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Upregulation of *Atg5* and *AIF* gene expression in synchronization with programmed cellular death events in integumental epithelium of *Bombyx mori* induced by a dipteran parasitoid infection

J. Anitha†, A.R. Pradeep†* and V. Sivaprasad

Proteomics Division, Seribiotech Research Laboratory, Central Silk Board, CSB-Kodathi Campus, Carmelram. P.O., Bangalore 560035, Karnataka, India

Abstract

Infection of the commercially important silkworm, *Bombyx mori* by a tachnid parasitoid, *Exorista bombycis* induced activation of genes and cellular responses associated with apoptosis in integumental epithelial cells. Composite cellular profile showed initial autophagy, intermediate endoplasmic reticulum degranulation and deformed nucleus as well as later DNA fragmentation indicating apoptosis. Two cell death-associated proteins, autophagy 5-like (Atg5L) and apoptosis-inducing factor (AIF), in addition to caspase, are identified from the infected integumental epithelium through mass spectrometric analysis. Genes encoding these proteins showed age-dependent activation after the infection as revealed by quantitative expression analysis. *Atg5* showed early upregulation in association with signs of autophagy whereas *AIF* showed late upregulation in association with DNA condensation and fragmentation. Expression of *AIF* showed negative correlation with that of *Atg5* after the infection. On the other hand, in control, *caspase* expression showed positive correlation with *AIF* expression indicative of regulated expression in normal larval epithelium, which was absent after infection. Activation of *Atg5*, *AIF* and *caspase* genes in close association with different cell death events revealed the synchronized differential expression of apoptosis-associated genes in response to the macroparasitism. Enhanced expression of *Atg5*, *AIF* and *caspase* genes coupled with the appearance of cell death symptoms indicate parasitism-induced activation of genetic machinery to modulate cell death events in the epithelium, which was hither to unknown in invertebrate systems.

Keywords: autophagy 5-like, apoptosis-inducing factor, caspase, *Bombyx mori*, parasitism

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Introduction

Type I programmed cell death (PCD) is characterized by DNA condensation and fragmentation and caspase-mediated cell death, whereas Type II PCD is characterized by autophagy, vacuolation and necrosis (Suzanne & Steller, 2013). It is well known that caspase mediates apoptosis, nevertheless regulation of autophagy is under scrutiny in different model systems. Though Types I and II PCD showed typical

*Author for correspondence
Phone: 91 + 80 + 28440651
Fax: 91 + 80 + 28439597
E-mail: arpradeepnair@gmail.com
†Both the authors contributed equally to this work.

Table 1. Key to the apoptosis-associated genes showing differential expression in integumental epithelium of *B. mori* larvae after infection with the parasitoid *E. bombycis*.

Protein	Gene name	Accession	Forward primer 5′..3′	Reverse primer 5′..3′
Autophagy 5-like (31,215 Da)[1]	*Atg5*	NM_001142487	TGATCCTCAACTACCCTGGA	CCATGGGACTCCATAAGACG
Apoptosis-inducing factor (67,453 Da)	*AIF*	NM_001202533	GAAGCAAATGTGGTGGAACC	CAGGAGCCAGTAAGCACCTC
Nedd2-like Caspase (49,974 Da)	*Caspase*[2]	NM_001195467	GCAATATGCAAGAGGAGCAC	AGCATTGTGAACGCCTGAG

[1] Calculated molecular weight as per NCBI website.
[2] Primer from Pradeep *et al*. (2012).

