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Gene discovery, comparative analysis and expression profile reveal the complexity of the *Crassostrea gigas* apoptosis system

Linlin Zhang[a,b], Li Li[a], Guofan Zhang[a,*]

[a] *Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Rd., Qingdao 266071, China*
[b] *Graduate School, Chinese Academy of Sciences, Beijing 100039, China*

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ABSTRACT

Apoptosis system was reported to play important role in organism immunity, but it was a currently understudied respect in molluscan immunity studies. Base on the recent generation of ESTs in the pacific oyster, *Crassostrea gigas*, a survey of apoptosis-related molecules was conducted in the assembled unigenes, we found that the basic genes and domains in apoptosis-associated proteins were conserved, the overall apoptotic machinery was complex in *C. gigas* and that the organism had an expanded number of putative baculovirus inhibitor of apoptosis repeat domains. Moreover, four typical apoptosis-related genes were cloned in *C. gigas* and compared with the sequences of these genes in *Drosophila melanogaster* and *Homo sapiens*. The expression level of these four apoptosis-related genes in the hemolymph increased dramatically in the presence of the bacteria, *Vibrio anguillarum*, indicating their role in bacterial defense. Our results suggest that the oyster apoptosis system is not simple and cannot be represented by model invertebrates.

1. Introduction

Apoptosis, or type I programmed cell death, plays a key role both in immune responses and during development (Kiss, 2010). It emerged in the primordial multicellular ancestors and has been well-studied in many model species, including *Mus musculus* (Enari et al., 1998), *Strongylocentrotus purpuratus* (Robertson et al., 2006), *Drosophila melanogaster* (Kornbluth and White, 2005) and *Caenorhabditis elegans* (Lettre and Hengartner, 2006), but apoptotic molecules have not been widely investigated in Lophotrochozoa. Despite conservation of the basic apoptotic machinery, recent studies have demonstrated that Lophotrochozoa have a unique apoptotic mechanism that cannot be represented by model invertebrates (e.g., *D. melanogaster* or *C. elegans*) (Kiss, 2010).

Molluscs (bilateria, Lophotrochozoa) are the second most diverse group of animals with about 93,000 extant species (Hedgecock et al., 2005). The apoptosis system has been reported to play an important role in molluscan immunity. Pathogen-induced modulation of apoptosis (Terahara and Takahashi, 2008; Hughes et al., 2010) and saliva release (Pirger et al., 2006; Pirger et al., 2009) are two relatively well-studied areas in molluscan studies of apoptosis. Typical apoptosis-related genes have been cloned in molluscs, such as an initiator caspase from variously colored abalones (*Haliotis diversicolor*) (Huang et al., 2010), a Fas ligand from disk abalones (*Haliotis discus discus*) (De Zoysa et al., 2009), a TNFR from Zhikong scallops (*Chlamys farreri*) (Li et al., 2009), and an inhibitor of apoptosis (IAP) from California sea hares (*Aplysia californica*) (Moroz and Kohn, 2010). Despite these findings, the basic molecular mechanism of apoptosis has not been well described in mollusks. The pacific oyster, *Crassostrea gigas*, is a widely used model for mollusc studies because of its economic, ecological and evolutionary importance (Hedgecock et al., 2005); additionally, 57,279 ESTs from *C. gigas* have been reported in the NCBI database as of October 1, 2010. These ESTs form the basis for apoptosis-related gene discovery and evolutionary analyses.

Apoptosis system is highly conserved and tightly regulated. Apoptotic signals are initiated and transduced via the intrinsic (mitochondrial) or extrinsic (receptor-mediated) pathway. Extracellular signals can activate the extrinsic pathway through death receptors (Fas/CD95, TNFR, DR4/DR5). These activated receptors recruit Fas-associated protein with Death domain (FADD) and caspase-8 to form the death-inducing signaling complex (DISC). Activated caspase-8 then directly or indirectly activates caspase-3, which plays a central role in the execution-phase of cell apoptosis. Organisms initiate the intrinsic apoptotic pathway in response to cytotoxic stimuli or environmental stressors. In vertebrates, mitochondria lie at the core of the intrinsic signaling pathway. When stressed, mitochondria become permeable and release cytochrome *c* into the cytosol (Armstrong, 2006). Released cytochrome *c* contributes to the formation of an apoptosome complex (Apaf-

* Corresponding author. Tel.: +86 532 8289 8701; fax: +86 532 8289 8701.
E-mail address: gfzhang@ms.qdio.ac.cn (G. Zhang).

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1/cytochrome *c*/caspase-9) and triggers caspase-3, which connects the intrinsic and the extrinsic pathways

The apoptosis network is usually connected by special protein-protein interaction domains that join the upstream and downstream element. These protein domains mainly consist of Peptidase_C14 (caspase), B-cell lymphoma 2 (Bcl-2), Caspase Recruitment Domain (CARD), Death, Death Effector Domain (DED), and Baculovirus IAP Repeat (BIR). Caspases are cysteinyl aspartate proteases that function at the core of the apoptotic apparatus. CARD, death and DED belong to a family of 6 alpha-helical "adaptor" domains, which transmit cell death signals to caspases via homotypic or heterotypic protein-protein interactions. BIR is a domain of tandem repeats separated by a variable length linker that likely to confer cell death-preventing activity and is mostly found in the IAPs (Deveraux and Reed, 1999). The Bcl-2 family also participates in the regulation of apoptosis by releasing apoptotic signals from the mitochondria (Deveraux and Reed, 1999; Benedict et al., 2002; Robertson et al., 2006; Orme and Meier, 2009)

These domains constitute the complexity of the apoptosis-related proteins. FADD, which consists of DED and Death domains, is an adaptor molecule in both the extrinsic and intrinsic apoptotic pathways (Eberstadt et al., 1998). IAP proteins contribute significantly to cell death regulation by suppressing apoptosis (Deveraux and Reed, 1999), activating death receptors (Krammer, 2000), promoting mitochondrial permeability (Green and Kroemer, 2004) and influencing other mediators of apoptosis (Salvesen and Duckett, 2002). Caspase proteins can be classified into two subfamilies based on their major functions as pro-inflammatory or pro-apoptotic. The pro-apoptotic subfamilies can also be divided into two subfamilies, initiator caspases with long prodomains and effector caspases lack long prodomains (Wolf and Green, 1999).

