotic member of the Bcl-2 family) regulates Caspase-9 activation through Apaf-1 in human cells (Hu et al., 1998). Therefore, Cytochrome c and Apaf-1 are thought to be the critical players in the mitochondrial signal transduction pathway (Reubold & Eschenburg, 2012). However, *cytochrome c* has only been identified in several lepidopteran insects such as *Bombyx mori* (Zhang et al., 2010), *Danaus plexippus* (Zhan et al., 2011), *M. sexta* (Swanson et al., 1984), and *S. litura* (Gu et al., 2012). In addition, *Apaf-1* has been identified in *B. mori* (Zhang et al., 2010) and *D. plexippus* (Zhan et al., 2011).

At the present time, it is not known whether apoptosis is triggered by various environmental stresses, such as heat stress, UV radiation, toxicant exposure and oxidative stress (Beumer et al., 1997; Richburg, 2000). The release of Cytochrome c from the mitochondria into cytosol under UV stress has been found in apoptotic *S. litura* cells (Shan et al., 2009), whilst ARK is found to be elevated upon UV treatment in *D. melanogaster* (Ujfaludi et al., 2007). The expression level of *Bm-Apaf-1* is up-regulated in midgut epithelial cells of *B. mori* larvae treated with Cry1Aa (an insecticidal protein produced by the bacteria, *Bacillus thuringiensis*) (Tanaka et al., 2012). Pretreatment with lithium and prostaglandin A_1, and in combination decrease the levels of *Apaf-1* in rats (Xu et al., 2007). However, knowledge about signal transduction and expression of the apoptosome has been limited in other insect species except *Drosophila*, in particular, in insecticide-resistant and -susceptible insect strains.

Plutella xylostella (diamondback moth, DBM) is a destructive cosmopolitan pest of cruciferous crops. In our previous works, resistance levels to several classical insecticides declined sharply during the summer in field populations of this pest moth species, but maintained a high level during spring and autumn (Wu & Jiang, 2002). Furthermore, the resistance level to methamidophos or avermectin declined significantly after DBM were reared at constant 33.5°C for one generation (Liu et al., 2008; Zhuang et al., 2010). Compared to insecticide-susceptible DBM, insecticide-resistant moths display significantly lower population growth tendency indices (*I*), fecundity and up-regulation expression of *hsp70* (Liu et al., 2008), and a higher up-regulation expression of *caspase-7* (Zhuang et al., 2011) under heat stress. It was earlier suggested (Liu et al., 2008; Zhuang et al., 2011) that low ecological and physiological fitness in insecticide-resistant DBM caused by high temperature might be involved in the sharp decline of insecticide resistance observed under heat stress, and that the development of insecticide resistance might indeed be affected by heat stress in this species. However, identifications of *cytochrome c* and *Apaf-1* genes in DBM and the study on the expression profiles of *cytochrome c* and *Apaf-1* genes in both insecticide-resistant and -susceptible insect strains have to date not be found. Because Cytochrome c and Apaf-1 are important components of the apoptosome in the mitochondrial-associated apoptosome pathway, perhaps also in *Drosophila*, the objective of this study was to identify the *cytochrome c* and *Apaf-1* genes, and to investigate their expression profiles under heat stress in order to evaluate the fitness cost as affected by heat stress in chlorpyrifos-resistant and -susceptible moths.

MATERIAL AND METHODS

Source of insect

A starting population of DBM was collected from the commercial crucifer fields located at Shangjie, Fujian, China in November 2005 and subsequently reared on *Brassica oleracea* in an insecticide-free field insectary at FAFU, Fujian, China for one year (i.e. about 18 generations). 800 pupae each were randomly chosen from the reared population in November 2006 and then reared separately in two field insectaries (A and B). These, of dimensions 4 m × 2 m × 4 m, were made of stainless-steel netting

with a glass roof to prevent contamination by external DBM populations. Insects in insectary A were not treated with any insecticides and were highly susceptible to chlorpyrifos after November 2008, and are hereafter defined as the chlorpyrifos-susceptible population, S_i. In insectary B, insects were treated with chlorpyrifos for about 36 generations (during November 2006–2008). Insects derived from this insectary were highly resistant to chlorpyrifos after November 2008, and are hereafter defined as being chlorpyrifos-resistant population, R_c. This genotype was selected on the basis of resistance to chlorpyrifos, so that successive generations maintained a high level of resistance in insectary B after November 2008. S_S was created by crossing a male and female insect randomly chosen from the S_i population. R_R was generated by treating R_c populations for several generations with a dose of chlorpyrifos that resulted in ~97% DBM mortality at 25°C. Based on our previous results, a total of 3 nucleotide substitutions of the *Acetylcholinesterase 1* (*Ace-1*) gene (resulting in three amino acid coding mutations, i.e., A201S, G227A and A441G) were found in resistant DBM from Fuzhou (GenBank: JQ085429 and JQ085428). Among the three sites, mutations A201S and G227A were located at the Acetylcholinesterase (AChE) active site, and are thought to be involved in the resistance of DBM to organophosphate insecticides (Lee et al., 2007). In contrast, the S_S population was homozygote susceptible with 100% of the *SS* genotype at mutation sites of A201S and G227A of the resistance allele *Ace-1*, while R_R population was homozygote resistant with 100% *RR* at A201S and G227A sites according to our analysis using a large number of individual DBM specimens (more than 1000 DBM individuals for each population). The difference in chlorpyrifos-resistance was >100-fold between R_R and S_S.

