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Super-induction of Dicer-2 expression by alien double-stranded RNAs. An evolutionary ancient response to viral infection?

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Abstract Dicer-2 is a ribonuclease involved in the insect RNAi pathway. On attempting to knockdown Dicer-2 expression in the insect *Blattella germanica* by RNAi, we found that treatment with Dicer-2 dsRNA up-regulated the targeted mRNA. This unexpected result was also observed after treating with a nucleopolyhedrovirus dsRNA. Experiments with this alien dsRNA showed an all-or-none response with a threshold for inducing Dicer-2 up-regulation between 0.4 and 0.04 µg in terms of dsRNA concentration and between 50 and 20 bp in terms of dsRNA length. The response seems specific of dsRNA given that equivalent experiments carried out with dsDNA did not affect dicer-2 expression. In insects, Dicer-2 is postulated to be a sensor of viral infections and a key antiviral defence element. The up-regulation of Dicer-2 expression after dsRNA administration fits well with this sensor role, and the occurrence of this mechanism in *B. germanica*, a phylogenetically basal insect, suggests that sensing alien RNAs might be an ancestral function of Dicer-2 proteins.

Key words Dicer – RNAi – microRNA – evolution of virus sensing – evolution of antiviral response – insect – *Blattella* - *Drosophila*

Introduction

One of the most profound recent discoveries of the biological sciences has been RNA interference (RNAi), by which introduced double-stranded RNA (dsRNA) is diced into a pool of 21-nucleotide small interfering RNAs (siRNAs) duplexes that select the target mRNA by base-pairing and destroy it (Meister and Tuschl 2004). Among other utilities, RNAi has become a powerful tool to unveil gene functions in non-model insects, thus breaking the *Drosophila* paradigm of functional genomics (Belles 2010). Of non-model insects, the cockroach *Blattella germanica* stands among the species most sensitive to RNAi. In addition, it is a hemimetabolan Polyneopteran insect that shows few derived characters, thus representing a "primitive" insect.

In the context of the high RNAi sensitivity *B. germanica*, we investigated the Dicer ribonuclease involved in the processing of dsRNAs into siRNAs. *Drosophila melanogaster*, a holometabolan Panorpidan species that shows many highly derived characters, has two Dicer ribonucleases. Dicer-1 is involved in the miRNA pathway, processing miRNA precursors into mature miRNAs, while Dicer-2 is involved in the RNAi pathway, processing dsRNAs into siRNAs (Lee et al. 2004). The occurrence of two Dicer ribonucleases in the same species has been reported in other holometabolan insects, which contrasts with the nematode *Caenorhabditis elegans*, another well studied model from a functional genomics point of view, which has a single Dicer protein that is involved in both RNAi and miRNA pathways (Ketting et al. 2001).

Previously, we characterized a Dicer-1 homolog in *B. germanica* and found, as expected, that it is involved in transforming miRNA precursors into mature miRNAs (Gomez-Orte and Belles 2009). For the present work, we first determined that *B. germanica* also has a Dicer-2 homolog that is distinct from Dicer-1. We then followed a

strategy of silencing Dicer-2 expression by RNAi, to demonstrate its involvement in the RNAi pathway. We expected that RNAi experiments targeting other mRNAs would not work in these specimens. The results obtained however, turned out to be quite contrary to those expected, as treatment with dsRNA targeting Dicer-2 elicited a fast and dramatic up-regulation of the targeted mRNA. The same was observed when using alien dsRNAs based on nucleopolyhedrovirus sequences, which suggested to us that the observed up-regulation of Dicer-2 expression is a first response to RNA infection.

Material and Methods

Insects

The specimens of *B. germanica* used in the experiments were obtained from a colony reared in the dark at 30 ±1ºC and 60-70% RH. They were anaesthetized with carbon dioxide prior to injection treatments and tissue sampling. If not stated otherwise, RNA extractions and transcript measurements were based on the whole body.