symptoms, both are inter-connected, atleast in some models. Biological functions of autophagy are defined to be protective in early stages of stress/diseased conditions, whereas it favours cellular collapse on extreme degradation. Under autophagy, after fusion of lysosomes with autophagosome membrane, engulfed cellular cargo is degraded and recycled back into cytoplasm (Mizushima *et al*., 2008). Autophagy is reported to activate the caspase-regulated apoptosis in different systems including *Drosophila* (Nezis *et al*., 2010), but a threshold level of caspase only could activate the PCD (Florentin & Arama, 2012). These processes are under regulation of genes such as *autophagy-related genes* (*Atg*) and apoptosis-associated genes (Jehn & Osborne, 1997; Lorenzo *et al*., 1999; He & Klionsky, 2009; Li *et al*., 2010; Ravikumar *et al*., 2010; Suganuma *et al*., 2011). The *Atg* were reported from yeast and many eukaryotic organisms including *Drosophila* and *Bombyx mori* (He & Klionsky, 2009; Zhang *et al*., 2009; Inoue & Klionsky, 2010), of which *Atg5* is associated with membrane genesis, inactivation of which suppressed autophagy, led to diseased conditions (Simonsen *et al*., 2007). However, *Atg5* performs not only the autophagic role but provokes apoptotic cellular death also (Yousefi *et al*., 2006). Overexpression of another proapoptotic gene *hid* activated autophagy under starvation in different tissues of *Drosophila;* however, Bruce protein promoted degradation of hid and negatively regulate autophagy (Hou *et al*., 2009) exhibiting the inter-relation between autophagy and apoptosis.

Apoptosis-inducing factor (AIF) is a flavoprotein originally locates in mitochondria and relocates to cytosol and nucleus under diseased or stress conditions (Susin *et al*., 1999). In the nucleus, AIF induces chromatin condensation and DNA fragmentation leading to apoptosis, in the absence of caspase (Daugas *et al*., 2000). However, in β-cells of islets of Langerhans, *AIF* gene is expressed and protects it from oxidation stress (Schulthess *et al*., 2009). But caspase triggers permeability of the mitochondrial membrane and release of cytochrome C and/or AIF (Guo *et al*., 2002; Van Loo *et al*., 2002). Recent studies showed a cross talk between apoptosis and autophagy, which ensures the balance between survival and death of cell (Gordy & He, 2012).

Gene activation associated with apoptosis in epithelial cells induced by macroparasitic infection is least examined in any invertebrate model systems. Though manipulations of host apoptosis by microbes were reported in mammalian model systems (Ubol *et al*., 1994; Sinai *et al*., 2004), reports from the invertebrate systems are scanty (Minguez *et al*., 2013). Recently, we have shown immunocompetence of integument in the *B. mori* larvae exhibited after infection by the eukaryotic parasitoid, *Exorista bombycis*. Under the parasitic influence, integumental epithelium showed cellular responses such as signs of autophagy and apoptosis and activation of humoral immune components (Pradeep *et al*., 2012). However, it is not known how parasitism regulates apoptosis in most model systems, particularly in invertebrates. In order to unravel the mechanism involved, we examined the activation of apoptosis-associated genes and its expression pattern in the larval integument of the lepidopteran model *B. mori* after infection by the tachnid parasitoid, *E. bombycis*.

Materials and methods

Materials

Larvae of mulberry silkworm, *B. mori* were reared under a photoregime of 13L: 11D, at 26 ± 1°C and humidity of 85% and fed with mulberry leaves. Day 1 fifth (final) instar larvae were exposed to mated gravid females of *E. bombycis* for an hour, for oviposition and is considered as the 0 h of infection.

Methods

The integumental tissue from control and infected larva of *B. mori* was dissected out and processed for transmission electron microscopy (TEM) as described earlier (Pradeep *et al*., 2012). From integument, protein was extracted using total protein extraction reagent (T-PER; Thermo-Pierce) and quantified (Lowry *et al*., 1951). The integumental protein was resolved by 10% SDS-PAGE and two-dimensional (2D) electrophoresis. Exclusive bands observed from parasitized tissue in the PAGE and 2D matrix were cut and processed for mass spectrometry and peptide mass fingerprinting (MASCOT). From another batch, integument was dissected out and kept in RNA stabilization reagent, RNA Later (Qiagen). Total RNA was extracted using a phenolic solution, RiboZol (Amresco). cDNA was synthesized from 1 µg total RNA using oligo d(T) primer (Protoscript; New England Biolabs) as per manufacture's protocol and amplified using gene-specific primers designed (Primer 3) from the mRNA sequences obtained from the NCBI database (table 1). For real time analysis, MESA GREEN qPCR Master Mix Plus for SYBR® Assay I Low ROX was used (Eurogentec, Belgium) on Agilent Stratagene Mx3005P qPCR system.

