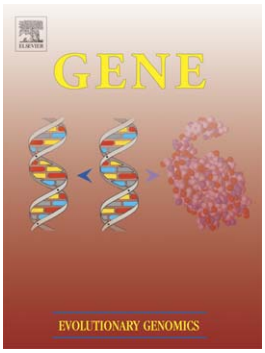

Functional divergence of six isoforms of antifungal peptide Drosomycin in *Drosophila melanogaster*

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Abbreviations:

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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;

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 multi-gene family on the 3rd chromosome of *D. melanogaster*. It is the first experimental demonstration that the 6 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 expression products was assayed using the Cerletti's method. The results showed difference among the six isoforms in antifungal activity against 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, Drs-lE and Drs-lF was effective to only three strains. Drs-lI had no activity to 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 the functional change. These results may explain the evolution of the *Drosomycin* multigene family and its functional divergence.

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 microorganism 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 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* a 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). Among characterized seven antimicrobial peptides in *Drosophila melanogaster*, some of them are encoded by multigene families, such as *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 Jim, 2005). Evolution of these multigene family has been well studies. *Cecropin* multigene family was clustered with four functional genes (*CecA1*, *CecA2*, *CecB* and *CecC*) and two pseudogenes (*CecY1* and *CecY2*) on chromosome 3 (99E) of *D. melanogaster* (Kylsten *et al*., 1990; Tryselius *et al*., 1992; Clark and Wang, 1997; Ramos-Onsins and Aguade, 1998). *Attacin* multigene family includs four members, *Attacin A*, *Attacin B*, *Attacin C* and *Attacin D*. The *Attacin A* and *Attacin B* are 96% and

97% identical at the nucleotide and amino acid levels respectively (Lazzaro and Clark, 2001), the *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 locats on chromosome 2, but the *Attacin D* is more divergent and locats on the different chromosome (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 seeds of Brassicaceae (Fehlbaum *et al*., 1994). Sequences of six genes are similary to *Drs* in *Drosophila melagnoster* genome. Jiggins and Jim (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 clusters 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 are corresponding 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 copies become spacers or pseudogenes (Daibo *et al*., 2001). *Drosomycin-like A/B* genes were also reported in the *D. 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 have not been functionally identified (Jiggins and Kim, 2005). It is unclear if each gene has anti-fungal activity, or these genes just become pseudogenes? To provide the 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 recombiant products were purified and the antifungal activity was assayed.

2. Material and methods

2. 1 Microorganisms

Filamentous fungi were grown on the 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*, *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'-GACTGCGCATATGGACTGCCTGTCCGGAAGATA-3') /Dros2 (with the *Bam* H I cleavage site, 5'-GCCGGATCCTTAGCATCCTTCGCACCAGCAC-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 1μL of vector DNA, 1μL of each primer, 2 μL of dNTPs (2.5mmol/L) (Takara, Japan), 2 μL of 10×PCR reaction buffer, 2 μL of Taq DNA polymerase (0.5U/μL) (Takara, Japan) and 11 μL of DD water. The PCR reaction mixtures was pre-denatured at 95 °C for 5 min and then followed by the 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The PCR reaction was ended by incubation at 72 °C for 10 min. PCR products were purified used E. Z. N. A. Cycle-Pure Kit (Omega, USA) according to the manufacturer's instructions and stored at -20°C for further use.

Drs-lD, *Drs-lE*, *Drs-lF*, *Drs-lG* and *Drs-lI* were amplified by two-steps PCR using the primers drs-N2/drs-N3 (with *Bam* HI cleavage site), drs-N1 (with *Nde* I cleavage site) and drs-N3 (Fig. 2, where N denotes D, E, F, G or I), in the first step, the 18 μL PCR reaction mixtures contained 3μL of drs-N2 (10μmol/L), 3μL of drs-N3 (10μmol/L), 2 μL of dNTPs (2.5mmol/L), 2 μL of 10×PCR reaction buffer, 8 μL of water. The mixture was kept in water both at 100 °C for 5 min, then stood on ice for 1 min, added with 2 μL of pfu Taq DNA polymerase (0.5U/μL) (Takara, Japan), followed by 72 °C for 10 min. In the second step, the 50 μL PCR reaction mixtures contained 20 μL of Step-One product, 2μL of drs-N1 (10μmol/L), 2μL of drs-N3 (10μmol/L), 2 μL of dNTPs (2.5mmol/L), 5 μL of 10×PCR reaction buffer, and 19 μL of water, 2μL of Taq DNA polymerase (0.5U/μL). The mixture was pre-denatured at 95 °C for 5 min and then followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The PCR reaction was ended by incubation at 72 °C for 10 min. PCR

products were purified using EZNACycle-Pure Kit (Omega, USA) according to the manufacturer's instructions and stored at -20°C for cloning.