Cloning of BgDcr2 cDNA

The *B. germanica* Dicer-2 homolog was obtained following a RT-PCR strategy using degenerate primers designed on the basis of conserved motifs from insect Dicer-2 sequences. As template, we used cDNA from five- to six-day-old adult ovaries obtained from a female that had been RNAi-treated targeting Dicer-1, as previously described (Gomez-Orte and Belles 2009). The sequence of the amplified fragment (1320 bp) was highly similar to the equivalent region in known insect Dicer-2 sequences. Then, the

sequence was completed by 5' and 3' RACE (5'- and 3'-RACE System Version 2.0; Invitrogen) using the same template. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Degenerate primers used in the first amplification and specific primers used in the 3'- and 5'-RACE experiments are available upon request.

RNA Extraction and retrotranscription to cDNA

All RNA extractions were performed using the GenElute Mammalian Total RNA kit (Sigma). A 500-ng sample from each RNA extraction was treated with DNAse (Promega) and reverse transcribed using the NCode Kit (Roche). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies).

Determination of mRNA levels by quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. The efficiency of the primer sets to measure Dicer-1 and Dicer-2 mRNA levels (primer sequences are available upon request) was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c (Accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA.

RNA interference

The detailed procedures for RNAi experiments were as described previously (Gomez-Orte and Belles 2009; Lozano and Belles 2011). A dsRNA encompassing a 391-bp fragment located between nucleotides 920 and 1310 of the Dicer-2 ORF sequence (Accession number HE647851) (dsDcr2 in Fig. 1a) was designed to carry out the RNAi experiments. The 343-bp dsRNA targeting Dicer-1 (dsDcr1 in Fig. 1a) was that described by Gomez-Orte & Belles (2009) as "dsBgDcr1-A". The primers used to generate the fragments to prepare dsDcr2 and dsDcr1 are available upon request. The fragments were amplified by PCR and cloned into the pSTBlue-1 vector. For the experiments using a dsRNA sequence from *Autographa californica* nucleopolyhedrovirus (Accession number K01149), the following dsRNA lengths were used: 300-bp (from nucleotide 236 to 535), 150-bp, 50 bp (from nucleotide 236 to 285) and 20 bp (from nucleotide 236 to 255). The dsRNAs were prepared as reported previously (Gomez-Orte and Belles 2009; Lozano and Belles 2011), except that of 20 bp, which was prepared by directly annealing the commercial oligonucleotides CCUACGUGUACGACAACAAG and CUUGUUGUCGUACACGUAGG, which encompass the chosen region. dsDNA of *Autographa californica* nucleopolyhedrovirus was prepared by PCR amplification of the 150 bp fragment described above (from nucleotide 236 to 385). A volume of 1 µL of dsRNA (or dsDNA) solution (4 µg/µL, if not stated otherwise) was injected into the abdomen of freshly emerged fifth instar female nymphs. Control specimens were treated with the same volume of water.

Sequence comparisons and phylogenetic analysis

We obtained the arthropod sequences labelled as Dicer from GenBank, and the list was enlarged by BLAST search using the *B. germanica* Dicer-1 and Dicer-2 sequences as queries. Two Dicer sequences (tetur19g00520 and tetur07g00990) were obtained from the *Tetranychus urticae* sequenced genome (http://bioinformatics.psb.ugent.be/webtools/bogas). Finally, the species and protein sequences of Dicer-1 (Dcr1) and Dicer-2 (Dcr2) included in the analysis were the following (the accession number or the bibliographic reference indicated in parenthesis; the annotation of Dcr1 or Dcr2 reflects the result of the phylogenetic analysis performed in the present work, not necessarily the annotation stated in the GenBank file). Insects: *Aedes aegypti* Dcr1 (XP_001659747.1) and Dcr2 (AAW48725.1), *Acromyrmex echinatior* Dcr1 (EGI60563.1) and Dcr2 (EGI69620.1), *Anopheles gambiae* Dcr1 (AAO73809.1) and Dcr2 (XP_320248.4), *Apis mellifera* Dcr1 (NP_001116485.1) and Dcr2 (XR_120636.1), *Acyrthosiphon pisum* Dcr1a and Dcr1b (Jaubert-Possamai et al. 2010) and Dcr2 (XP_001945890.2), *Blattella germanica* Dcr1 (CAX68236.1) and Dcr2 (CCF23094.1), *Bombus impatiens* Dcr1 (XP_003493408.1) and Dcr2 (XP_003485689.1), *Bombus terrestris* Dcr1 (XP_003401955.1) and Dcr2 (XP_003394821.1), *Bombyx mori* Dcr2 (NP_001180543.1), *Culex quinquefasciatus* Dcr1 (XP_001844757.1) and Dcr2 (XP_001855187.1), *Drosophila melanogaster* Dcr1 (NP_524453.1) and Dcr2 (NP_523778.2), *Danaus plexippus* Dcr1 (EHJ64690.1) and Dcr2 (EHJ65725.1), *Nasonia vitripennis* Dcr1 (XP_001605287.1) and Dcr2 (XP_001602524.2), *Pediculus humanus* Dcr1 (XP_002429494.1) and Dcr2 (XP_002430037.1), *Tribolium castaneum* Dcr1 (EFA11550.1) and Dcr2 (NP_001107840.1). Crustaceans: *Daphnia pulex* (EFX72380.1) Dcr1, Dcr2a