At least two replications of quantitative expression analysis (qPCR) experiments using independent templates from control and parasitized samples were performed at five different time points at 24 h intervals after infection. After performing the qPCR, the reaction mixture was resolved on 1.5% agarose gel and confirmed the single amplicon of predicted molecular weight and primer specificity.

Statistical analyses

Quantitation of gene expression in the parasitized tissue relative to the calibrator (defined as 1.0) was calculated using Mx3500P qPCR software (Agilent). Average threshold cycle (C_t) value of transcript expression was calculated by $\Delta\Delta$ C_t method (Livak & Schmittagen, 2001) from the duplicates

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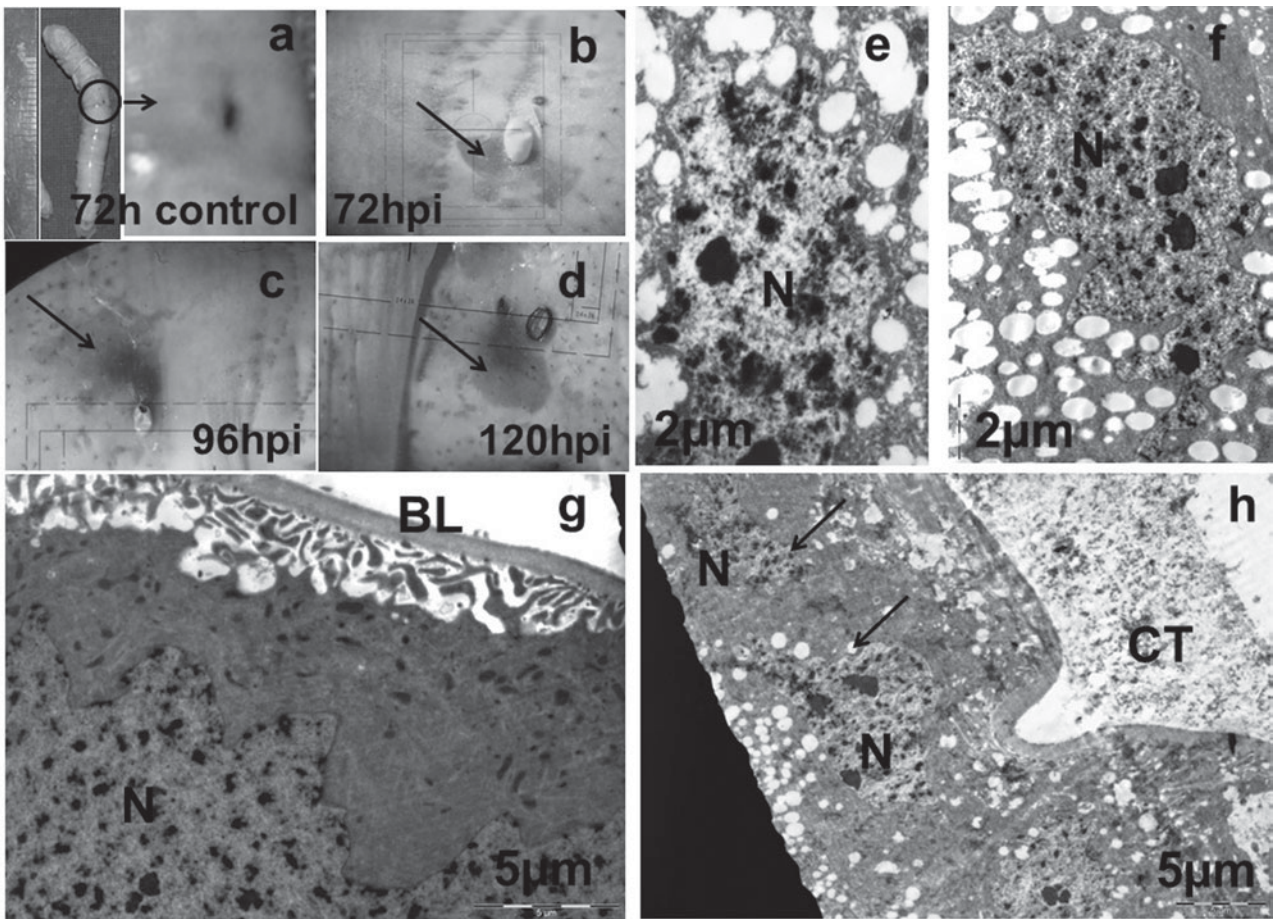