Cloning and nucleotide sequencing of *cytochrome c* and *Apaf-1*

Initial fragments cloning of *cytochrome c* and *Apaf-1*

Total RNA was extracted from individual whole bodies of adult DBM using an RNA Simple Total RNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). The first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™III First-Strand System (Life Technologies, Carlsbad, California, USA). For cloning *cytochrome c*, an initial fragment was amplified using PCR using degenerate primers (cyt c-F1 and cyt c-R1) (Table 1), based on conserved sequences of several lepidopteran insects (*B. mori*: ACF41193.1; *D. plexippus*: EHJ77081.1; *M. sexta*: AAA29308.1 and *S. litura*: AFS31125.1). PCR conditions involved a 94°C denaturation step for 5 min, 15 cycles in a touch down program (94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a 0.6°C decrease of annealing temperature per cycle), followed by 25 cycles of 94°C for 30 s, 51°C for 30 s, an extension step at 72°C for 30 s, and a final extension step at 72°C for 7 min. For cloning the *Apaf-1*, fragment 1 and fragment 2 were amplified by PCR using specific primers: Apaf-1-F1 and Apaf-1-R1 (for fragment 1) and Apaf-1-F2 and Apaf-1-R2 (for fragment 2) (Table 2) based on the whole genome of DBM available in GenBank (AHIO01014284.1 and AHIO01014285.1). PCR conditions for fragment 1 were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 3 min, finally 72°C for 10 min. The PCR conditions for fragment 2 were the same as for fragment 1, except the primer annealing at 52°C.

Rapid Amplification of cDNA Ends (RACE) of *cytochrome c* and *Apaf-1*

For 3'- and 5'-RACE, the first-strand cDNAs were separately constructed from 1 μg of total RNA according to the SMART™ RACE cDNA Amplification Kit (TaKaRa Bio Inc., Otsu, Japan). For *cytochrome c*, 3'-RACE was performed using cyt c 3'-1 and a Universal Primer A Mix (UPM) (Table 1). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, finally 72°C for 10 min. 5'-RACE was performed using UPM and cyt c 5'-1 (Table 1). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, finally 72°C for 7 min. Subsequently, the PCR product was used for re-amplification using the nested universal primer (NUP) and cyt c 5'-2 (Table 1). PCR conditions were the same, except for the annealing step (57.5°C). For *Apaf-1*, 3'-RACE was performed using Apaf-1 3'-1 (Table 2) and UPM. PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 45 s, finally 72°C for 7 min. Subsequently, the PCR product was used for re-amplification using Apaf-1 3'-2 (Table 2) and NUP. Fragment 3 (from *Apaf-1a*) and fragment 4 (from *Apaf-1b*) were obtained by nested PCR under the conditions, except for the annealing step (54°C). 5'-RACE was performed using UPM and Apaf-1 5'-1 (Table 2). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, finally 72°C for 10 min. Subsequently, the PCR product was used for

Table 1. Sequences of primers used for cloning *cytochrome c* cDNA and qPCR of DBM (*Plutella xylostella*).

Primers	Sequences (5'-3')	Product size (bp)
For cDNA sequencing		
cyt c-F1	5'-ATGGGTGTMCCTGCAGGAAAC-3'	
cyt c-R1	5'-TTTKAGRTAGGCRATRAGRTCWGC-3'	312
cyt c 3'-1	5'-CGAAGGGAATCTCATGGAAT-3'	570
cyt c 5'-1	5'-CTTCTTGGGGTTCTCCAGGTA-3'	
cyt c 5'-2	5'-GCCAAAGAAGCCATTTAGGTT-3'	248
UPM-Long	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'	
UPM-Short	5'-CTAATACGACTCACTATAGGGC-3'	
NUP	5'-AAGCAGTGGTATCAACGCAGAGT-3'	
cyt c-F	5'-GACAATTATTTCGTCGTC-3'	
cyt c-R	5'-CCACAGAATGAATATCTAAC-3'	720
For qPCR		
β-actin-F	5'-ACCGGTATCG TGCTGGACTC-3'	
β-actin-R	5'-GCCATCTCCTGCTCGAAGTC-3'	239
qcyt c-F	5'-CCCAGTGCCACACAGTTG-3'	
qcyt c-R	5'-GCCTTCTTCAGACCAGCG-3'	220

Fig. 1. The sequences alignment of fragment 3 (from *Apaf-1a*) and fragment 4 (from *Apaf-1b*) of *Plutella xylostella* (DBM). The stop codon TAG was boxed.

re-amplification using NUP and Apaf-1 5'-2 (Table 2). Fragment 5 was obtained by nested PCR whose conditions were the same.

Cloning ORF of *cytochrome c* and *Apaf-1*

For 3'-RACE of *Apaf-1*, Fragment 3 (from *Apaf-1a*) and fragment 4 (from *Apaf-1b*) were obtained. Therefore, the full-length cDNAs of *cytochrome c*, *Apaf-1a* and *Apaf-1b* were edited and assembled using the initial fragments and the fragments obtained from 3'- and 5'-RACE. Because fragment 3 from *Apaf-1a* was 73 nucleotides longer than fragment 4 from *Apaf-1b* at 3'-untranslated region (3'-UTR) (Fig. 1), *Apaf-1a* and *Apaf-1b* shared the same region of the open reading frame (ORF). To confirm the full-length of sequences, cyt c-F, cyt c-R (Table 1) and Apaf-1-F, Apaf-1-R (Table 2) were used to amplify the ORF of *cytochrome c* and *Apaf-1a* (or *Apaf-1b*), respectively. For *cytochrome c*, PCR conditions used were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 1 min, finally 72°C for 7 min. For *Apaf-1a* (or *Apaf-1b*), PCR conditions used were 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 5 min, finally 72°C for 10 min.

The initial fragments, 3'- and 5'-RACE fragments and the ORF fragments were cloned and sequenced by Shanghai Biosune Biotechnology Co., Ltd., Shanghai, China.

Real-time quantitative PCR (qPCR) for expression of *cytochrome c* and *Apaf-1* after heat stress

Temperature shock

DBM adults emerged at 25°C and were incubated at 33.5, 37, 42, 47 or 50°C for 3 h, respectively. 33.5°C was chosen because the life-table parameters of both insecticide-resistant and -susceptible DBM were significantly different at this temperature as shown in our previous experiments (Liu et al., 2008). 47°C and 50°C were chosen to determine the response of DBM to the extreme high temperature. After heat-stress treatment, the survival rates of adults were 100% (at 42°C or lower than 42°C for 3 h), about 70% (at 47°C for 3 h) or 40% (at 50°C for 3 h). Living adults surviving after heat stress were allowed to recover for 1 h at 25°C before they were used in the experiments involving detection of mRNA expression (as below).