In this study, we provided a molecular view of the apoptosis-related unigenes in the pacific oyster, *C. gigas*, and additional analysis of typical genes. We firstly describe the overall apoptotic molecules existing in the apoptotic network of the pacific oyster. Then we compared the unigenes that contained apoptosis-related domains with other species and selectively cloned four of these genes according to their biological and molecular function to highlight the evolutionary lineage of apoptosis. We also characterized the genomic organization of the selected genes. Finally, *C. gigas* were challenged with a bacterial infection, and an analysis of the expression pattern of the genes was conducted to prove the essential role of the selected genes in immunity and defense.

2. Materials and methods

2.1. Database, EST processing and contig assembly

C. gigas ESTs (57,279) were downloaded from the NCBI taxonomic EST database (http://www.ncbi.nlm.nih.gov/guide/taxonomy/, up to October 1, 2010) and filtered with a cut-off length of ≥100 bp. Vector sequences were trimmed from ESTs using Cross match software (http://www.phrap.org) and based on UniVec (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). Poly-A/T stretches were removed under conditions when the T nucleotide was repeated at least five times in a 5′ window of 50 bp, with the same conditions applied to the A nucleotide in the 3′ region. The pre-processed ESTs were first clustered into groups of similar sequences using Unicluster software (https://genome.uiowa.edu/pubsoft/software.html). These clusters were then separately assembled into contiguous sequences (contigs) using Phrap software with the parameter "revise_greedy". The EST sequences that did not cluster were considered singletons. The contigs and singletons were combined and formed the processed EST database (pEST) of *C. gigas* used in this study.

2.2. Database searching

To identify ESTs that contained complete open reading frames (ORFs) or short fragments of genes, the processed ESTs were translated in six-frame. The result was the generation of 46,815 ORFs that consisted of ≥75 amino acids each. The obtained ORFs were searched mainly with Pfam (Protein Families Database) using HMMER3.0 (http://hmmer.org/) with an *E*-value threshold of 0.1 to identify proteins containing typical apoptosis-related domains, including Peptidase_C14 (PF00656); BIR (PF00653); Bcl-2 (PF00452); Death (PF00531); DED (PF01335), CARD (PF00619), TNFR_c6 (PF00020), BID (PF06393), Smac_DIABLO (PF09057) TNF (PF00229), NB_ARC (PF00931), NACHT (PF05729) and MATH (PF00917). Blast, SMART and ScanProsite programs (http://smart.embl-heidelberg.de/; http://www.expasy.ch/tools/scanprosite/) were also use in some cases. For the blast analyses, the *E*-value was set to 1e-5 to acquire reliable predictions.

Genes containing apoptosis-related domains from other model species were also annotated using HMMER3.0. The filtered annotated genome of *C. elegans* and *Nematostella vectensis* were downloaded from WormBase (http://www.sanger.ac.uk/Projects/C_elegans/WORMBASE) and StellaBase (www.stellabase.org), and genome sequence information from *S. purpuratus*, *D. melanogaster* and *H. sapiens* was downloaded from NCBI.

2.3. Cloning of the cDNA and genomic DNA from four typical apoptosis-related genes

Based on the Pfam annotation information, four clusters were selected as apoptotic homologs for cloning. First, total RNA was extracted from *C. gigas* gills and hemocytes using TRIzol reagent (Invitrogen, USA). Complementary DNA primers (Supplementary Table 1) were then designed to verify the selected homologs. Race primers (Supplementary Table 1) were used to obtain the transcripts containing complete ORFs according to the manufacturer's instructions (Invitrogen, USA). Genomic DNA was isolated from the gill using a genomic DNA purification kit (Promega, USA). Primers were designed to obtain genomic DNA covering the full coding region (Supplementary Table 1). All of the purified PCR products were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced in both directions.

2.4. Sequence analysis

Multiple alignments of the complete BIR domains were conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and grayed with BoxShade server v. 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Three-dimensional modeling of the oyster CgIAP BIR2 structure was performed using the SWISS-MODEL protein modeling server (http://swissmodel.expasy.org/). Nucleotide and deduced amino acid sequences from the four cloned apoptosis-related genes were analyzed using DNAman software (version 5.2.2). Deduced protein domains were predicted using SMART and ScanProsite programs. The amino acid similarity between *C. gigas*, *D. melanogaster* and *H. sapiens* was calculated with the Blastp program. Genomic architecture of *D. melanogaster* and *H. sapiens* was predicted using UCSC's genome bioinformatics system (http://genome.ucsc.edu/).

2.5. Animals, bacterial challenge and tissue collection

The pacific oysters, *C. gigas*, averaging 110 mm in shell height, were taken from an oyster farm located in Qingdao, China, and acclimatized in seawater tanks at a temperature of 18 ± 1.0°C and a salinity of 30‰. In the challenge experiment, oysters were chal-

lenged by filing the shell and injecting 100 μL of phosphate buffered saline (PBS, pH 7.2) or 100 μL of live *Vibrio anguillarum* (strain no. MVM425) into the adductor muscle. The bacteria were suspended in PBS, and the concentration was determined by reading the optical density at wavelength 550 nm (1 unit at OD 550 equaled 5×10^8 bacteria/mL). The hemolymph from three oysters was individually taken from the control (injected with PBS) and challenge groups (injected with bacteria) with a 1 mL syringe from the adductor muscle at 0, 3, 6, 12, 24 and 48 h after injection according to Itoh and Takahashi (2009). Hemolymph was also collected from the pericardial cavity with sterile syringes and immediately centrifuged at 1000 × *g* for 10 min at 4 °C according to Gueguen et al. (2003). Other tissues were surgically obtained. There were three replicates for each tissue and all the oysters were also individually collected.

