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Functional divergence of six isoforms of antifungal peptide Drosomycin in *Drosophila melanogaster*

Wan-Ying Yang [a], Shuo-Yang Wen [b], Ya-Dong Huang [c], Ming-Qiang Ye [a], Xiao-Juan Deng [a], Dong Han [a], Qin-You Xia [d], Yang Cao [a,*]

[a] *Department of Sericulture Science, College of Animal Science, South China Agricultural University, 510642, Guangzhou, PR China*
[b] *Department of Entomology, College of Natural Resource and Environment, South China Agricultural University, 510642, Guangzhou, PR China*
[c] *Biopharmaceutical Research and Development Center, Jinan University, 510632, Guangzhou, PR China*
[d] *Key Sericultural Laboratory of Agriculture Ministry, College of Sericulture and Biotechnology, Southwest University, 400716, Chongqing, PR China*

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Abstract

Drosomycin (*Drs*) gene encodes a 44-residue inducible antifungal peptide, Drosomycin, in *Drosophila melanogaster*. Six genes, *Drs-lC*, *Drs-lD*, *Drs-lE*, *Drs-lF*, *Drs-lG* and *Drs-lI*, show homology to the *Drs* form in a multigene family on the 3rd chromosome of *D. melanogaster*. It is the first experimental demonstration that the six members in the Drs family act as functional genes. To further delineate the functional divergence of these six members, their cDNA sequences were cloned respectively into the pET-3C vector and expressed in the *E. coli*. The antifungal activity of the expression products was assayed using the Cerletti's method. The results showed a difference among the six isoforms in antifungal activity against the tested fungal strains: in which Drs was most effective and showed antifungal activity to all seven fungal strains, whereas isoform Drs-lC was effective to six strains, Drs-lD was effective to five strains, Drs-lG was effective to four strains, and Drs-lE and Drs-lF were effective to only three strains. Drs-lI had no activity against any tested fungal strains. By comparing the variable residue sites of these six isoforms to that of Drosomycin in the three-dimensional structure, we suggested that the reduction in the antifungal activity was due to the variable residues that were not in the α-helix. In addition, two inserted residues (RV) in Drs-lI may affect the dimensional structure and resulted in a functional change. These results may explain the evolution of the *Drosomycin* multigene family and its functional divergence.

Keywords: *Drosomycin*; Multigene family; Antifungal peptide; *Drosophila melanogaster*; Functional identification

1. Introduction

Insects respond to microbial challenge by the rapid and transient synthesis of a large number of potent antimicrobial peptides (Cociancich et al., 1994; Hoffmann, 2003). Inducible antimicrobial peptides are active against many different microorganisms and they play a critical role in the humoral reactions of insect innate immunity for surviving in the microorganism-rich environment. This is one of the reasons why insects become the most prosperous class within the animal kingdom (Cociancich et al., 1994). The lack of adaptive immune system but with potent antimicrobial responses makes *Drosophila* particularly well suited for the study of innate immunity (Hoffmann and Reichhart, 2002). To date, seven distinct inducible antimicrobial peptides (or peptide families), Drosomycin, Metchnikowin, Defensin, Attacin, Cecropin, Drosocin and Diptericin, have been identified (Hoffmann, 2003). Their activity spectra are different. Only Drosomycin and Metchnikowin are effective to fungi. Antimicrobial peptide genes are typically organized in small, but closely related clusters. These clusters appear to be in a dynamical steady-state where new genes are continuously produced by gene duplication while others are lost by mutation (Hedengren et al., 2000).

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethylsulfoxide; *Drs*, *Drosomycin*; *Drs-lC*, *Drosomycin-like C*; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, PA-gel electrophoresis.

* Corresponding author. Tel.: +86 20 8528 0367; fax: +86 20 8528 1459.
E-mail address: caoyang@scau.edu.cn (Y. Cao).

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Among the seven characterized antimicrobial peptides in *Drosophila melanogaster*, some of them are encoded by multigene families, such as the *Cecropin* multigene family (Clark and Wang, 1997; Date et al., 1998; Ramos-Onsins and Aguade, 1998; Quesada et al., 2005), *Attacin* multigene family (Asling et al., 1995; Dushay et al., 2000; Hedengren et al., 2000; Lazzaro and Clark, 2001), *Diptericin* multigene family (Hedengren et al., 2000) and *Drosomycin* multigene family (Daibo et al., 2001; Jiggins and Kim, 2005). The evolution of these multigene families has been well studied. *Cecropin* multigene family was clustered with four functional genes (*CecA1*, *CecA2*, *CecB* and *CecC*) and two pseudogenes (*Cec Ψ1* and *Cec Ψ2*) on chromosome 3 (99E2) of *D. melanogaster* (Kylsten et al., 1990; Tryselius et al., 1992; Clark and Wang, 1997; Ramos-Onsins and Aguade, 1998). The *Attacin* multigene family includes four members, *Attacin A*, *Attacin B*, *Attacin C* and *Attacin D*. *Attacin A* and *Attacin B* are 96% and 97% identical at the nucleotide and amino acid levels respectively (Lazzaro and Clark, 2001), and *Attacin C* shows only 67% nucleotide and 70% amino acid identity to *Attacin A* (Hedengren et al., 2000; Lazzaro and Clark, 2001). These three genes are located in chromosome 2, but the *Attacin D* is more divergent and is located in different chromosomes (Hedengren et al., 2000). The *Diptericin* gene family includes two members, *Diptericin* and *Diptericin B* which are linked in tandem (Wicker et al., 1990; Hedengren et al., 2000).