(EFX69538.1), Dcr2b (EFX86072.1) and Dcr2c (EFX87988.1), *Lepeophtheirus salmonis* Dcr1 (JP311757.1) and Dcr2 (assembly JP310136.1+JP312169.1), *Litopenaeus vannamei* Dcr1 (ACF96960.1) and Dcr2 (AEB54796.1), *Marsupenaeus japonicus* Dcr1 (ADB44075.1). Arachnids: *Ixodes scapularis* Dcr1 (XP_002408100) and Dcr2 (XP_002408099), *Penaeus monodon* Dcr1 (ABR14013.1), *Tetranychus urticae* Dcr1 (tetur19g00520) and Dcr2 (tetur07g00990). The unique Dicer sequence from the nematode *Caenorhabditis elegans* (NP_498761.1), was used as out-group.

The protein sequences were aligned using the MAFFT program (http://mafft.cbrc.jp/alignment/software), with the E-INS-I parameter, which is recommended for multidomain proteins. The model of protein evolution that best fits the data was determined using ProtTest 2.4 (http://darwin.uvigo.es/software/prottest.html). The LG+I+G+F evolutionary model was preferred by the ProtTest program, and was implemented in the maximum-likelihood analyses. These were carried out by using the PHYML version 3.0 program (http://www.atgc-montpellier.fr/phyml/), with the above evolutionary model. Data were bootstrapped for 100 replicates using the same program.

Results and discussion

Blattella germanica has two Dicer ribonucleases

Cloning of Dicer-2 cDNA in *B. germanica* was accomplished by obtaining a partial sequence by RT-PCR using degenerate primers based on Dicer-2 conserved motifs, from which extended sequence was generated by 5'-RACE and 3'-RACE. These amplifications rendered a cDNA of 5429 bp (GenBank accession number HE647851).

The putative start and stop codons are preceded and followed respectively by in-frame stop codons, suggesting that a full-length open reading frame had been obtained. Database BLAST searches suggested that it encoded an orthologue of Dicer-2. The conceptual translation rendered a 1649 amino acid sequence, and a ScanProsite search revealed that the protein is organized as a typical Dicer sequence (de Jong et al. 2009) with an N-terminal DEAD box, an RNA helicase domain, a divergent dsRNAs-binding domain, a Piwi-Argonaute-Zwille (PAZ) domain, two ribonuclease (RNase III) domains and an additional dsRNAs-binding domain (desrm) (Fig. 1a). The *B. germanica* Dicer-1 protein previously described (Gomez-Orte and Belles 2009) has the same organization (Fig. 1a), but very different sequence, showing only 27.1% identity compared with the Dicer-2 reported herein.

The landmark paper of Lee et al. (2004) demonstrated that the two Dicer proteins of *D. melanogaster* were involved in different functions, Dicer-1 in miRNA biogenesis and Dicer-2 in dsRNA processing. Later, Dicer-1 and Dicer-2 proteins have been found to be present in other insects, and our present work reveals, thus, that *B. germanica* also has a Dicer-2 homolog.