2.6. Quantitative analysis of transcription of four apoptosis-related genes

Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on an ABI 7500 real-time thermal cycler (Applied Biosystems, USA). The gene expression of elongation factor (EF) (GenBank accession number AB122066) was used as an internal control with the EFF-real forward and EFR-real reverse primers (Montagnani et al., 2007). RNA was extracted using TRIzol reagent (Invitrogen, USA), and DNase I (Promega, USA) was included to eliminate contaminating genomic DNA. Reverse transcription was conducted with Oligo dT_{18} and random primers (N_6). SYBR Green I was used as a fluorescence dye, and PCR was performed according to the *Premix Ex* Taq™ protocol (TaKaRa, Japan). Primers for the four genes are shown in Supplementary Table 1. Gonad was used as a reference sample (called the calibrator) in the tissue expression experiment, and time 0 h was used as the baseline value in the challenge experiment. The ΔCt for each sample was subtracted from the ΔCt of the calibrator; the difference was the ΔΔCt value. The expression level of the target genes was calculated by $2^{-\Delta\Delta Ct}$. Statistical differences between the control and treatment groups were determined by Student's *t*-test using SPSS v. 13.0 software, and differences were considered significant at $P < 0.05$

3. Results

3.1. EST sequence analysis and apoptosis-related gene annotation

Expressed sequence tags from *C. gigas* were downloaded from NCBI and assembled into 11,453 contigs and 15,038 singletons, producing a total of 26,491 unique sequences. The average contig length was 921 bp, and the average singleton length was 583 bp.

To ascertain the pacific oyster apoptotic machinery and compare it to that of other model organisms, we annotated the apoptosis-related ORFs generated from the unigenes of the pacific oyster with the conserved apoptosis-related domains determined by the Pfam database. Overall, 46,815 six-frame ORFs produced 165 protein fragments containing apoptosis-related domains. The predicted unigenes and correlative ESTs are showed in Supplementary Table 2.

The basic apoptotic molecules are showed to be conserved in the *C. gigas* (Fig. 1). Major ORFs identified contained a caspase domain (17), at least a BIR domain (22), a Bcl-2 domain (2), a Death domain (20), a DED (7), and a CARD (20) (Table 1). Besides, we also found ORFs containing Smac_DIABLO (1), TNF (19) and MATH (6). These apoptosis-related domains interact with each other and form the complex apoptosis network. Pfam and blast analyses in the oyster putative EST ORFs gave significant hits to major apoptosis-related genes such as TNF, TRAIL, FASL, TNFR, TRAFs, FADD, Smac/DIABLO, caspase-9, caspase-3/7, IAP and Bcl-2/xL.

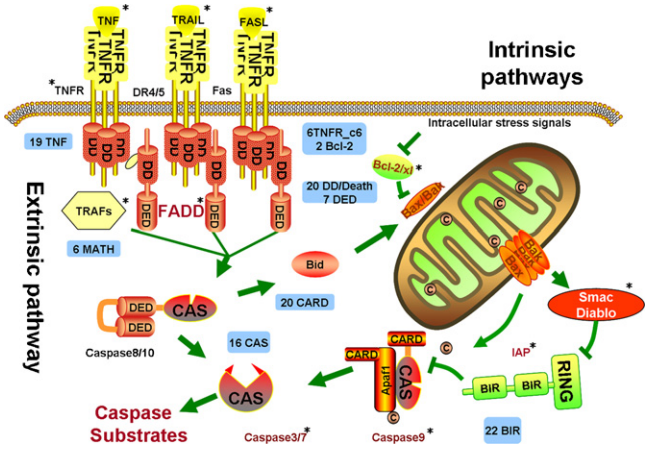

Fig. 1. Simpled schematic illustrating representative genes and domains of apoptotic signaling pathway. The signaling of vertebrate extrinsic pathway (left) begins with death receptors, requires interactions of their death/DD domains and the downstream adapters FADD. The interactions activate the caspase-8 and then lead to caspase-3/7 activation. Mitochondrion lies in the core of vertebrate intrinsic pathway (right) and IAPs plays a role as apoptosis inhibitor. When stressed, mitochondria become permeable and release cytochrome *c* into the cytosol. Released cytochrome *c* contributes to the formation of an apoptosome complex (Apaf-1/cytochrome *c*/caspase-9) and triggers caspase-3/-7. "*" symbols show molecules also identified in molluscan EST putative ORFs. The colors used for different domains have no special meanings. Numbers in blue textbox mean the unigenes identified containing corresponding domain, for example, 22 BIR means 22 unigenes were predicted containing BIR domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Comparative analysis of apoptosis-associated domains

Comparisons with other animals suggested that the complexity of apoptosis-associated domains in the *C. gigas* and *N. vectensis* was a little higher than that of nematodes and arthropods. The apoptotic complexity in oysters was particularly apparent in BIR domains; cnidarias, nematodes, arthropods, echinoderms and vertebrates contained 3, 4, 12, 8 and 14 ORFs, respectively, whereas our investigation in the pacific oyster has revealed 22 ORFs. Except for Bcl-2 and caspase, the ORFs from other domains in the pacific oyster (unigenes) were more numerous than those found in *D. melanogaster* and *C. elegans* (Table 1).

The PFAM analysis predicted 36, 6, 8, 26, 14 and 29 BIR domains in oysters, cnidarias, nematodes, arthropods, echinoderms and vertebrate, respectively (Table 1). Such comparison also suggested that the BIR domain expanded in the pacific oyster ORFs. All the identities of the BIR domains with each other were no more than 90%. The functional zinc ion-binding motif, $CX_2CX_{16}HX_6C$, was completely conserved in 87.5% of BIR domains in the pacific oyster unigenes, and all of them contained most of the conserved residues (Fig. 2). The predicted 3D structure of second BIR domain of cloned CgIAP was similar to that of the third BIR domain from the mammalian IAP homolog B (Supplementary Fig. 1), displaying a unique hydrophobic fold that is stabilized by a zinc residue that is tetrahedrally bound by three cysteine residues and one histidine residue.