2.3 Construction of recombined expressive vector and expression of peptide

The PCR products were cloned into pGEM®-T Vector System (Promega) and sequenced with BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) in an ABI 377 automated sequencer. Target clones were digested with *Nde* I and *Bam* HI and separated on 2.0% agarose and purified with the QIAquick Gel Extraction Kit (Qiagen, USA). The purified products were ligated to pET-3C Vectors (Novagen, USA) which were digested using *Nde* I and *Bam* HI. The recombinant expression vectors, pET-Drs, pET-C, pET-D, pET-E, pET-F, pET-G, pET-I were extracted by EZNAPlasmid Miniperps Kit (Omega, USA), and then used to transform *E. coli* origami (DE3) (Novagen, USA). The transformant cells were induced with 0.02% IPTG for 3 hours. The products were checked using Tricine-SDS-PAGE as described previously (Schagger and von Jagow, 1987).

2.4 Purification of expression products

The protein inclusion body was collected from the ultrasonicated cells and rinsed respectively with rinsing buffer I (pH 8.0, 0.5% TritonX-100, 5% glycerol), rinsing buffer II (pH 8.0, 5 mmol/L EDTA, 100mmol/L NaCl, 100mmol/L HAc, 3 mol/L Urea), rinsing buffer III (pH 8.0, 5 mmol/L EDTA, 100mmol/L NaCl, 100mmol/L HAc, 4 mol/L Urea), rinsing buffer IV (pH 8.0, 5 mmol/L EDTA, 100mmol/L NaCl, 100mmol/L HAc) and finally centrifuged at 4 °C, 4000 rpm for 15 min to collect inclusion body. The inclusion body was resolved in the 10 vol (w/v) resolving buffer (100 mmol/L KH_2PO_4, 10 mmol/L Tris-HCl, 8 mol/L Urea, 50 mmol/L DTT), homogenized under a rapid dispersive homogenater for 30 min, centrifuged at 25 °C, 12000 rpm for 20 min.

The supernatants were adjusted to pH 5.5 and filtrated using a 0.45 μm filter (Millipore). The protein were then purified with an *ÄKTA* FPLC system (Amersham Pharmacia Biotech) equipped with an UPC-900 monitor, P-920 pump and Frac-950 fraction collector. The column effluent was monitored by absorbance at 280nm. The supernatants were applied to a CM-Sepharose Fast Flow column (18mL, Amersham Pharmacia Biotech) equilibrated with Cation-Buffer I (100 mmol/L HAc-NaOAc, pH 4.0), eluted with Cation-buffer II (0.8 mol/L NaCl, 0.1mol/L L-Arg, pH=9.5). Fractions with target peptides were collected and further purified by Sphacryl S-100 (Amersham Biosciences), equilibrated with Gel-buffer I (0.1mol/L PBS, 0.05 mol/L NaCl, pH 7.0) eluted with Gel-buffer II (0.2 mol/L NaCl, 0.2 mol/L L-Arg). The fractions with target peptides were dried in a vacuum system (Freeze Dry System/Freezone® 4.5, LABCONCO).

To retrieve the dimensional structure of the peptides, the purified peptides were retrieved for antifungal activity by Cerletti's method (Cerletti, 2000) with modification. The fractions with target peptides were treated with a solution containing 15mmol/L Glutathione, 2 mol/L NaCl, 30 mmol/L CHAPS and 20% DMSO at 4 °C for overnight. The fractions were then concentrated using Sephadex™ G-25 (Qiagen) with Desalt-buffer I (0.1 mmol/L K_2PO_4, pH 9.0) and Desalt-buffer II (0.6 mol/L NaCl, 0.2 mol/L sodium citrate, pH 5.0), followed by ultra-filtrate (Ulracel-YM 10 kD, 10mL, Millipore). The fractions were centrifuged at 4 °C, 4000 rpm for 20 min. The supernatants were checked on Tricine-SDS-PAGE gels, and were stored in -20°C for antifungal activity assay.