Drosomycin can be induced by bacteria and exhibits potent antifungal activity. Fehlbaum et al. (1994) reported firstly that the bacteria challenge can also induce the synthesis of a 44-residue peptide of Drosomycin which is processed from a 70-residue precursor molecule. The Drosomycin contains 8 cysteines engaged in intramolecular disulfide bridges and shows a significant homology with a family of 5-kDa cysteine-rich plant antifungal peptides of the seeds of Brassicaceae (Fehlbaum et al., 1994). Sequences of six genes are similar to *Drs* in the *Drosophila melagnoster* genome. Jiggins and Kim (2005) reconstructed the patterns of the *Drosomycin* multigene family in their study on the evolution of antifungal peptides in *Drosophila*. In this study, we found that *Drs* and these six similar genes cluster along the 3rd chromosome. We tentatively named them *Drosomycin-like C* (*Drs-lC*) (GenBank accession no. AY225091), *Drosomycin-like D* (*Drs-lD*) (GenBank accession no. AY351397), *Drosomycin-like E* (*Drs-lE*) (GenBank accession no. AY351398), *Drosomycin-like F* (*Drs-lF*) (GenBank accession no. AY351399), *Drosomycin-like G* (*Drs-lG*) (GenBank accession no. AY351400) and *Drosomycin-like I* (*Drs-lI*) (GenBank accession no. AY351402) (Fig. 1). Their corresponding products were named Drosomycin-like C (Drs-lC), Drosomycin-like D (Drs-lD), Drosomycin-like E (Drs-lE), Drosomycin-like F (Drs-lF), Drosomycin-like G (Drs-lG) and Drosomycin-like I (Drs-lI), which correspond to Dro1, Dro2, Dro3, Dro4, Dro5, and Dro6 respectively (Jiggins and Kim, 2005). These genes encode putative antifungal peptides duplicated at least several times and some of the copies become spacers or pseudogenes (Daibo et al., 2001). *Drosomycin-like A/B* genes were also reported in the *Drosophila triauraria*. Two genes that are shown to be upregulated in diapausing *D. triauraria* have similarity to *Drosomycin* (Daibo et al., 2001). Six *Drosomycin* genes were also found in *D. yakuba*, seven in *D. simulans*, *D. ereca* and four in *D. ananassae* (Jiggins and Kim, 2005). Neither *Drosomycin-like* in *D. melanogaster* nor *Drosomycin-like* in *D. triauraria* and in other *Drosophila* species has not been functionally identified (Jiggins and Kim, 2005). It is unclear if each gene has antifungal activity, or if these genes just become pseudogenes. To provide experimental evidences for the antibacterial function of the novel 6 members of *Drs* multigene family, we amplified these genes by two-steps PCR and expressed in the *E. coli*. The recombinant products were purified and the antifungal activity was assayed.

2. Material and methods

2.1. Microorganisms

Filamentous fungi were grown on a standard potato medium. Spores and hypha were harvested as described by Broekaert et al. (1990). The following fungal strains were used: *Alternaria longipe*, *Neuropora crassa*, *Fusarium culmorum Sacc* (purchased from Institute of Microbiology, Chinese Academy of Sciences, Beijing), *Botrytis cinereapers*, *Fusarium oxysporum*, *Colletotrichum capsici*, and *Rhizoctonia solani* (gifts from Dr. Zi-De Jiang and Dr. Ping-Gen Xi, Lab of Mycology, South China Agricultural University).

2.2. Amplification of genes of Drs multigene family by PCR

Drs and *Drs-lC* genes were amplified from the recombined vector pHIL-S1-dro (with *Drs* gene) and pET-21d-dro (with *Drs-lC* gene) (Zhong et al., 2004) by using the primer pair Dros1 (with the *Nde* I cleavage site, 5′-GACTGCGCATATG-GACTGCCTGTCCGGAAGATA-3′)/Dros2 (with the *Bam* HI cleavage site, 5′-GCCGGATCCTTAGCATCCTTCGCAC-CAGCAC-3′). Recombined vector DNA was extracted using the EZNAPlasmid Miniperps Kit (Omega, USA) according to the manufacturer's instructions. The 20 μL PCR reaction mixtures contained 1 μL of vector DNA, 1 μL of each primer,

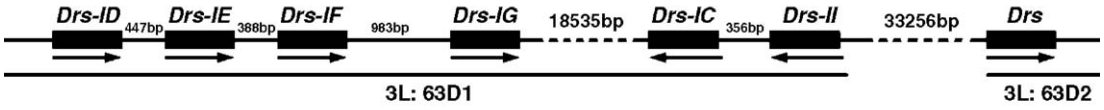

Fig. 1. The genomic structure of the *Drosomycin* multigene family. The black boxes indicated the members of the multigene family; the thick lines with numbers indicated the numbers of nucleotide between the members of the multigene family; the fine lines with the codes indicated the location of the members in the chromosome; the arrows indicated the transcription direction.