3.3. cDNA and deduced amino acid sequence of four typical apoptosis-associated genes

To gain a better understanding of the apoptotic machinery of the pacific oyster, four typical apoptosis-related genes (FADD, IAP, initiator caspase and effector caspase) were selected for detailed analysis (gene cloning, genomic structure and gene expression pattern). Cluster19036, which was assembled from EST AM865894 and FP006478, yielded a full-length predicted transcript of FADD. Using 3′RACE on cluster19036, a 1022-bp cDNA fragment was identified

Table 1
Complexity of selected apoptosis-related genes in *C. gigas* EST and other various animal genomes.

Domain	Cnidaria (Nv)[a]		Mollusca (Cg)[b]		Nematodes (Ce)[c]		Arthropods (Dm)[d]		Echinoderms (Sp)[d]		Vertebrates (Hs)[d]	
	Gene	Domain	Unigene	Domain	Gene	Domain	Gene	Domain	Gene	Domain	Gene	Domain
Caspase	15	19	16	19	17	18	9	11	53	60	24	38
BIR	3	6	**22**	**36**	4	8	12	26	8	14	14	29
Bcl-2	14	14	2	3	3	10	3	4	15	18	19	29
Death	22	40	20	25	17	19	18	23	172	268	67	76
DED	16	25	7	10	4	4	4	6	10	16	21	36
CARD	11	15	20	24	6	11	1	2	26	32	48	58
Total	81	119	87	117	51	70	47	72	284	408	193	266

The expansion of putative baculovirus inhibitor of apoptosis repeat domains are marked in bold.
[a] Numbers were annotated by PFAM search in the filtered annotated genome of *Nematostella vectensis* at StellaBase (www.stellabase.org).
[b] Numbers were identified in *Crassostrea gigas* assembled EST open reading frames.
[c] Numbers were annotated by PFAM in the *Caenorhabditis elegans* protein database at WormBase (http://www.sanger.ac.uk/Projects/C_elegans/WORMBASE).
[d] Numbers were annotated by PFAM search in annotated genome of *D. melanogaster*, *Strongylocentrotus purpuratus*, and *Homo sapiens* download from NCBI.

(named CgFADD, GenBank accession number HQ425699) that contained 726-bp ORFs codes for a 241 aa peptide (Supplementary Fig. 2A). Cluster 6104 and Cluster 10162, which were obtained from the AJ565467 and EW777636 ESTs, were annotated as IAP homologues. Analysis using 3′ and 5′RACE identified the full-length cDNA of CgIAP (GenBank accession number HQ425701), which consisted of 1967 bp and 1746-bp predicted ORFs (Supplementary Fig. 2B). Cluster 18973 (assembled from the ESTs FP005108 and AM853628) and Cluster 20647 (assembled from the ESTs CU984422, AM860863 and CU989565) were annotated by Pfam to contain effector and initiator caspases. The identified cDNA of the effector caspase (named CgCaspase1, GenBank accession number HQ425703) and initiator caspase (named CgCaspase2, GenBank accession number HQ425705) contained ORFs encoding putative proteins of 303 and 428 amino acids in length, respectively (Supplementary Fig. 2C–D).

3.4. Domain architecture of the four selected apoptosis-related genes

Domain architecture of the four apoptosis-related genes was predicted using ScanProsite and is illustrated in Fig. 3. CgFADD had a protein structure similar to that of *H. sapiens* FADD, but it was different from that of BG4, a FADD ortholog of *D. melanogaster*. CgIAP had domain organization predicted to be similar to that of IAP-1 of *D. melanogaster* (Fig. 3A). We searched all eight IAPs identified in human and did not find one containing two BIR domains and a RING domain. The most similar were XIAP (three BIR and a RING) and MLIAP (a BIR and a RING) (Fig. 3B). Similar to all of the caspases, caspases cloned from *C. gigas* had a conservative pentapeptide active site, QACXG (where X could be R, Q or D). CgCaspase1 was a typical effector caspase lacking the long prodomain, whereas CgCaspase2 was an initiator caspase with a long CARD prodomain. Figure 3C shows that the effector caspases were conserved in *C. gigas*, *D. melanogaster* and *H. sapiens*. They all consisted of a caspase domain and lacked long prodomains. The deduced amino acid sequence of CgCaspase1 showed 41% and 43% identity with the DCP-1 and DRICE of *D. melanogaster*, respectively, and 44% identity with caspase-7 of *H. sapiens*. The *C. gigas* initiator caspase showed lower sequence identity with *D. melanogaster* and *H. sapiens* and were only estimated at 23% and 26%, respectively. In fact, the ScanProsite analysis of DRONC did not predict the CARD, and we instead found this domain annotation from UniProt (Fig. 3D). Thus, it should be noted that the CARD in DRONC was a little different from FADDs in *C. gigas* and *H. sapiens*.

3.5. Genomic sequences of selected apoptosis-related genes

Introns located within the coding sequences of the pacific oyster genome were calculated and compared with those of fly and

Fig. 2. Multiple sequence alignments of the complete BIR domains obtained from the assembled unigenes of the pacific oyster. For shading, 0.5 was designated as the fraction of sequences that agreed. The conserved amino acid residues in the functional zinc ion-binding motif ($CX_2CX_{16}HX_6C$) are marked with an asterisk.