Table 2. Sequences of primers used for cloning *Apaf-1* cDNA and qPCR of DBM (*Plutella xylostella*).

Primers	Sequences (5'-3')	Product size (bp)
For cDNA sequencing		
Apaf-1-F1	5'-CGTGGACACTCTACAGAAGG-3'	
Apaf-1-R1	5'-CACTTTATTGCCCAGGAA-3'	1396
Apaf-1-F2	5'-ATTTGAATCTGAAGTTCCTGGG-3'	
Apaf-1-R2	5'-TTGTCCACGGTGACGAAA-3'	3019
Apaf-1 3'-1	5'-GTCGGGAATGAACGCTAAG-3'	
Apaf-1 3'-2	5'-AGTTCTGCTGTTTCGTCACC-3'	315 and 242
Apaf-1 5'-1	5'-CCTTATTCTCGCACAGTTGA-3'	
Apaf-1 5'-2	5'-CATAGTGGTCGGGCAGTC-3'	524
Apaf-1-F	5'-TGTGGATTGTGAATAAATAACC-3'	
Apaf-1-R	5'-ATAAAAACGCTAGGCTATGC-3'	4806
For qPCR		
qApaf-1-F [a]	5'-CTTCTGGCTGAACTTGGGC-3'	
qApaf-1-R [a]	5'-GCTAGGGAATGGCTGTCGT-3'	168
qApaf-1a-F [b]	5'-TCATAGCACACACTGCCACAC-3'	
qApaf-1a-R [b]	5'-GAGGGTTAATGTCCAATCATT-3'	182

[a] The full-length cDNAs of *Apaf-1a* and *Apaf-1b* were 5,054 and 4,981 bp long, respectively. The sequences of the 5'-UTR for the two genes were the same. The nucleobase position of ORF was 173–4,819 bp for both *Apaf-1a* and *Apaf-1b*. The qApaf-1-F and qApaf-1-R, which were used for detecting the mRNA expression of the two genes, were designed based on the ORF sequences of the two genes at the nucleobase position 691–709 bp (for qApaf-1-F) and 840–858 bp (for qApaf-1-R), respectively. [b] qApaf-1a-F and qApaf-1a-R designed based on the different 3'-UTR of the two genes were at the nucleobase position 4,814–4,834 bp (for qApaf-1a-F) and 4,975–4,995 bp (for qApaf-1a-R) of *Apaf-1a*, respectively. Because *Apaf-1a* showed 73 bp longer than *Apaf-1b* in the region of 3'-UTR of cDNA, the primer qApaf-1a-R could only be found in *Apaf-1a*. Therefore, the primers qApaf-1a-F and qApaf-1a-R were used for detecting the mRNA expression of *Apaf-1a*.

Fig. 2. The sequences alignment of Cytochrome c in *Plutella xylostella* with its homologs from several species. The protein alignment was created using Lasergene v7.1 (DNASTAR, Inc., Madison, Wisconsin, USA) with the following sequences: *P. xylostella* (KC507801); *Bombyx mori* (ACF41193.1); *Spodoptera litura* (AFS31125.1); *Danaus plexippus* (EHJ77081.1); *Papilio polytes* (BAM19277.1); *Papilio xuthus* (BAM18013.1); *Drosophila melanogaster* (NP_477176.1); *Mus musculus* (NP_031834.1); *Homo sapiens* (NP_061820.1). Solid black box represented residues of homologs identical to those of *Plutella xylostella* exactly. White box represented residues of homologs different from those of *Plutella xylostella*.

Determination of mRNA expression

The primers used for qPCR of *cytochrome c* and *Apaf-1* are listed in Tables 1 and 2. Because two *Apaf-1*, i.e., *Apaf-1a and Apaf-1b*, were identified, the mRNA expression of *Apaf-1a* and *Apaf-1a* plus *Apaf-1b* were determined, respectively, in order to compare the contributions of the two *Apaf-1* in gene expression. The full-length cDNA of *Apaf-1a* and *Apaf-1b* was 5,054 bp and 4,981 bp, respectively. The two genes had the same sequence in ORF (4,647 bp encoding a putative protein of 1,548 amino acids) and 5'-untranslated region (5'-UTR) of cDNA, but different sequence in 3'-UTR of cDNA, i.e., the 3'-UTR of *Apaf-1a* was 73 bp longer than that of *Apaf-1b* (Fig. 1). Therefore, two pairs of primers, qApaf-1-F and qApaf-1-R, were designed for the assay of *Apaf-1a* plus *Apaf-1b* mRNA expression and qApaf-1a-F and qApaf-1a-R for the assay of *Apaf-1a* mRNA expression (Table 2). qApaf-1-F and qApaf-1-R were designed based on the ORF sequences because the two genes had the same ORF sequence. qApaf-1a-F and qApaf-1a-R were designed based on the alone sequence of 3'-UTR of *Apaf-1a*, where the sequence of *Apaf-1a* was of 73 bp longer than that of *Apaf-1b.*

The total RNA was extracted using the RNA Simple Total RNA Extraction Kit (Tiangen Biotech Co., Ltd.), and cDNA was synthesized from 1 μg of total RNA using the SuperScript™III First-Strand System (Life Technologies). The mRNA expression was detected by qPCR using SYBR Premix Ex Taq™ Kit (TaKaRa Bio Inc.). The mRNA expression level of target gene (*cytochrome c*, *Apaf-1a*, and *Apaf-1a* plus *Apaf-1b*) was normalized to that of *β*-actin (housekeeping gene). The qPCR was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) with each sample prepared in triplicates. The program for qPCR was as follows: 95°C for 10 s; 40 cycles of 95°C for 6 s, 60°C for 25 s, read plate 10 s. Lastly, a melting curve analysis was performed by heating the PCR products from 75°C to 95°C (0.2°C per cycle hold 2 s). The expression level of each gene was calculated according to the Ct (threshold cycle) and equation of the standard curve. Hence, the normalized expression value of the target gene was calculated by comparing the expression value of the target gene with that of a reference gene (i.e. *β*-actin) (Larionov et al., 2005; Zhuang et al., 2011). All data thus obtained from qPCR were analyzed using the Statistical Product and Service Solutions (SPSS) program. mRNA expression was replicated at least three times with 12 insect individuals per replication.