Fig. 1. Induction of apoptosis in the epithelium followed by lysis of integument of final instar larvae of *B. mori* after infection through the parasitoid *E. bombycis*: (a) Saline injected 72 h control larvae showed a spot of melanization at the point of needle injection; no lysis was observed in the surrounding area of cuticle. However at 72, 96 and 120 h post infection (hpi) (b–d), progressive increase in lysis area was observed (arrows) under stereo zoom microscope (magnification: 400× each). TEM observation on the changes in nucleus of integumental epithelium: Nucleus of 24 h control larvae (e) and 24 h post infected (hpi) larvae (f) showed branched nuclear membrane and uniformly distributed chromatin material however did not show variations in nuclear morphology. In 48 hpi larvae of *B. mori* (g), the cytoplasm was free of organelles, but with network of cytoplasmic channels. At 96 hpi (h), the nuclei (arrows) become deformed, pyknotic, lost ramifications and with condensed chromatin. BL, basal lamina; CT, cuticle; N, nucleus.

and normalized with the house-keeping gene, β-*actin*. Comparative C_t values of the gene of interest were standardized by C_t values for the control gene β-*actin*. C_t values were standardized relative to the average value for the control, yielding the delta C_t value, and these values were standardized to make the average control value '1' (the $\Delta\Delta$ C_t values) (Gerardo *et al*., 2010). Fold change in gene expression relative to the calibrator was calculated, which allowed to display the down regulated relative quantities as negative values. The data (mean ± SE) represented is the gene expression induced after parasitism, excluding the changes in control introduced by injection or developmental processes. Moreover, this calculation allowed displaying the downregulated relative quantities as negative values when fold change in gene expression was calculated.

Significance of variation between control and infected samples was tested by Student's *t*-test. Correlation in expression levels between the genes, *Atg 5*, *AIF* and *caspase* was analysed by linear regression analysis.

Results and discussion

Infection of *B. mori* larvae by *E. bombycis* maggot induced signs of apoptosis in the integumental epithelial cells. Formation of autophagic vesicles, accumulation of protein in vacuoles, dilation and degranulation of cisterne and emptying of rough endoplasmic reticulum in to vacuoles were noticed in a temporal manner after the infection. During infection, the epithelial nuclear morphology showed gradual variation from branched morphology in the early stages to an unorganized one in the later stages (fig. 1). Moreover genomic DNA showed condensation and fragmentation in the later stages of infection. Similar signs of autophagy, apoptosis and cellular death were noticed during PCD occurred during metamorphosis of insects (Misch, 1965; Li *et al*., 2010). A composite TEM profile of the integument of the parasitized *B. mori* larva demarcated the infection-induced autophagy in the early stage, its progress in the middle stage followed by apoptosis in the later stage of infection.