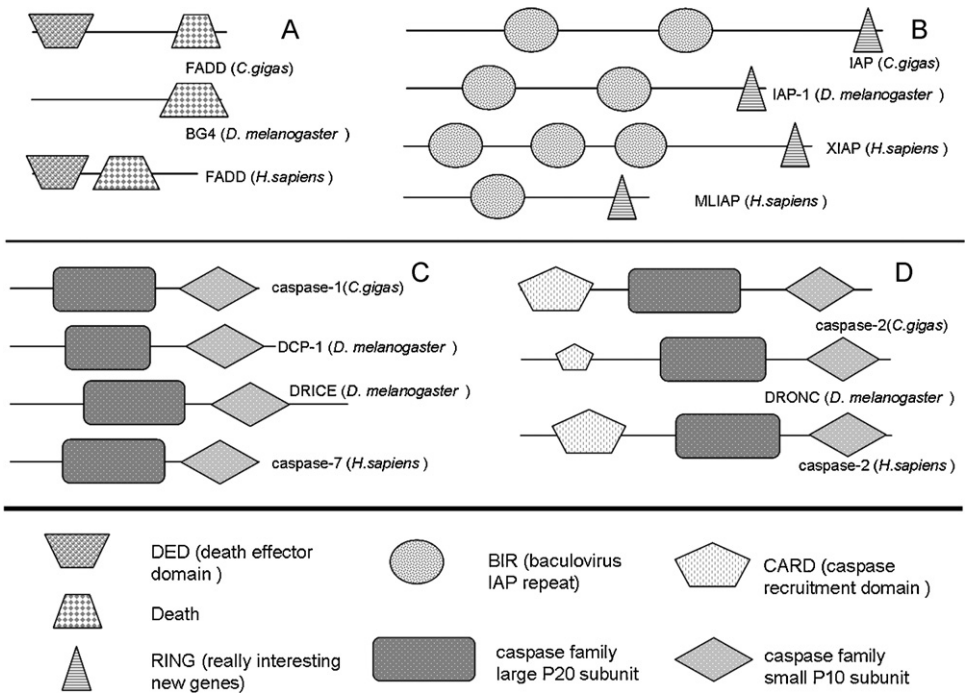

Fig. 3. Predicted domain architecture for each selected gene. Protein domains were predicted using ExPASy (http://www.expasy.ch/). Proteins were obtained from Uniprot, and the accession numbers are as follows: *D. melanogaster*: FADD (Q9V3B4), IAP-1 (Q24306), DROME (O02002), DRICE (O01382), DRONC (Q9XYF4); *Homo sapiens*: FADD (Q13158), XIAP (P98170), MLIAP (Q96CA5), caspase-7 (P55210) and caspase-2 (P42575).

human. The cloned genomic DNA of CgFADD (GenBank accession no HQ425700), CgIAP (GenBank accession no HQ425702), CgCaspase1 (GenBank accession no HQ425704) and CgCaspase2 (GenBank accession no HQ425706) contained 2, 4, 5 and 7 introns, respectively, in the coding regions (Table 2). All of the introns satisfied the consensus GT/AG rule. Table 2 shows that the rate of intron gain or loss in the selected apoptosis genes differed significantly between *C. gigas*, *D. melanogaster* and *H. sapiens*. *D. melanogaster* underwent extensive intron loss, whereas *C. gigas* and *H. sapiens* accumulated and retained many introns (Table 2). There was a positive correlation between the intron density and the generation time of each organism in the genes analyzed in our study. The generation times are about 7 days, 1 year and 20 years in *D. melanogaster*, *C. gigas* and *H. sapiens*, respectively, and the average intron density of selected genes showed an increasing pattern with 0.6, 4.5 and 5.4 introns per gene, respectively.

3.6. Tissue expression of CgFADD, CgIAP, CgCaspase1 and CgCaspase2

Tissue expression pattern was assessed in six tissues of the pacific oyster, including hemolymph, gill, mantle, muscle, digestive gland and gonad (Supplementary Fig. 3). The highest expression of caspases (CgCaspase1 and CgCaspase2) transcripts were found in gill and mantle, and the lowest expression were found in gonad and digestive gland. CgIAP was expressed at the highest levels in hemolymph, followed by gill and muscle. The highest expression of CgFADD was also in hemolymph.

3.7. Temporal expression of CgFADD, CgIAP, CgCaspase1 and CgCaspase2 challenged with V. anguillarum

To examine whether the four genes functioned in the pacific oyster defense system, we employed qRT-PCR to test the expres-

Table 2
Genomic architecture for the selected gene.

Species_gene	Length	No. exon	No. intron	Genomic architecture
Cg_FADD	2729	3	2	**E1**:430; **I1**:1,394; **E2**:27; **I2**:607; **E3**:269;
Dm_BG4	720	1	0	**E1**:720;
Hs_FADD	3014	2	1	**E1**:286; **I1**:2387; **E2**:341;
Cg_IAP	2491	5	4	**E1**:236; **I1**:415; **E2**:928; **I2:**108; **E3**:190; **I3**:97; **E4**:195; **I4**:125; **E5**:197;
Dm_IAP-1	1317	1	0	**E1**:1,317;
Hs_XIAP	21,519	6	5	**E1**:877; **I1**:2,079; **E2**:100; **I2**:2,519; **E3**:79; **I3**:1,414; **E4**:43; **I4**:7,719; **E5**:201; **I5**:6,294; **E6**:194;
Hs_MLIAP	3509	6	5	**E1**:449; **I1**:1,457; **E2**:100; **I2**:395; **E3**:81; **I3**:91; **E4**:46; **I4**:547; **E5**:72; **I5**:124; **E6**:248;
Cg_Caspase1	4053	6	5	**E1**:34; **I1**:511; **E2**:73; **I2**:1,190; **E3**:254; **I3**:414; **E4**:176; **I4**:188; **E5**:142; **I5**: 838; **E6**:233;
Dm_DCP-1	1458	3	2	**E1**:152; **I1**:426; **E2**:563; **I2**:60; **E3**:257;
Dm_DRICE	1020	1	0	**E1**:1,020
Hs_caspase-7	31,097	6	5	**E1**:110; **I1**:23,478; **E2**:137; **I2**:482; **E3**:129; **I3**:2,582; **E3**:176; **I4**:767; **E5**:130; **I5**:2,876; **E6**:230
Cg_Caspase2	4749	8	7	**E1**:135; **I1**:190; **E2**:199; **I2**:392; **E3**:83; **I3**:220; **E4**:207; **I4**:574; **E5**:220; **I5**:1,036; **E6**:168; **I6**:611; **E7**:110; **I7**:439; **E8**:165;
Dm_DRONC	1862	2	1	**E1**:397; **I1**:459; **E2**:906;
Hs_caspase-2	16,565	12	11	**E1**:13; **I1**:3,010; **E2**:151; **I2**:609; **E3**:168; **I3**:149; **E4**:82; **I4**:1,331; **E5**:95; **I5**:272; **E6**:177; **I7**: 5,149; **E8**:129; **I8**:152; **E9**:91; **I9**:3,489; **E10**:150; **I10**:740; **E11**:110; **I11**:156; **E12**:132;

The abbreviations are as follows: E: Exon; I: Intron; Cg: *Crassostrea gigas*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens*.