RESULTS

Sequence and analysis of *cytochrome c*, *Apaf-1a* and *Apaf-1b*

Based on the initial fragment and 3'- and 5'-RACE amplification fragments, a full-length cDNA sequence of *cytochrome c* was edited and assembled. *Cytochrome c*

Table 3. The positions of three functional domains in amino acid sequences of Apaf-1.

	CARD	NOD	WD40 repeats
P. xylostella	1–83	121–431	632–668, 671–711, 1091–1131, 1264–1303, 1309–1350
B. mori	48–135	164–473	629–670, 1034–1073, 1260–1298
D. plexippus	1–88	120–424	623–659, 850–885, 888–937, 1044–1084, 1086–1124, 1268–1306, 1309–1367
D. melanogaster	1–95	129–397	638–679, 754–792, 848–886, 1178–1216, 1221–1259
C. elegans	2–89	133–440	
H. sapiens	6–90	118–403	593–632, 635–674, 677–718, 721–760, 776–814, 817–856, 898–935, 939–977, 979–1017, 1020–1059, 1062–1101, 1113–1149

Notes: Sequences included *Plutella xylostella* (DBM) (KC588901); *Bombyx mori* (NP_001186937.1); *Danaus plexippus* (EHJ68576.1); *Drosophila melanogaster* (AAF07207.2); *Caenorhabditis elegans* (CCD66782.1); *Homo sapiens* (EAW97605.1). CARD – the N-terminal caspase recruitment domain; NOD – the central nucleotide-binding oligomerization domain and the C-terminal multiple WD40 repeats.

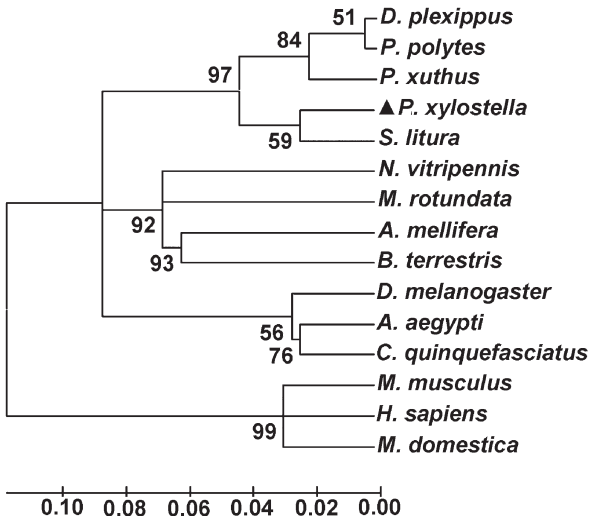

Fig. 3. The phylogenetic analysis of Cytochrome c in *Plutella xylostella* and other species using the neighbour-joining method (MEGA 4.0). The bootstrap cut-off value was 50%. The Cytochrome c of *P. xylostella* identified in this study was labelled with ▲. Sequences were *Aedes aegypti* (XP_001649236.1); *Apis mellifera* (NP_001170961.1); *Bombus terrestris* (XP_003394648.1); *Culex quinquefasciatus* (XP_001869210.1); *Drosophila melanogaster* (NP_477176.1); *Danaus plexippus* (EHJ77081.1); *Homo sapiens* (NP_061820.1); *Megachile rotundata* (XP_003701289.1); *Monodelphis domestica* (XP_001363248.1); *Mus musculus* (NP_031834.1); *Nasonia vitripennis* (XP_001606272.1); *Papilio polytes* (BAM19277.1); *Papilio xuthus* (BAM18013.1); *Plutella xylostella* (KC507801); *Spodoptera litura* (AFS31125.1).

(KC507801) had a full length of 873 bp, including a 327 bp ORF encoding a putative protein of 108 amino acids with the predicted molecular mass of 11.74 kDa and isoelectric point of 9.62. For 3'-RACE of *Apaf-1*, two fragments (fragment 3 from *Apaf-1a*: 315 bp; fragment 4 from *Apaf-1b*: 242 bp) were obtained (Fig. 1). Therefore, based on the initial fragments (fragment 1: 1,396 bp; fragment 2: 3,019 bp) and fragments obtained from 3'- and 5'-RACE (fragment 5: 524 bp), two full-length cDNA sequences of *Apaf-1* (*Apaf-1a* and *Apaf-1b*) were obtained by editing and assembling those fragments. *Apaf-1a* (KC588901) had a full length of 5,054 bp, including a 4,647 bp ORF encoding a putative protein of 1,548 amino acids with the predicted molecular mass of 175.96 kDa and isoelectric point of 6.85. Similarly, *Apaf-1b* (KF318722) had a full length of 4,981 bp with a 4,647 bp ORF encoding a putative protein also of 1,548 amino acids with a molecular mass of 175.96 kDa and isoelectric point of 6.85. The deduced amino acid sequences from *Apaf-1a* and *Apaf-1b* shared 100% identity.

Multiple protein alignment of Cytochrome c in *P. xylostella* with its homologs showed that among other insects, it showed 93.5% identity to Cytochrome c of *B. mori* and *S. litura*, 90.7% to *D. plexippus* and *Papilio polytes*, 86.3% to *P. xuthus*, 85.2% to *D. melanogaster*, whilst in other higher eukaryotes, it shared 80.8% identity to the house mouse, *Mus musculus* and 74.0% to humans, *Homo sapiens* (Fig. 2). Phylogenetic analysis revealed that Cytochrome c of *P. xylostella* formed a sister subgroup with other lepidopteran insects (Fig. 3).