2.5 Antifungal activity Assay

The antifungal activity of the purified expressional products of the members of the *Drosomycin* multigene family was assayed using ultra sensitive radial diffusion method on thin potato plates

seeded with filamentous fungi (Lambert *et al*., 1989). Briefly, 9-cm plates were poured with the underlay potato medium, filamentous fungi on 2 cm diameter potato medium was seeded on the center of the plates, the plates were then incubated at 28 ℃ until the filamentous fungi grew to 4 cm diameter. Forty μL of test sample (50 μg/mL) was dropped beside the filamentous fungi, and then plates were incubated at 28 ℃ for 24 h. The size of clear area around the filamentous fungi was measured.

3. Result

3.1 Amplification, cloning and expression of seven members of Drosomycin multigene family

Drs and *Drs-lC* were successfully amplified from the combined vector pHIL-S1-dro (with *Drs* gene) and pET-21d-dro (with *Drs-lC* gene) (Zhong *et al*, 2004). *Drs-lD*, *Drs-lE*, *Drs-lF*, *Drs-lG* and *Drs-lI* were amplified by two-steps PCR using primer pairs drs-N2/drs-N3 and drs-N1/drs-N3. The PCR products of the seven genes were checked on 2% agarose gel (Fig. 3) and by sequencing. The size of *Drs-lI* was 163 bp and other products were 157 bp. All sequences are consistent with the sequences in GenBank.

Recombinant proteins were obtained from the transformant *E. coli* origami (Fig. 4-a). The size of the proteins are all predicted being 4.901 kDa (Fig. 4-b).

3.2 Antifungal activity assay

After purification by CM-Sepharose Fast Flow column and Sphacryl S-100 column, further treated desalted to retrieve the dimensional structure and the antifungal activity, the products were also checked on Tricine-SDS-PAGE gel (Fig. 4-c). The antifungal activity assay for the recombinant

products was carried out with the seven strains of pathological fungi. As illustrated in Fig. 5, growth of the tested filamentous fungal strain, *Alternaria longipe*, was inhibited by Drs, Drs-lC, Drs-lD, Drs-lF and Drs-lG, but it can grow normally with Drs-E, Drs-lI and the control Desalt-buffer. The results of antifungal activity assay with other tested fungal strains are showed in Table 1. Different peptides have different antifungal spectra. *Rhizoctonia solani* was inhibited by all expressive products, excepted the Drs-lI. Drs showed the strongest activity against all tested fungal strains. Drs-lC had activity to six strains, Drs-lD to five strains, Drs-lG to four strains, Drs-lE and Drs-lF to three strains. Drs-lI has no antifungal activity to any tested fungal strains.

4. Discussion

4.1 The functional divergence and peptide structure

The three-dimensional structure of Drosomycin in solution has been determined by Landon *et al* (1997). The sequence of 44-residue peptide includes eight cysteine residues engaged in the formation of four internal disulfide bridges: Cys1-Cys8, Cys2-cys5, Cys3-Cys6 and Cys4-Cys7 (Fig. 6A). The fourth disulfide bridge connects the N-terminal short strand of β-sheet to the C-terminal cysteine residue and forms the βαββ scaffold with three β-sheets and one α-helix (Landon *et al*., 1997; Bulet *et al*., 1999) (Fig 6B). The six isoforms of the Drosomycin family have 12 conservative residues with the Drosomycin (marked with black asterisks in Fig. 6A). Eight cysteine residues were among these conservative residues. These conservative residues maintain the similar four internal disulfide bridges as in the Drosomycin, therefore the 3-D structure of the isoforms may be also identical to that of Drosomycine. Comparing the variable residues of the isoforms with those of the Drosomycin, most of variable residues were within the α-helix, whereas only 6 variable residues were in 3 β-sheets area (marked with green asterisks in Fig. 6 A, green letters in Fig. 6 B). In the first β-sheet, L (Leu) of