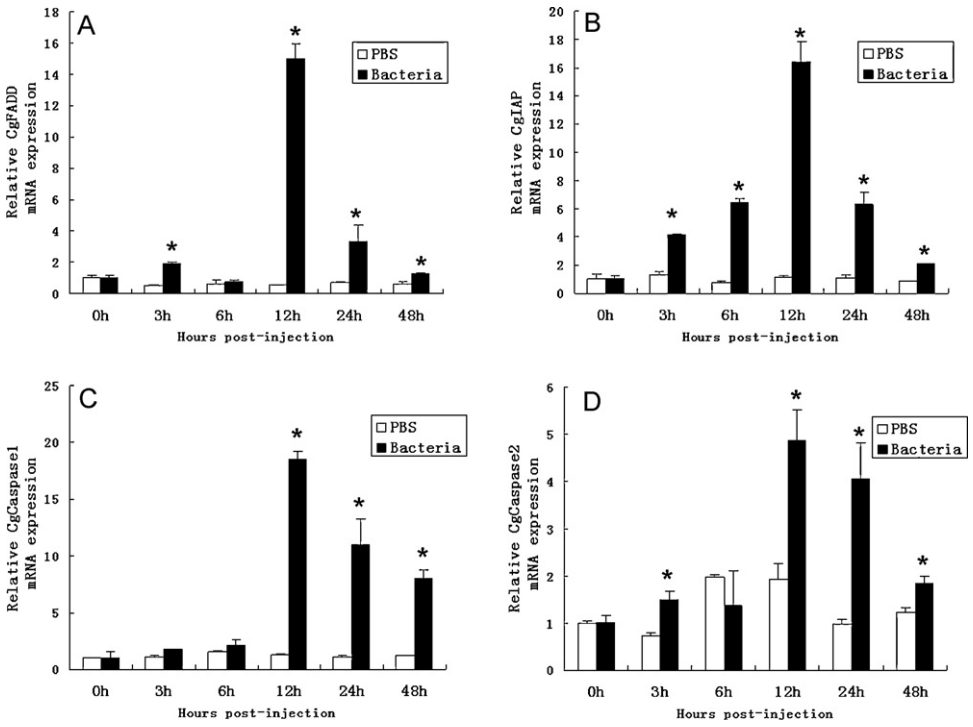

Fig. 4. CgFADD, CgIAP, CgCaspase1 and CgCaspase2 mRNA expression in oyster hemolymph following *V. anguillarum* infection. Elongation factor (EF) gene expression is used as an internal control, and 0 h is used as the reference time. Vertical bars represent the mean ± SD ($N = 3$). White indicates organisms that were injected with LPS (negative control), and black indicates organisms that were injected with *V. anguillarum*. *$P < 0.05$ between challenged and control organisms at the same time point.

sion level of the genes after challenge with *V. anguillarum* (Fig. 4). Compared with the control group, the levels of each of the gene transcripts increased gradually at early time points after challenge and reached the highest level at 12 h, then decreased gradually from 12 h to 48 h. Significant differences in expression of each of the four genes were found between the control group and the challenged group ($P < 0.05$).

4. Discussion

Expressed sequence tags are a valuable resource for gene discovery; however, ESTs are incomplete and do not cover the full-length coding sequences. In this study, we used a domain-based method to search for molecules with apoptotic features in the assembled unigenes of the pacific oyster. To the best of our knowledge, this study is the first to report the overall apoptotic molecular pattern in mollusca. Additionally, neither cDNA nor gDNA of FADD and effector caspases have been identified in molluscs; thus, the CgFADD and CgCaspase1 genes cloned in this study are the first FADD-like and effector caspase-like ones isolated in molluscs.

Fig. 1 showed the basic apoptotic molecules were conserved in the pacific oyster. Homologs of FADD, IAP, capase-3/7 and caspase-9 were present and domains joining in the protein interaction were all conserved. Although caspase-8 were not predicted in the oyster putative EST ORFs, homolog in other mollusks (*Haliotis discus discus*) has been identified (De Zoysa et al., 2009), it may also exist in the oysters. Despite presence of major apoptotic molecules, whether the vertebrate signal transduction pathway exists in *C. gigas* need further demonstration (Yuan et al., 2010).

Comparative analysis of apoptosis-related domains suggests two major findings; first, from an evolutionary and comparative point of view, the apoptotic machinery in the *C. gigas* and *N. vectensis* is more complex than that observed in Ecdysozoa, and second, the BIR domain copy number is greater in the pacific oyster.

It has been reported that cnidarians, echinoderms and vertebrates have a greater complexity of apoptotic signals than nematodes and arthropods (Sullivan et al., 2006; Robertson et al., 2006). Herein, we found that complexity in the pacific oyster was intermediate between that of ecdysozoa and deuterostomes (Table 1), suggesting that gene loss occurred in the ecdysozoa during evolution or that the apoptotic machinery expanded in the deuterostome lineages leading to echinoderms and vertebrates but shrunk in the ecdysozoa lineages leading to *C. elegans* and *D. melanogaster*. Such divergence is in agreement with Oberst et al. (2008) who indicated that the well-characterized apoptotic pathway requiring mitochondrial outer membrane permeability upstream of caspase activation arose in chordates or was lost in ecdysozoa during evolution. Apoptosis is a gene-directed cellular self-destruction program that is ubiquitous in virtually all cells of eukaryotes and serves to remove cells during development and in adulthood that are no longer needed, damaged, or infected (Baehrecke, 2002; Benedict et al., 2002). The flexibility of the apoptotic system might help the pacific oyster adapt to complicated and volatile living conditions, such as harmful algae, heavy metals, pesticides and parasites. Such flexibility might also function in mediating oyster development.