Protein structure prediction (http://smart.embl-heidelberg.de/) showed that Apaf-1a or Apaf-1b in *P. xylostella* comprised three functional domains: the N-terminal caspase recruitment domain (CARD) (1-83 aa), the central nucleotide-binding oligomerization domain (NOD) (121–431 aa), and the C-terminal multiple WD40 repeats (Table 3). Multiple protein alignment of Apaf-1a or Apaf-1b in *P. xylostella* with its homologs showed that it had 55.8% identity to Apaf-1 of *B. mori*, 54.9% to *D. plexippus*, 15.5% to *D. melanogaster*, 20.6% to *M. musculus* and 20.0% to *H. sapiens* (Fig. 4). Phylogenetic analysis revealed that Apaf-1a or Apaf-1b of *P. xylostella* formed a sister subgroup with *B. mori* and *D. plexippus* (Fig. 5).

Expression of *cytochrome c*, *Apaf-1a* and *Apaf-1b* under heat stress

In comparison to the situation at 25°C for 3 h, no great variations of the expression of *cytochrome c* were found after heat stress (33.5–50°C for 3 h) in R_R DBM, although the mRNA transcript level increased slightly at 42°C for 3 h. Similar situations were found in the mRNA expression levels of *cytochrome c* of S_S DBM, although the mRNA expression levels of *cytochrome c* at 25, 37 and 42°C for 3 h were higher than those at 33.5, 47 and 50°C for 3 h. The mRNA expression levels of *cytochrome c* in R_R and S_S DBM were similar in general, except for higher mRNA expression levels in S_S DBM than that in R_R DBM at 25 and 37°C for 3 h (Fig. 6). In the case of *Apaf-1a* expression, compared to the control (25°C for 3 h), *Apaf-1a* showed similar mRNA transcript levels after exposure to 33.5, 37 and 42°C for 3 h, but increased significantly after exposure to 47 and 50°C for 3 h in R_R DBM. In contrast, in S_S DBM and compared to the control (25°C for 3 h), *Apaf-1a* mRNA transcript levels did not change greatly at 33.5 and 37°C but increased significantly after exposure to 42, 47 and 50°C for 3 h (Fig. 6). In the case of the expression of *Apaf-1a* plus *Apaf-1b* and compared to the control (at 25°C for 3 h), mRNA transcript levels of the two *Apaf-1* genes increased with the increase of treatment temperature and were significantly higher at 37, 42, 47 and 50°C than at 25 and 33.5°C in R_R DBM. Contrastingly, the extent of up-regulation expression of the two *Apaf-1* genes in S_S DBM was slight under heat stress, except at 42°C. In general, significantly higher up-regulation expression of *Apaf-1a* plus *Apaf-1b* was found in R_R DBM compared with that in S_S DBM (Duncan's-test, $P \leq 0.05$) (Fig. 6). To study the contributions of *Apaf-1a* and *Apaf-1b* in mRNA expression induced by heat stress, the ratios of the mRNA expression between the high temperature treatment and control (25°C) were calculated. Compared to that at 25°C, the mRNA expressions of *Apaf-1a* plus *Apaf-1b* increased 3.2-fold at 42°C and 3.9-fold at 47°C in R_R DBM, and 3.6-fold at 42°C and 1.7-fold at 47°C in S_S DBM, respectively. On the other hand, compared to the situation at 25°C, mRNA expressions of *Apaf-1a* did not increase at 42°C but increased some 1.8-fold at 47°C in R_R DBM, and 2.8-fold at 42°C and 2.5-fold at 47°C in S_S DBM, respectively (Fig. 6).