Drosomycin changed to P (Pro) of Drs-lF; In the second β-sheet, P of Drosomycin changed to G (Gly) of Drs-lE, A (Ala) of Drs-lF and Drs-lI; S (Ser) of Drosomycin changed to A of Drs-lE and R (Arg) of Drs-lI; L (Leu) of Drosomycin changed to M (Met) of Drs-lE; K (Lys) of Drosomycin changed to Q (Gln) of Drs-lI; In the third β-sheet, G (Gly) of Drosomycin changed to Q (Gln) of Drs-lF. The variable residues in the three β-sheet only occur in Drs-lE, Drs-lF and Drs-lI which showed the lower antifungal activity. Drs-lE and Drs-lF were active to only 3 fungi strains, while Drs-lI couldn't inhibit any tested fungi strains. these results suggests that the β-sheet structure is very important for the antifungal activity function and the residue change in the β-sheet might reduce antifungal activity. In addition to the residues change in the β-sheet, one remarkable change in the amino acid sequence divergence of Drs-lI is that two residues, R and V, were inserted between 29^{th} and 30^{th} amino acid of Drosomycin (orange asterisks in Fig. 6 A and orange arrow in Fig. 6 B). Whether or not this insertion, which is located in the outside of the β-sheet and α-helix, results in the reduction of the antifungal function is not clear. If this is the case, it might cause the 3-D structure change of Drs-lI. Other variable residues of Drs-E and Drs-F were located outside of β-sheets, as that happened in Drs-lC, Drs-lD and Drs-lG. these residues are almost inside the α-helix. Although Drs-lC had the most variable residues (12 residues) in α-helix, but its antifungal activity was the strongest among the other isoforms except the Drs of the Drs family. This reveals that the residues change in the α-helix may not affect the antifungal function.

4.2 The biological consequence of the functional divergence of the multigene family

Lynch and Conery (2000) suggest that three alternative outcomes in the evolution of duplicate genes: (1) one copy may simply become silenced by degenerative mutations (nonfunctionalization); (2) one copy may acquire a novel and beneficial function and become preserved by natural selection,

with the other copy retaining original function (neo-functionalization); or (3) both copies may become partially compromised by mutation accumulation to the point at which their total capacity is reduced to the level of single copy ancestral gene (subfunctionalization). The function of duplicate genes may be predicted by the bioinformatics approach. The evolution of some antimicrobial peptide multigene family, such as *Cecropin*, *Attacin*, *Defencin* and *Drosomycin* multigene families, has been well studied as described earlier, but these approaches were lack of the experimental support. Change of amino acid residues could alter conformation of a protein, resulting in change of biological activity. Interestingly, in the Drosomycin family, only a few changes of amino acid residues in the β-sheets cause lose of antifungal activity. However, changes of amino acid residues in the α-helix were not critical. Each gene of the *Drs* multigene family did express after microbial infection, but they showed different expression patterns depending upon the microbial sources (Deng *et al*., preparing). Our result demonstrates a functional divergence in the antimicrobial peptides, and provides evidence for predicting crucial functional sites for engineering new antimicrobial regents.

4.3 Refolding dimensional structure of the peptide after purification

The biological activity of a peptide is related to it's three dimensional structure. Dimensional structure of the expressive AMP gene product might change and lost the biological activity during purification. It happened in our experiment. To refold the three dimensional structure of a peptide, we tried different conditions to get highest antifungal activity for each peptide using the modified Cerletti's method (Cerletti, 2000). However, we couldn't clearly exclude such a possibility that there may be a difference in extent of configurational refolding among seven Drs products. In the future study, we will measure the proportion with the right configuration, and use much more fungal strains to test whether or not the Drs-I lost the function or change the function to resistant other fungal

strains.

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Table 1 Antifungal activity of purified recombinant proteins against the seven strains of fungi

Fungi strain	Drs	Drs-lC	Drs-lD	Drs-lE	Drs-lF	Drs-lG	Drs-lI	CK
Alternaria longipe	+*	+	+	-**	+	+	-	-
Botrytis cinerea pers	+	+	+	+	-	+	-	-
Colletotrichum capsici	+	+	+	+	+	+	-	-
Fusarium culmorum	+	+	+	-	-	-	-	-
Fusarium oxysporum	+	+	-	-	-	-	-	-
Neurospora crassa	+	-	-	-	-	-	-	-
Rhizoctonia solani	+	+	+	+	+	+	-	-
No. of strains inhibited	7	6	5	3	3	4	0	0

* With clear area around the tested filamentous fungi, as in Fig. 5-1, indicating antifungal activity; ** without clear area around the tested filamentous fungi, as in Fig. 5-4, indicating no antifungal activity.