Most BIR domains were discovered in IAP proteins (Uren et al., 1998; Wu et al., 2000). The expansion of BIR domains may illustrate a complex regulatory mechanism in the apoptotic process of the pacific oyster. It is known that IAPs typically inhibit apoptosis through interaction with caspases, but caspase expansion was not found in the unigenes of the pacific oyster. The evolutionary lineage of BIRs and caspase domains is an interesting area for further studies when the genome sequence information is available.

Unlike human FADD gene consists of DED and Death domain, BG4 (FADD of *D. melanogaster*) was found to lack a DED but had a death-inducing domain (DID) instead (Hu and Yang, 2000). CgFADD consisted of DED and Death domains that were more similar to the human than the fly (Fig. 3A), indicating that FADD gene might undergo converging evolution. It is also possible that an ancestral FADD gene underwent mutation in *D. melanogaster*. CgIAP had two BIR domains and a RING finger, but we did not find this structure in

the *H. sapiens* IAPs (Orme and Meier, 2009; Yang and Li, 2000); the human IAPs most resembling CgIAP were XIAP and MLIAP (Fig. 3B). Importantly, different BIR domains selectively interact with various caspases. In humans, the XIAP BIR1 domain is able to inhibit caspase-3 (Sun et al., 1999), BIR2 directly associates with both pro-caspase-3 and activated caspase-3 (Sun et al., 1999), and the BIR3-RING region binds and inhibits caspase-9 (Deveraux et al., 1999). This selectivity was also reported in the IAP-1 gene of *D. melanogaster* (Orme and Meier, 2009). How the BIR domains function in CgIAP requires further studies

Alignment of the CgCaspase amino acid sequences with the *D. melanogaster* and *H. sapiens* sequences showed that the conservation of effector caspases was higher than initiator caspases, which is in accordance with a report in mammals indicating that downstream caspases evolve more slowly than upstream caspases in the caspase-cascade signaling system (da Fonseca et al., 2010). In this study, initiator caspases showed low alignment identities between oysters and other model animals (23% with *D. melanogaster* and 26% with *H. sapiens*), which was also seen in the initiator caspase8 from *Mytilus californianus* (GenBank accession number ADB80147). The McCaspase8 presented 29% and 28% identities with that of *D. melanogaster* and *H. sapiens*. Initiator caspases have been reported to own a higher positive selection and rapid mutation to keep the efficient apoptosis machinery (da Fonseca et al., 2010), which might lead to the mentioned low identities. However, whether the molluscan initiator caspases play similar roles with that of model speices need further functional studies, in consideration of such low identities.

There was extensive intron gain or loss during evolution, and it has been reported that the intron density of eukaryotic genomes varies by more than three orders of magnitude (Jeffares et al., 2006). Reproduction is a major feature of life, and the generation time of organisms undergoes strong selective pressure. The presence of introns delays mRNA processing time (Neugebauer, 2002) and could be crucial in organisms with shorter generation times (Jeffares et al., 2006). In agreement with this hypothesis, the generation times in *D. melanogaster*, *C. gigas* and *H. sapiens* showed an increasing average intron density in the selected genes (Table 2).

Hemocytes are reported to be effective cells in the immune responses of mollusks (Matozzo et al., 2007), which are often affected by vibriosis, a major bacterial disease in bivalves (Gomez-Leon et al., 2005). It has been reported that caspases from variously colored abalones (*H. diversicolor*) are upregulated after bacterial challenge (Huang et al., 2010). The clear time-dependent upregulated expression of the four apoptosis-related genes after infection with *V. anguillarum* suggested the involvement of regulatory mechanisms and an essential role of the four apoptosis-related genes in the immune defense of the pacific oyster (Fig. 4).

Oysters live in coastal tidal zones and often encounter volatile conditions. Physical factors (e.g., salinity change caused by rainfall; temperature change between days and nights), harmful algal blooms and disease (caused by parasites, viruses and bacteria) require that oysters possess a defense mechanism to adapt to such environments. Apoptosis plays a significant role in the molluscan defense system; however, few studies have focused on this important process, particularly at the molecular and evolutionary levels (Kiss, 2010). Although apoptosis is highly conserved among species, some molluscan apoptotic pathways and their key genes appear to be sufficiently different from those in model invertebrates. It will be interesting to find more apoptosis-related genes in molluscs and explore their roles in defense.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.01.005.