Arthropods have Dicer-1 and Dicer-2 ribonucleases

Maximum-likelihood phylogenetic analysis of arthropod Dicer protein sequences, using the Dicer orthologue from the nematode *C. elegans* as out-group, rendered a tree (Fig. 1b) that separates Dicer-1 and Dicer-2 sequences into two different groups. The tree clearly shows that the sequence of *B. germanica* reported herein clusters in the Dicer-2 group, while the Dicer-1 previously reported (Gomez-Orte and Belles 2009) clusters in the other group. The data show that arachnids like the blacklegged tick *Ixodes*

scapularis and the red spider mite *Tetranychus urticae* (Acari) possess Dicer-1 and Dicer-2 genes. The same occurs in crustaceans like the whiteleg shrimp *Litopenaeus vannamei* (Malacostraca, Decapoda) the sea louse *Lepeophtheirus salmonis* (Maxillopoda, Copepoda) and the water flea *Daphnia pulex* (Branchiopoda, Cladocera). Interestingly, *D. pulex* has three paralogues of Dicer-2, which should derive from two Dicer-2 duplications in the Branchiopoda lineage. We presume that Dicer-2 must also occur in those crustaceans where only Dicer-1 has been searched and reported (the decapodans *Penaeus monodon* and *Marsupenaeus japonicus*). In insects, both Dicer-1 and Dicer-2 are generally present in the same species. Of note, and as reported by Jaubert-Possamai et al. (2010), the pea aphid *Acyrtosyphon pisum* has two paralogues of Dicer-1. The functional significance of Dicer-1 and Dicer-2 paralogues remains unknown. With few exceptions, the topology of the respective Dicer-1 and Dicer-2 groups approximately follows the currently-established phylogenetic relationships of the represented arthropod classes and orders, especially in Dicer-1 group, where, in addition, the sequences have diverged less.

Dicer proteins constitute a widely conserved family that occur in many organisms including plants, fungi and metazoans. A recent study by de Jong et al. (2009) showed that “basal” (early branching) metazoans like Placozoans and Poriferans have five Dicer proteins, Cnidarians have two, whereas “higher” metazoans have only one, with the exception of insects, which possess two (de Jong et al. 2009). To explain the present Dicer diversity within different groups, these authors postulated an ancient duplication event of a “Proto-Dicer” gene at the origin of metazoans followed by successive lineage-specific duplications. De Jong et al. (2009) did not include non-insect arthropods in their analysis, and proposed that the occurrence of the Dicer-1 and Dicer-2 genes in insects should be explained by a duplication that occurred in the

lineage leading to this metazoan class (de Jong et al. 2009). In our phylogenetic study, we have considered all data available in arthropods, and found that there are Dicer-1 and Dicer-2 sequences not only in insects, but also in crustaceans (sampled in Malacostraca, Maxillopoda and Branchiopoda) and in arachnids (sampled in Acari). No Dicer sequences have yet been described from miriapods. However, if the Mandibulata hypothesis is followed (Giribet and Edgecombe 2012), by which the Myriapoda clusters with the Crustacea + Hexapoda while the Chelicerata is a sister group of them all, then the most parsimonious prediction is that miriapods would have Dicer-1 and Dicer-2 genes, and that the gene duplication traces to the origin of the arthropods during the Cambrian.

Injection of dsRNA to target Dicer mRNA induces Dicer-2 up-regulation

As an initial Dicer-2 RNAi experiment, we performed a “conventional” treatment of 4 µg of a 391-bp dsRNA targeting Dicer-2 mRNA (dsDcr2) injected into freshly emerged fifth instar female nymphs. Specimens of the same sex and age treated with the same volume (1 µL) of water were used as controls. We then quantified Dicer-2 mRNA at defined intervals during the entire instar in treated and control samples. Unexpectedly, we observed a dramatic (five-fold) up-regulation of Dicer-2 (the targeted mRNA) 6 h after the treatment (Fig. 2a). One day after the treatment, Dicer-2 mRNA levels were similar to controls and when subsequently measured every 24 h, the levels were also approximately similar to controls.