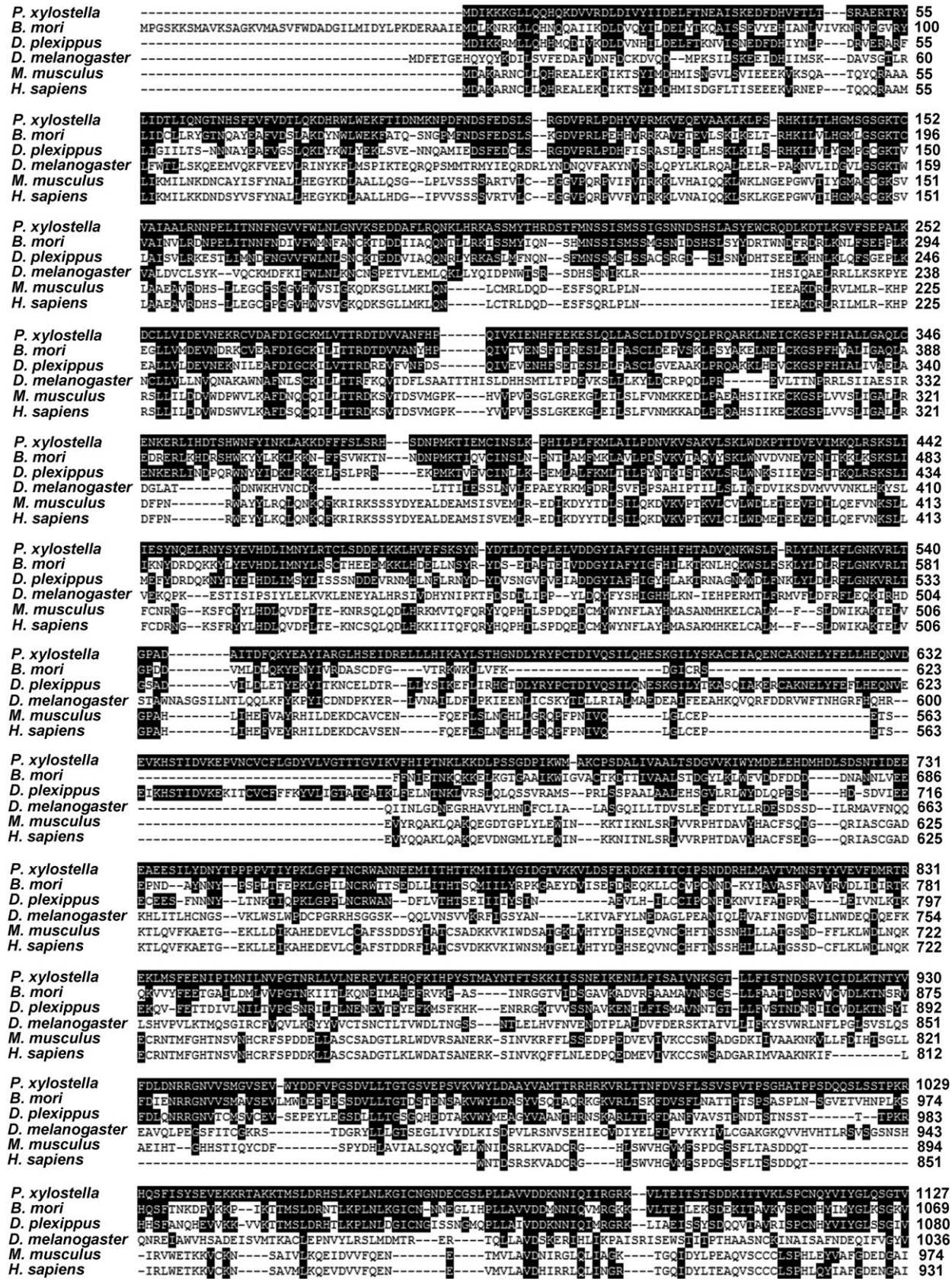

Fig. 4 (continued on next page). The sequences alignment of Apaf-1a (or Apaf-1b) in *Plutella xylostella* with its homologs from several species. The protein alignment was created using Lasergene v7.1 (DNASTAR, Inc.) with the following sequences: *P. xylostella* (KC588901); *Bombyx mori* (NP_001186937.1); *Danaus plexippus* (EHJ68576.1); *Drosophila melanogaster* (AAF07207.2); *Mus musculus* (AAC62458.1); *Homo sapiens* (EAW97605.1). The deduced amino acid sequences from *Apaf-1a* and *Apaf-1b* shared 100% identity. Solid black boxes represent residues of homologs identical to those of *Plutella xylostella* exactly. White boxes represent residues of homologs different from those of *Plutella xylostella*.

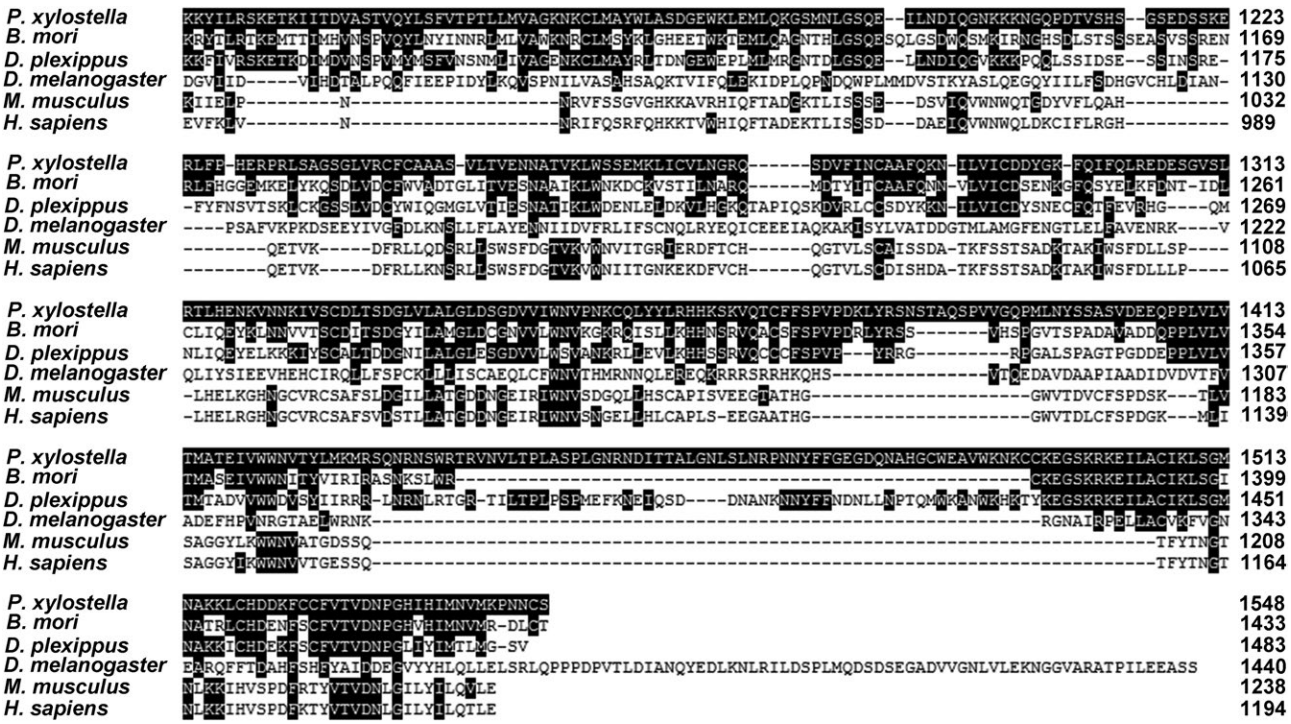

Fig. 4 (continued).