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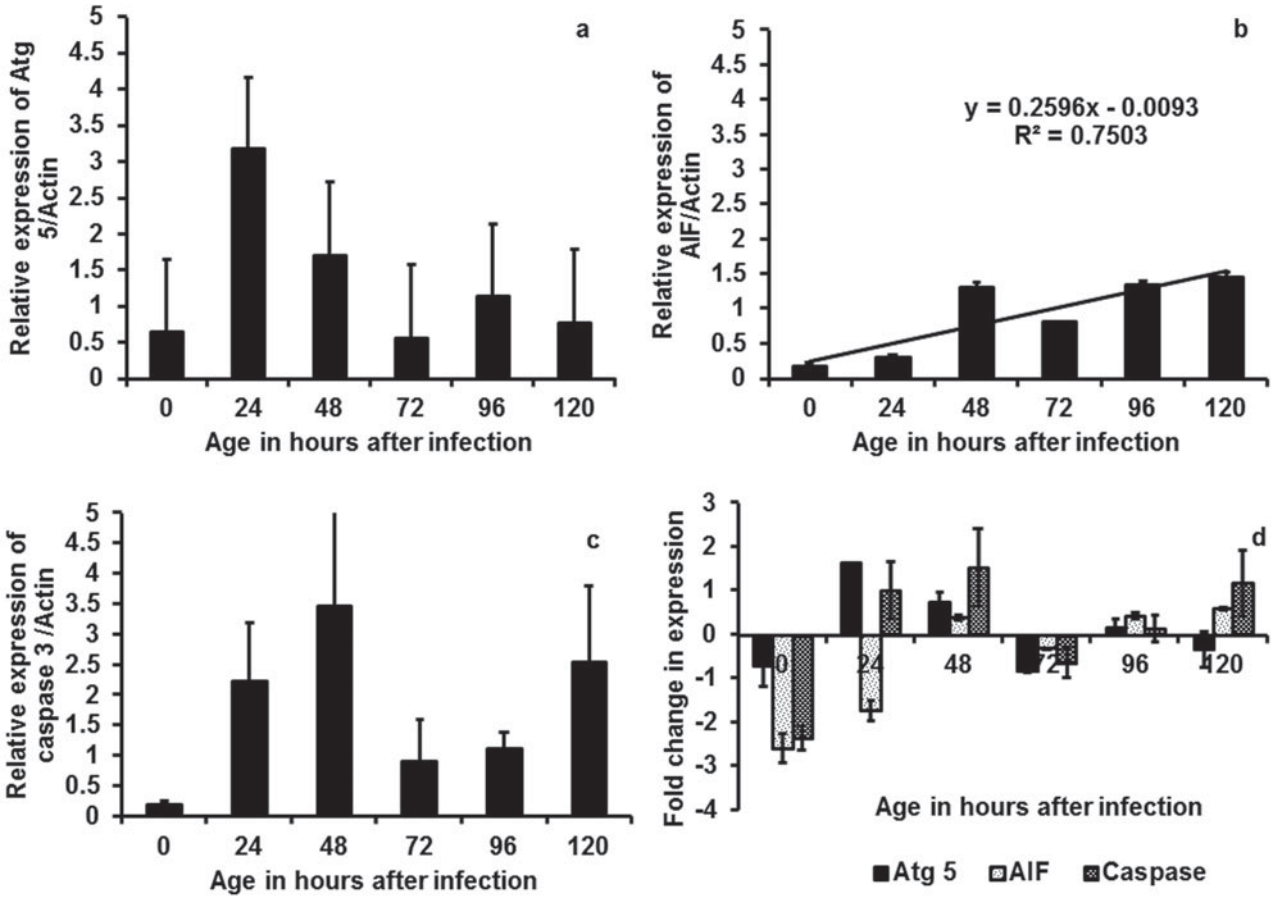