To check whether a similar dsRNA would induce the same effect on Dicer-2 expression, we tested 4 µg of a 343-bp dsRNA targeting Dicer-1 mRNA (dsDcr1), following the procedure of the previous experiment. This time, as expected, mRNA

levels of Dicer-1 tended to decrease 6 h after the treatment, and were already significantly lower than in control (water-treated) specimens 1 day later (Fig. 2b). When Dicer-2 mRNA levels were quantified in the same samples, results were similar to those observed in the treatment with dsDcr2: a five-fold up-regulation of Dicer-2 6 h after the treatment and a return to approximately normal levels 1 day later (Fig. 2b).

Super-induction of Dicer-2 expression by alien dsRNA

At this point, we wanted to test whether alien dsRNAs would induce a similar fast up-regulation of Dicer-2. For this purpose, we carried out the same experiments, but this time used a 300-bp dsRNA sequence from *Autographa californica* nucleopolyhedrovirus (dsPolyH). This sequence is currently used in our laboratory as a control in RNAi experiments (Lozano and Belles 2011). The 300-bp dsPolyH was injected at a dose of 4 µg in freshly emerged fifth instar female nymphs, as in previous experiments, and 6 h after the treatment Dicer-2 mRNA levels showed a five-fold increase compared to water-treated controls (Fig. 3a), also as in previous experiments. When the dose of this dsPolyH was reduced to 0.4 µg, the induction of Dicer-2 expression was very similar, but no induction was observed when the dsPolyH dose was further reduced to 0.04 µg (Fig. 3a).

dsRNA length threshold for Dicer-2 response and specific effect of dsRNA

Once we established that a 300-bp alien dsRNA such as dsPolyH induced the up-regulation of Dicer-2, we investigated the minimum size of the dsRNA that could elicit such a response. To address this issue, we tested dsPolyHs of different lengths (150, 50

and 20 bp) injected respectively at a dose of 4 µg in freshly emerged fifth instar female nymphs. Results showed that the lengths of 150 and 50 bp induced a five-fold up-regulation of Dicer-2 (Fig. 3b), which was similar to that observed in the equivalent experiments using the 300-bp dsPolyH at a dose of 4 µg (Fig. 3a). Conversely, the dsPolyH of 20 bp did not elicit any significant response (Fig. 3b).

Finally, we wondered whether the property of up-regulating dicer-2 expression was specific of dsRNAs, or also dsDNAs could elicit the same effect. To answer this question, we tested a dsDNA of *Autographa californica* nucleopolyhedrovirus whose sequence corresponded to the 150 bp fragment tested as dsRNA and described above. The experiment methodology and the dose used was the same as in the dsRNA experiments, but the results (Fig. 3c) showed that dsDNA did not induce dicer-2 expression.

An evolutionary ancient response to viral infection?

In insect RNA-based antiviral immunity, double-stranded RNAs are recognized as molecules associated with pathogens and, as a defence, are processed into siRNAs by host Dicer-2 (Ding 2010; Galiana-Arnoux et al. 2006). Moreover, striking evidence for a role of Dicer-2 as a sensor of viral infection and as a key antiviral defence element beyond the RNAi pathway has recently been demonstrated in *D. melanogaster*, where Dicer-2 mediates the induction of the anti-viral gene *Vago* (Deddouche et al. 2008). Interestingly, Dicer-2 belongs to the same DExD/H-box helicase superfamily as the RIG-I-like receptors that sense viral infection and mediate interferon induction in mammals (Deddouche et al. 2008). This suggests that DExD/H-box helicase is an evolutionary old superfamily of sensors devoted to the detection of viral infections to induce defence responses by the host.

The up-regulation of Dicer-2 expression after an exogenous administration of a dsRNA fits well with this sensor role. Its occurrence in *B. germanica* suggests that sensing alien RNAs might be an ancestral function of Dicer proteins, and it is even still operative in an insect that is not particularly prone to viral infections. Indeed, only densoviruses, which are single-stranded DNA viruses, are considered genuine cockroach viruses, and a densovirus has recently been characterized in *B. germanica* (Mukha et al. 2006). This reasoning leads to the prediction that the mechanism of up-regulation of Dicer-2 after exogenous dsRNA administration is present in other arthropods, especially if they are prone to viral infections. Consistent with this prediction, a very recent report on the crustacean *L. vannamei* (Chen et al. 2011), which is typically prone to viral infections, shows that expression levels of Dicer-2 increase ca. seven-fold 9 h after treatment with the commercial double-stranded homopolymer Poly(C-G) (Sigma-Aldrich P4038) that is used as control in RNAi experiments. Of note, up-regulation of Dicer-2 was also observed in *L. vannamei* following infection with white spot syndrome virus (WSSV) (Chen et al. 2011).