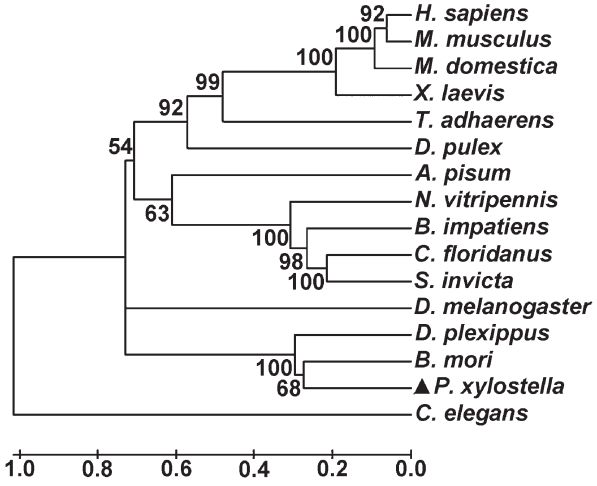

Fig. 5. The phylogenetic analysis of Apaf-1a (or Apaf-1b) in *Plutella xylostella* and other species using the neighbour-joining method (MEGA 4.0). The bootstrap cut-off value was 50%. The Apaf-1a of *P. xylostella* identified in this study was labeled with ▲. Sequences were *Acyrthosiphon pisum* (XP_003246649.1); *Bombus impatiens* (XP_003488903.1); *Bombyx mori* (NP_001186937.1); *Caenorhabditis elegans* (CCD66782.1); *Camponotus floridanus* (EFN60982.1); *Danaus plexippus* (EHJ68576.1); *Daphnia pulex* (EFX74325.1); *Drosophila melanogaster* (AAF07207.2); *Homo sapiens* (EAW97605.1); *Monodelphis domestica* (XP_001365091.1); *Mus musculus* (AAC62458.1); *Nasonia vitripennis* (XP_003427041.1); *Plutella xylostella* (KC588901); *Solenopsis invicta* (EFZ20566.1); *Trichoplax adhaerens* (XP_002117644.1); *Xenopus laevis* (NP_001085834.1).

DISCUSSION

In this study, we have cloned and identified the full-length cDNAs of *cytochrome c*, *Apaf-1a* and *Apaf-1b* from the diamondback moth, *P. xylostella*. Although amino acid sequences of *Apaf-1a* and *Apaf-1b* shared 100% identity, their 3´-UTRs were different (Fig. 1). In many organisms, especially higher eukaryotes, because of the presence of multiple polyadenylation sites, most of their genes (more than 50% of human genes) can produce multiple mRNA isoforms derived from alternative polyadenylation (APA) (Dickson & Wilusz, 2010; Proudfoot, 2011). According to the locations of multiple polyadenylation sites, APA can be divided into two types: coding region-APA (CR-APA) and untranslated region-APA (UTR-APA). In the first type, the locations of multiple polyadenylation sites were found to be in internal introns (or exons), and therefore this type will generate different protein isoforms. In the second type, the locations of multiple polyadenylation sites were always in the 3´-UTR, and thus resulted in mRNA isoforms differing only in terms of the length of 3´-UTR, but encoding the same protein (Di Giammartino et al., 2011). Therefore, *Apaf-1a* and *Apaf-1b* were mRNA isoforms derived from UTR-APA.

In addition to the nematode *C. elegans*, Apaf-1 comprises three functional domains, i.e., the N-terminal caspase recruitment domain (CARD), the central nucleotide-binding oligomerization domain (NOD), and the C-terminal multiple WD40 repeats (Qi et al., 2010; Reubold et al., 2011). The CARD is responsible for recruiting Procaspase-9, whilst the NOD domain is involved in dATP/ATP-dependent oligomerization, and the WD40 repeats contribute to the binding of Cytochrome c (Fadeel et al., 2008). Three functional domains of Apaf-1 shown in Table 3 indicate

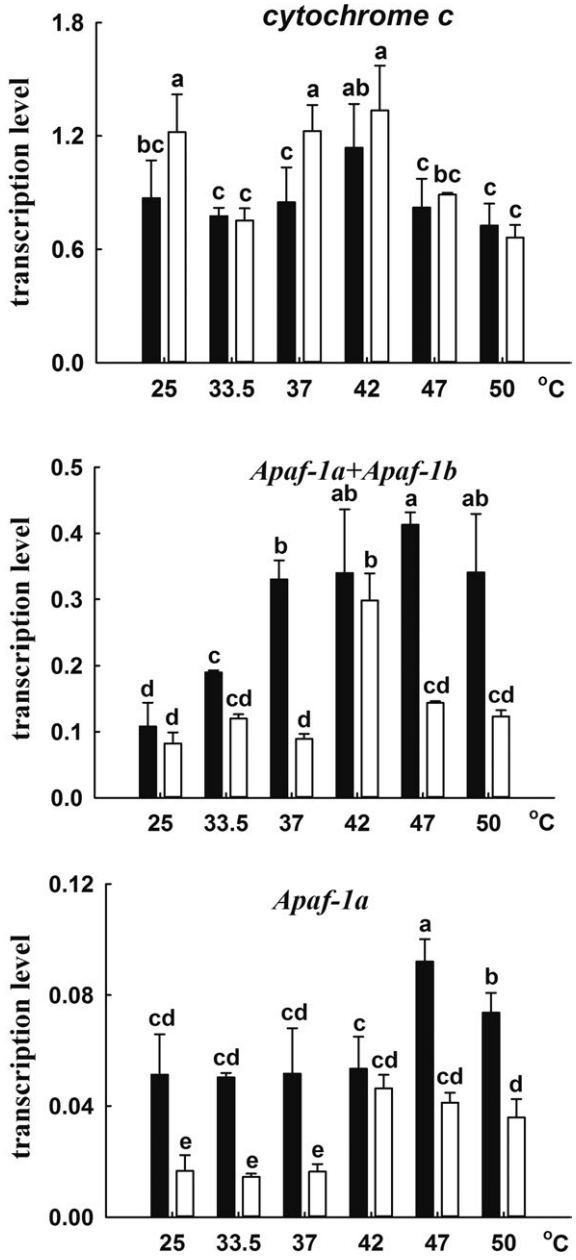

Fig. 6. Effects of high temperature on expression of *cytochrome c*, *Apaf-1a* plus *Apaf-1b* and *Apaf-1a* in R_R (black) and S_S (white) DBM (*Plutella xylostella*). Samples of RNA were extracted from R_R or S_S DBM after the insects were reared at 25, 33.5, 37, 42, 47 or 50°C for 3 h. *Apaf-1a* plus *Apaf-1b* represent the mRNA expressions contributed by both *Apaf-1a* and *Apaf-1b*, while *Apaf-1a* represent the mRNA expressions contributed by *Apaf-1a* alone. Each bar represents the mean ± SE of three independent experiments. Different lowercase letters indicate significant differences among the expressions of mRNA (Duncan's-test, $P \leq 0.05$).

that the numbers of WD40 repeats were different among *P. xylostella*, *B. mori*, *D. plexippus*, *D. melanogaster*, *C. elegans* and *H. sapiens*. The more WD40 repeats, the higher organisms were in an evolutionary sense. These repeats may provide an additional level of complexity to the regulation of Apaf-1 function (Srinivasula et al., 1998). Hence, an increase in the number of WD40 repeats accompanies the evolution of species complexity from lower to higher organisms (Liu et al., 2012a). In addition, *C. elegans* CED-4 (Apaf-1 ortholog) lacks the WD40 repeats domain, but the oligomerization of CED-4 could facilitate CED-3 activation (Yang et al., 1998).