Fig. 2. Relative expression of genes encoding apoptosis-associated proteins in the integumental epithelium of final instar larvae of *B. mori* after infection through the parasitoid *E. bombycis*. (a) *Atg5* showed upregulation in the early stages whereas (b) *AIF* showed upregulation in the later stages of infection. Allometric line represents the linear relation between AIF expression and larval age. (c) *Caspase* showed consistent expression throughout infection with a dip at 72 h post infection. (d) Fold change in expression of *Atg5*, *AIF* and *caspase* at various time points after infection. *Caspase* was upregulated before the *AIF* increase and also in the later stages of infection, indicating genetic regulation of organized cell death events induced by the parasitic infection. The transcript level was normalized with that of the house keeping gene, β-*actin*. Each point represents mean of two independent analysis (mean ± SE) performed at five time points (age in hours) after infection. Quantitation of gene expression in the parasitized tissue relative to the calibrator (defined as 1.0) was calculated using Mx3500P qPCR software (Agilent). Average C_t value of transcript expression was normalized with the house-keeping gene, β-*actin*. The C_t values were standardized relative to the average value for the control treatment, yielding the delta C_t value, and these values were standardized to make the average control treatment value '1' (the $\Delta\Delta$ C_t values) (Gerardo *et al.*, 2010). Thus the gene expression induced after parasitism is presented here, excluding the changes in control introduced by injection or developmental processes. Moreover, this calculation allowed displaying the down regulated relative quantities as negative values when fold change (d) in gene expression was calculated.

Infection of *B. mori* larva by *E. bombycis* has induced an enhancement in production of immune- and host-response proteins in the integument as revealed earlier by 2D electrophoresis and SDS-PAGE coupled with mass spectrometry (Pradeep *et al.*, 2012). The analysis showed activation of signalling, melanization, proteolysis and humoral immunity components. Genes encoding these immune-associated proteins showed expression in a co-regulatory way in the control larvae whereas the co-regulation was lost in different sets of genes after the infection (Anitha *et al.*, 2013).

Advanced analysis showed activation of proteins associated with autophagy and apoptosis in the integumental epithelium. Three key proteins associated with Types I and II apoptosis viz. autophagy 5-like (Atg5L), AIF and Nedd2-like caspase were identified by mass spectrometry from the infected larval integument. Activation of genes encoding these apoptosis-associated proteins in the integumental epithelium after the infection was examined by qPCR. In early stages of infection, *Atg5* gene showed upregulation till 48 h after infection, followed by significant ($P < 0.045$) decrease (fig. 2a). On the other hand, *AIF* gene showed lower level of relative expression in the early stages of infection followed by gradual increase to significantly ($P < 0.001$) greater expression at 96 h and 120 h after infection and correlation ($R^2 = 0.75$) with increase in age (fig. 2b). Consequently in the infected larvae, relative expression of *AIF* showed negative correlation ($R^2 = 0.4698$) with that of *Atg5* (table 2). However, *Atg5* showed larger level of quantitative expression than *AIF* gene.

In the earlier stages of infection, upregulation of *Atg5* gene coincides with appearance of autophagic vacuoles at 24 h after infection, unswerving with its role in initiation of autophagy (Sakoh-Nakatogawa *et al.*, 2013; Tian *et al.*, 2013),

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Table 2. Correlation between relative expression of apoptosis-associated genes in the integumental epithelium of *B. mori* final instar larvae infected by the parasitoid, *E. bombycis*.

Gene pair	Parasitized		Control	
	Linear regression equation	Correlation coefficient	Linear regression equation	Correlation coefficient
Caspase 3–AIF	$y=0.0503x+0.9342$	$R^2=0.0161$	$y=0.7403x+0.3385$	$\mathbf{R^2=0.7597}$
Caspase 3–Atg5	$y=0.3654x+0.7169$	$R^2=0.1348$	$y=0.3869x+0.3271$	$R^2=0.3601$
AIF–Atg5	$y=-1.7204x+3.2563$	$\mathbf{R^2=0.4698\ (-)}$	$y=0.1713x+0.4459$	$R^2=0.0509$

AIF, apoptosis-inducing factor; Atg5, autophagy-related gene 5; (−) indicates negative correlation.
Bolded values indicate higher correlation coefficients.

which supports the viability of cell (Palmer *et al*., 1986; Schaub & Sehnitker, 1988; Pinheiro *et al*., 2010). However, when the parasitic influence is intensified on the epithelial cells, relative expression of *Atg5* was decreased in the *B. mori* larvae (fig. 2a). It is reported that *Atg5* enhances cellular susceptibility towards apoptosis showing that *Atg5* is required not only for inducing autophagosome formation (Scott *et al*., 2004; Berry & Baehrecke, 2007), but also acts as a molecular link between autophagy and apoptosis (Yousefi *et al*., 2006). However, in the lepidopteran *Galleria mellonella*, *Atg8* expression was noticed in different tissues during larval-pupal transformation and before the PCD (Khoa & Takeda, 2012).