Finally, it is tempting to speculate that the ancestral Dicer that gave rise to Dicer-1 and Dicer-2 in the arthropod lineage would possess the dual ability to process miRNAs and siRNAs, with the latter function being related to antiviral immunity. Indeed, this dual function operates today in the single Dicer protein of *C. elegans* (Ketting et al. 2001). After the duplication, Dicer-1 might specialize in dicing miRNA precursors in the miRNA pathway, while Dicer-2 might specialize in dicing dsRNAs in the RNAi pathway. These are the respective basic functions that we observe today in those species that have been functionally studied, and that possess the two Dicer genes.

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FIGURE LEGENDS

Fig. 1. Organization of *Blattella germanica* Dicer-2 and phylogenetic relationships with other insect orthologues. (a) Protein organization of *B. germanica* Dicer-2 (BgDcr2) compared with that of Dicer-1 (BgDcr1) reported by Gomez-Orte and Belles (Gomez-Orte and Belles 2009), indicating the typical Dicer domains: an N-terminal DEAD box, an RNA helicase, a divergent dsRNAs-binding domain, a PAZ domain, two ribonuclease (RNase III) domains and an additional dsRNAs-binding domain (desrm), as well as the region encompassed by the dsRNAs (dsDcr1 and dsDcr2) used. Organization of *Drosophila melanogaster* Dicer-1 (DmDcr1) and Dicer-2 (DmDcr2) is also represented for comparison; the DEAD box in DmDcr1 is not recognized by ScanProsite but conserved residues are identified in the alignments. (b) Maximum-likelihood tree of the Dicer protein sequences of arthropod species and the nematode *Caenorhabditis elegans* used as outgroup; the complete binomial nomenclature of all species is indicated in Materials and Methods; the *B. germanica* Dcr2 sequence described herein is squared; blue and green backgrounds indicate Dicer- 2 and Dicer-1 sequences, respectively; arachnid, crustacean and insect species are indicated with orange, yellow and magenta backgrounds, respectively; bootstrap values >50 are indicated on the corresponding node; the scale bar represents 0.7 substitutions per position.

Fig. 2. Expression of Dicer-2 after exogenous application of dsDcr in *Blattella germanica*. (a) Expression of Dicer-2 in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 µg of dsDcr2; they were treated as freshly emerged and examined 6 h later, and then every day (D) during the instar. (b) Expression of Dicer-1 (left) and of Dicer-2 (right) in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 µg of dsDcr1; they were treated as freshly emerged and

examined 6 h and 1 D later. Results represent the mean ± SEM (n = 5-9) and are expressed as copies of Dicer-1 (left) or Dicer-2 (left) mRNA per 1000 copies of BgActin-5c mRNA; the asterisk indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant ($p < 0.05$), according to the REST software tool (Pfaffl et al. 2002).

Fig. 3. Expression of Dicer-2 in specimens of *Blattella germanica* treated with dsPolyH or dsDNA. (a) Effect of the dose of dsPolyH. (b) Effect of the length of the dsPolyH. (c) Effect of a dsDNA whose sequence corresponds to the 150 bp dsPolyH. Specimens were treated as freshly emerged fifth instar female nymphs and examined 6 h later; results represent the mean ± SEM (n = 8-12) and are expressed as copies of Dicer-2 mRNA per 1000 copies of BgActin-5c mRNA; the asterisk indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant ($p < 0.05$), according to the REST software tool (Pfaffl et al. 2002).