Legends of figures

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 nucleotide sequences 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.

Fig. 2 The positions and sequences of the primers for amplifying *Drs-lD*, *Drs-lE*, *Drs-lF*, *Drs-lG* and *Drs-lI*. The sequence of *Drs-lD* is taken as a representative and indicated in the box. The fine lines indicate the sequences of drs-N1 and drs-N3, the thick line indicate the sequence of drs-N2. To obtain the entire sequences of *Drs-lD*, *Drs-lE*, *Drs-lF*, *Drs-lG* and *Drs-lI*, drs-N2 was designed to overlap part sequences of drs-N1 and drs-N3. N denotes D, E, F, G or I. The sequences outside the box are included to indicate the *Bam* H I and *Nde* I cleavage sites (in italic)

Fig. 3 PCR products of the seven genes. M: DNA marker, 1: *drs*, 2: *Drs-lC*, 3: *Drs-lD*, 4: *Drs-lE*, 5: *Drs-lF*, 6: *Drs-lG*, 7: *Drs-lI*.

Fig. 4 The Tricine-SDS-PAGE profile and the molecular weight of the expressive product of Drs. A: The rinsed expressive products (1: pET-3C, 2: pET-Drs, M: molecular marker); B: Scanning results showed the highest peak as 4.901 kDa; C: Purified recombinant product of pET-Drs (M: low molecular weight marker).

Fig. 5 Antifungal activity assay for the purified recombinant proteins (50 μg/mL) against *Alternaria longipe*. 1: Drs, 2: Drs-lC, 3: Drs-lD, 4: Drs-lE, 5: Drs-lF, 6: Drs-lG, 7: Drs-lI, CK: Desalt-buffer

Fig. 6 Alignment of amino acid sequences of the Drosomycin family and the three-dimensional structure of Drosomycin. A: alignment of amino acid sequences of the Drs family members, Drs vs. isoforms of the Drs family using Mega 3.0 (Kumar *et al*., 2004). Highly conservational residues are

indicated with black asterisks. Variable residues in Drs-lE, Drs-lF and Drs-lI are indicated with green asterisks. The insertion of two residues (R and V) of Drs-lI is indicated with orange asterisks. Eight cysteine residues involved in the formation of the four internal disulfide bridges are linked using black lines and marked with red letters. B: Three-dimensional structure of Drosomycin is presented using the Swiss PDBviewer v3.7 (http://www.expasy.org/spdbv/). The α-helix is located above the pink line; The three β-sheets is located below the pink line. The four internal disulfide bridges are marked with yellow letters. Variable residues in Drs-lE, Drs-lF and Drs-lI are marked with green letters. The inserting sites of two residues (R and V) of Drs-lI are indicated by an orange arrow.