References

Armstrong, J.S., 2006. Mitochondrial membrane permeabilization: the sine qua non for cell death. Bioessays 28, 253–260.
Baehrecke, E.H., 2002. How death shapes life during development. Nat. Rev. Mol. Cell Biol. 3, 779–787.
Benedict, C.A., Norris, P.S., Ware, C.F., 2002. To kill or be killed: viral evasion of apoptosis. Nat. Immunol. 3, 1013–1018.
da Fonseca, R.R., Kosiol, C., Vinar, T., Siepel, A., Nielsen, R., 2010. Positive selection on apoptosis related genes. FEBS Lett. 584, 469–476.
De Zoysa, M., Nikapitiya, C., Moon, D.O., Whang, I., Kim, G.Y., Lee, J., 2009. A novel Fas ligand in mollusk abalone: molecular characterization, immune responses and biological activity of the recombinant protein. Fish Shellfish Immunol. 27, 423–432.
Deveraux, Q.L., Leo, E., Stennicke, H.R., Welsh, K., Salvesen, G.S., Reed, J.C., 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 18, 5242–5251.
Deveraux, Q.L., Reed, J.C., 1999. IAP family proteins--suppressors of apoptosis. Genes Dev. 13, 239–252.
Eberstadt, M., Huang, B., Chen, Z., Meadows, R.P., Ng, S.C., Zheng, L., Lenardo, M.J., Fesik, S.W., 1998. NMR structure and mutagenesis of the FADD (Mort1) death-effector domain. Nature 392, 941–945.
Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S., 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.
Gomez-Leon, J., Villamil, L., Lemos, M.L., Novoa, B., Figueras, A., 2005. Isolation of Vibrio alginolyticus and Vibrio splendidus from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. Appl. Environ. Microbiol. 71, 98–104.
Green, D.R., Kroemer, G., 2004. The pathophysiology of mitochondrial cell death. Science 305, 626–629.
Gueguen, Y., Cadoret, J.P., Flament, D., Barreau-Roumiguiere, C., Girardot, A.L., Garnier, J., Hoareau, A., Bachere, E., Escoubas, J.M., 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene 303, 139–145.
Hedgecock, D., Gaffney, P.M., Goulletquer, P., Guo, X., Reece, K., Warr, G.W., 2005. The case for sequencing the Pacific oyster genome. J. Shellfish Res. 24, 429–441.
Hu, S., Yang, X., 2000. dFADD, a novel death domain-containing adapter protein for the Drosophila caspase DREDD. J. Biol. Chem. 275, 30761–30764.
Huang, W.B., Ren, H.L., Gopalakrishnan, S., Xu, D.D., Qiao, K., Wang, K.J., 2010. First molecular cloning of a molluscan caspase from variously colored abalone (*Haliotis diversicolor*) and gene expression analysis with bacterial challenge. Fish Shellfish Immunol. 28, 587–595.
Hughes, F.M., Foster, B., Grewal, S., Sokolova, I.M., 2010. Apoptosis as a host defense mechanism in *Crassostrea virginica* and its modulation by *Perkinsus marinus*. Fish Shellfish Immunol. 29, 247–257.
Itoh, N., Takahashi, K.G., 2009. A novel peptidoglycan recognition protein containing a goose-type lysozyme domain from the Pacific oyster, *Crassostrea gigas*. Mol. Immunol. 46, 1768–1774.
Jeffares, D.C., Mourier, T., Penny, D., 2006. The biology of intron gain and loss. Trends Genet. 22, 16–22.
Kiss, T., 2010. Apoptosis and its functional significance in molluscs. Apoptosis 15, 313–321.
Kornbluth, S., White, K., 2005. Apoptosis in Drosophila: neither fish nor fowl (nor man, nor worm). J. Cell Sci. 118, 1779–1787.
Krammer, P.H., 2000. CD95's deadly mission in the immune system. Nature 407, 789–795.
Lettre, G., Hengartner, M.O., 2006. Developmental apoptosis in *C. elegans*: a complex CEDnario. Nat. Rev. Mol. Cell Biol. 7, 97–108.
Li, L., Qiu, L., Song, L., Song, X., Zhao, J., Wang, L., Mu, C., Zhang, H., 2009. First molluscan TNFR homologue in Zhikong scallop: molecular characterization and expression analysis. Fish Shellfish Immunol. 27, 625–632.
Matozzo, V., Rova, G., Marin, M.G., 2007. Haemocytes of the cockle *Cerastoderma glaucum*: morphological characterisation and involvement in immune responses. Fish Shellfish Immunol. 23, 732–746.
Montagnani, C., Avarre, J.C., de Lorgeril, J., Quiquand, M., Boulo, V., Escoubas, J.M., 2007. First evidence of the activation of Cg-timp, an immune response component of Pacific oysters, through a damage-associated molecular pattern pathway. Dev. Comp. Immunol. 31, 1–11.
Moroz, L.L., Kohn, A.B., 2010. Do different neurons age differently? Direct genome-wide analysis of aging in single identified cholinergic neurons. Front. Aging Neurosci., 2.

Neugebauer, K.M., 2002. On the importance of being co-transcriptional. J. Cell Sci. 115, 3865–3871.
Oberst, A., Bender, C., Green, D.R., 2008. Living with death: the evolution of the mitochondrial pathway of apoptosis in animals. Cell Death Differ. 15, 1139–1146.
Orme, M., Meier, P., 2009. Inhibitor of apoptosis proteins in Drosophila: gatekeepers of death. Apoptosis 14, 950–960.
Pirger, Z., Elekes, K., Kiss, T., 2006. Electrical properties and cell-to-cell communication of the salivary gland cells of the snail, Helix pomatia. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 145, 7–19.
Pirger, Z., Racz, B., Kiss, T., 2009. Dopamine-induced programmed cell death is associated with cytochrome c release and caspase-3 activation in snail salivary gland cells. Biol. Cell. 101, 105–116.
Robertson, A.J., Croce, J., Carbonneau, S., Voronina, E., Miranda, E., McClay, D.R., Coffman, J.A., 2006. The genomic underpinnings of apoptosis in *Strongylocentrotus purpuratus*. Dev. Biol. 300, 321–334.
Salvesen, G.S., Duckett, C.S., 2002. IAP proteins: blocking the road to death's door. Nat. Rev. Mol. Cell Biol. 3, 401–410.
Sullivan, J.C., Ryan, J.F., Watson, J.A., Webb, J., Mullikin, J.C., Rokhsar, D., Finnerty, J.R., 2006. StellaBase: the *Nematostella vectensis* genomics database. Nucleic Acids Res. 34, D495–499.
Sun, C., Cai, M., Gunasekera, A.H., Meadows, R.P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S.C., Fesik, S.W., 1999. NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature 401, 818–822.
Terahara, K., Takahashi, K.G., 2008. Mechanisms and immunological roles of apoptosis in molluscs. Curr. Pharm. Des. 14, 131–137.
Uren, A.G., Coulson, E.J., Vaux, D.L., 1998. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem. Sci. 23, 159–162.
Wolf, B.B., Green, D.R., 1999. Suicidal tendencies: apoptotic cell death by caspase family proteinases. J. Biol. Chem. 274, 20049–20052.
Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X., Shi, Y., 2000. Structural basis of IAP recognition by Smac/DIABLO. Nature 408, 1008–1012.
Yang, Y.L., Li, X.M., 2000. The IAP family: endogenous caspase inhibitors with multiple biological activities. Cell Res. 10, 169–177.
Yuan, S., Liu, H., Gu, M., u, L.X., Huang, S., Ren, Z., Xu, A., 2010. Characterization of the extrinsic apoptotic pathway in the basal chordate amphioxus. Sci. Signal. 3, ra66.