Continuous presence of the parasite in vicinity of integument induced prolonged autophagy followed by integumental lysis (fig. 1a–d), which was accompanied by increase in *AIF* and *caspase* expression (fig. 2). Both the genes are encoding enzymes that stimulate apoptosis. *Caspase* showed increase in expression at 48 h followed by decrease at 72 h after infection. Subsequent increase was noticed in the later stage of infection, at 120 h after infection. The initial rise in *caspase* expression is associated with cellular protection while the later enhancement is related with cellular death (Martin & Baehrecke, 2004; Liu *et al*., 2007; LeBlanc & Saleh, 2009; Suganuma *et al*., 2011). In the control *B. mori* larvae, relative expression of *caspase* showed marginal positive correlation with that of *Atg5* gene ($R^2=0.36$), whereas it showed larger positive correlation ($R^2=0.76$) with *AIF* expression (table 2). When the cells were under stress, AIF, located in the mitochondria is translocated to cytosol and then to nucleus, and induced DNA fragmentation leading to cell death (Lorenzo *et al*., 1999; Joza *et al*., 2001; Candé *et al*., 2002*a*, *b*, 2004). Moreover, the DNA fragmentation is accompanied by upregulation of *caspase* gene expression, in the later stages of parasitism (fig. 2c) confirming that apoptosis is mediated by a combined action of AIF and caspase. However, the relation and/or regulation between *caspase* expression and other genes become void after the infection (table 2) showing derailing of regulation on caspase activity under the parasitic influence (Castino *et al*., 2005; LeBlanc & Saleh, 2009). Similar loss of co-regulation of other host-response genes with caspase expression was reported (Anitha *et al*., 2013). Co-regulated caspase expression seems essential to prevent surge in caspase activity in the final larval instar, which is under the endocrine control during larval-pupal transformation (Liu *et al*., 2009). The deregulatory gene activities induced by the parasitism in *B. mori* shows the requirement of an effector caspase to control both autophagy and apoptosis as noticed in *Drosophila* (Hou *et al*., 2009).

Fold increase in expression of cell death-associated genes at 24 h after infection showed upregulation of *caspase* gene and downregulation of *AIF* gene in the early stage of infection (fig. 2d). This revealed an early activation of *caspase* gene before the upregulation of *AIF*, confirming the observations in *Drosophila* (Susin *et al*., 2000; Gabriel *et al*., 2003). Apoptosis preceded by autophagy was also reported during larval–pupal transformation of midgut in *B. mori* (Franzetti *et al*., 2012). But no *caspase* activation was observed during PCD of larval fat body in *Manduca sexta* (Muller *et al*., 2004). Present observations confirm the co-occurrence of both autophagy and apoptosis and an organized PCD (Zhang *et al*., 2009, 2010) induced by the parasitoid infection in the integument of *B. mori*.

In summary, we observed early upregulation of *Atg5* gene in association with appearance of signs of autophagy and late upregulation of *AIF* gene in association with nuclear deformation, DNA condensation and fragmentation. *Caspase* gene was active throughout the infection period. The expression profile of *Atg5* showed negative correlation with that of *AIF* in the infected larvae led to a novel observation of regulated expression of these apoptosis-associated genes in the parasitized epithelium. Enhanced expression of *Atg5, AIF* and *caspase* genes coupled with the appearance of cell death symptoms indicate parasitism-induced activation of genetic machinery to modulate cell death events in the epithelium, which was hither to unknown in invertebrate systems.

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