Homolog analysis revealed that the amino acid sequences of Cytochrome c were conserved, and that Cytochrome c from *P. xylostella* shared an identity from 74.0% to 93.5% with other species (Fig. 2). Phylogenetic tree analysis showed that in addition to mammals, Cytochrome c of lepidopteran (*Danaus plexippus*, *Papilio polytes*, *P. xuthus*, *P. xylostella* and *Spodoptera litura*) and hymenopteran (*Apis mellifera*, *Bombus terrestris*, *Megachile rotundata* and *Nasonia vitripennis*) as well as dipteran (*Aedes aegypti*, *Culex quinquefasciatus* and *Drosophila melanogaster*) species formed three distinct subgroups (Fig. 3).

Homolog analysis of the putative amino acid sequences revealed that Apaf-1a or Apaf-1b from *P. xylostella* shared high identity with lepidopteran insects, but low identity with distantly related species (Fig. 4). In the phylogenetic tree, we found that *P. xylostella* and *B. mori* as well as *D. plexippus* were phylogenetically close, and formed a subgroup (Fig. 5).

Expression levels of mRNA isoforms derived from UTR-APA depended on the length of 3′-UTR. In general, longer 3′-UTRs were associated with a relatively lower expression level, as they had a higher probability of possessing more miRNA binding sites that had the ability to inhibit translation. On the other hand, mRNA isoforms with shorter 3′-UTRs could produce higher levels of protein (Sandberg et al., 2008; Di Giammartino et al., 2011; Barrett et al., 2012). In this study, the extent of up-regulation expression of *Apaf-1a* plus *Apaf-1b* under heat stress was higher than that of *Apaf-1a* alone in both R_R and S_S DBM. This proved that *Apaf-1b* is more sensitive to heat stress than *Apaf-1a*. Therefore, *Apaf-1b* with shorter 3′-UTR plays a more important role in the up-regulation expression induced by heat stress.

Apoptosis is a normal physiological cell suicide program, and may well be induced by environmental stress. Apoptosis-related genes are regulated by different mechanisms to meet different cellular needs during growth and development process under conducive or stress environments. Different *Apaf-1*'s (such as *Apaf-1a* and *Apaf-1b*) are probably selected by heat stress in relation to thermal adaptation of the particular life stage in question.

Cell apoptosis induced by heat shock, and the mitochondrial-associated apoptotic pathway appears to be critical for heat-induced apoptosis, in which apoptosome plays important functions (Lue et al., 1999; Xu et al., 2000; Vera et al., 2004). As aforementioned, heat stress induces Cytochrome c to be released from mitochondria into the cytosol, where it bound to Apaf-1. The binding of Cytochrome c to Apaf-1 results in the activation of the initiator Caspase-9, which in turn eventually activates the effector caspases. It is known, as also earlier stated, that the effector Caspase-3 and -7 are "executioners" of apoptosis in terms of the downstream caspase cascade and play a critical role in final cell death (Vera et al., 2006; Wang et al., 2012).

The expression levels of *caspase-3* and/or *caspase-7* probably reflect the apoptotic procession (Cooper & Granville, 2009; Zhuang et al., 2011).

Our previous results suggested that significantly lower biological and physiological fitness (lower thermal tolerance and higher *caspase-7* expression) in insecticide-resistant DBM under heat stress is associated with their higher *caspase-7* expression (executioner of apoptosis) and lower *hsp70* expression (Liu et al., 2008; Zhuang et al., 2011). Based on the results of the present study, we here suggest that significantly higher expression of *caspase-7* and lower biological fitness in R_R DBM under heat stress relates to their higher up-regulation expression of *Apaf-1a* and *Apaf-1b*. This is because Apaf-1 is an important component of apoptosome and the activated apoptosome activates the effector Caspase-7 downstream in the caspase-mediated apoptotic pathway. The individual DBM with high insecticide resistance levels in a given population are most probably eliminated under heat stress because of their significantly lower fitness. Certainly, as shown in this study, a sharp declines of insecticide resistance of DBM occurs during the summer in the field or when reared under heat stress conditions for one generation in the laboratory (Wu & Jiang, 2002; Liu et al., 2008).

To avoid the unrelated resistant and susceptible strains being used, S_S and R_R DBM were selected from a same starting population, but developed (by selection) to yield two different strains. AChE was the target of organophosphate insecticides (OPs). The mutations A201S and G227A, which result in insensitive AChE, are located at the AChE active site, and are doubtless involved in the resistance of DBM to organophosphates (OPs) (Lee et al., 2007).

In conclusion, our results provided clear evidence that significantly higher expression of *caspase-7* and lower biological fitness in OP-resistant insects under heat stress, is associated with their higher up-regulation expression of *Apaf-1a* and *Apaf-1b*, compared to that in OP-susceptible insects. Possibly global warming may affect the evolution of the level of insecticide resistance in DBM, because the insect's physiological and ecological fitness is affected by induced heat stress. This being so, understanding insect adaptation to high temperature is surely important in the study of the development of DBM insecticide resistance, more especially in programs involving combating these pests using integrated pest management (IPM) approaches.

ACKNOWLEDGEMENTS. This study was supported by the National Natural Science Foundation of China (No. 30971924 and 31272049), the National Basic Research Program of (No. 2013CB127604) and Natural Science Foundation of Fujian Province (No. 2012J01085). We sincerely thank H.D. Loxdale for his most helpful editing of the manuscript.

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Received November 25, 2013; revised and accepted April 25, 2014
Prepublished online August 15, 2014