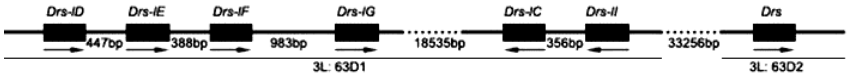

Fig. 1

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Fig. 2

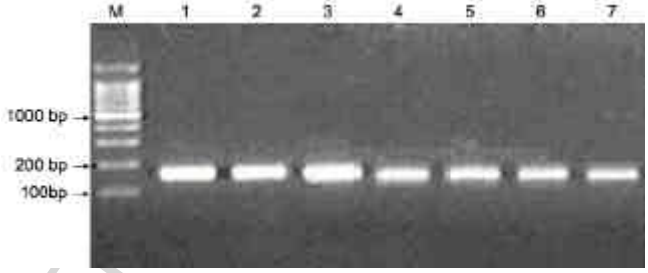

Fig. 3

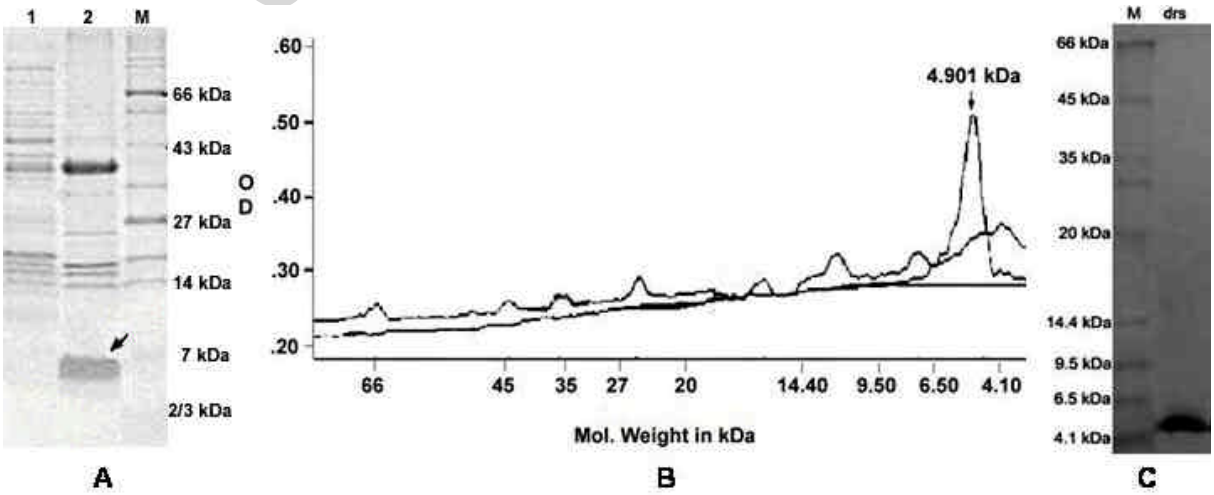

Fig. 4

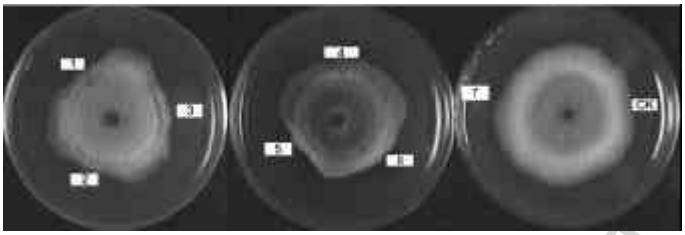

Fig. 5

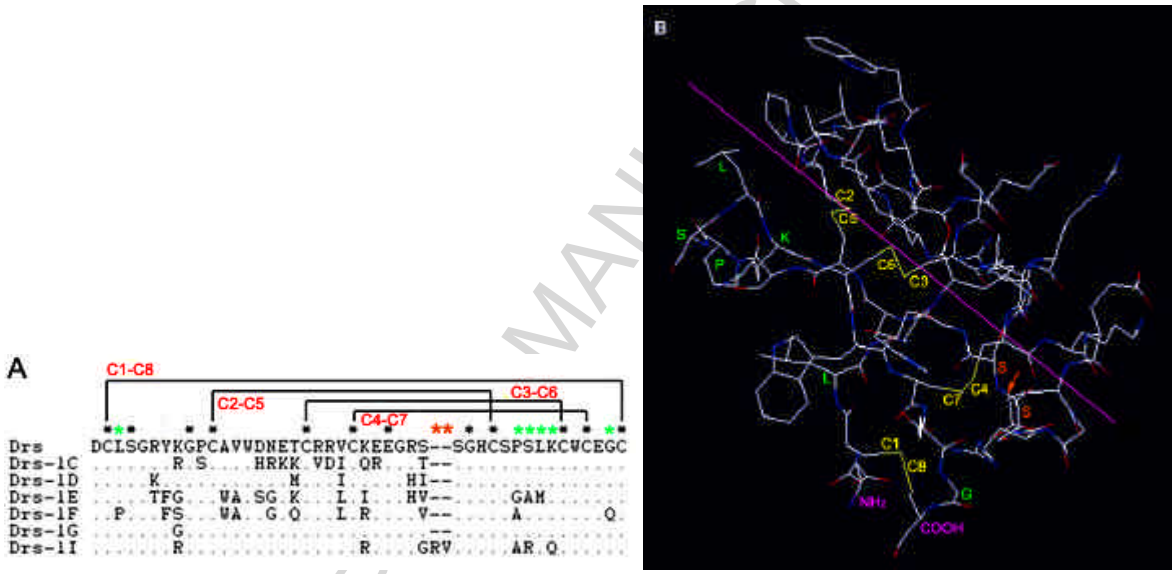

Fig. 6