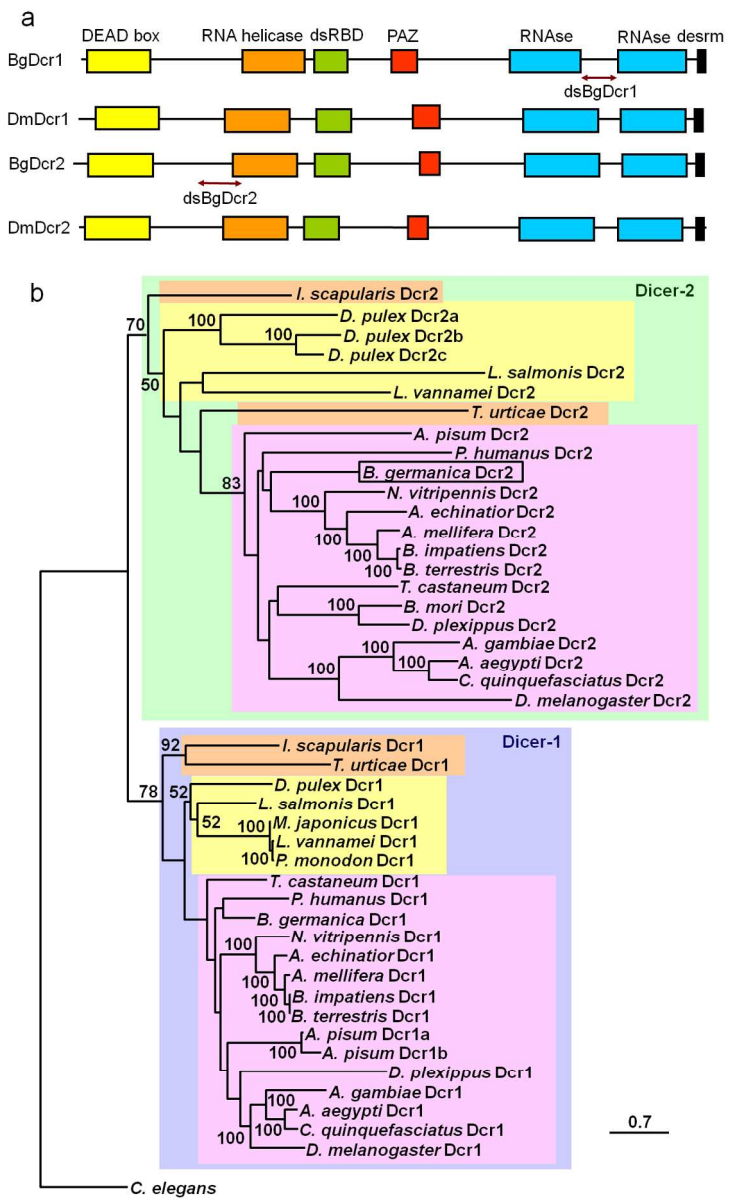

Fig. 1. Organization of Blattella germanica Dicer-2 and phylogenetic relationships with other insect orthologues. (a) Protein organization of B. germanica Dicer-2 (BgDcr2) compared with that of Dicer-1 (BgDcr1) reported by Gomez-Orte and Belles (Gomez-Orte and Belles 2009), indicating the typical Dicer domains: an N-terminal DEAD box, an RNA helicase, a divergent dsRNAs-binding domain, a PAZ domain, two ribonuclease (RNase III) domains and an additional dsRNAs-binding domain (desrm), as well as the region encompassed by the dsRNAs (dsDcr1 and dsDcr2) used. Organization of Drosophila melanogaster Dicer-1 (DmDcr1) and Dicer-2 (DmDcr2) is also represented for comparison; the DEAD box in DmDcr1 is not recognized by ScanProsite but conserved residues are identified in the alignments. (b) Maximum-likelihood tree of the Dicer protein sequences of arthropod species and the nematode Caenorhabditis elegans used as outgroup; the complete binomial nomenclature of all species is indicated in Materials and Methods; the B. germanica Dcr2 sequence described herein is squared; blue and green backgrounds indicate Dicer- 2 and Dicer-1 sequences, respectively; arachnid, crustacean and insect species are indicated with orange, yellow and magenta backgrounds, respectively; bootstrap values >50 are indicated on the corresponding node; the

scale bar represents 0.7 substitutions per position.
117x189mm (300 x 300 DPI)

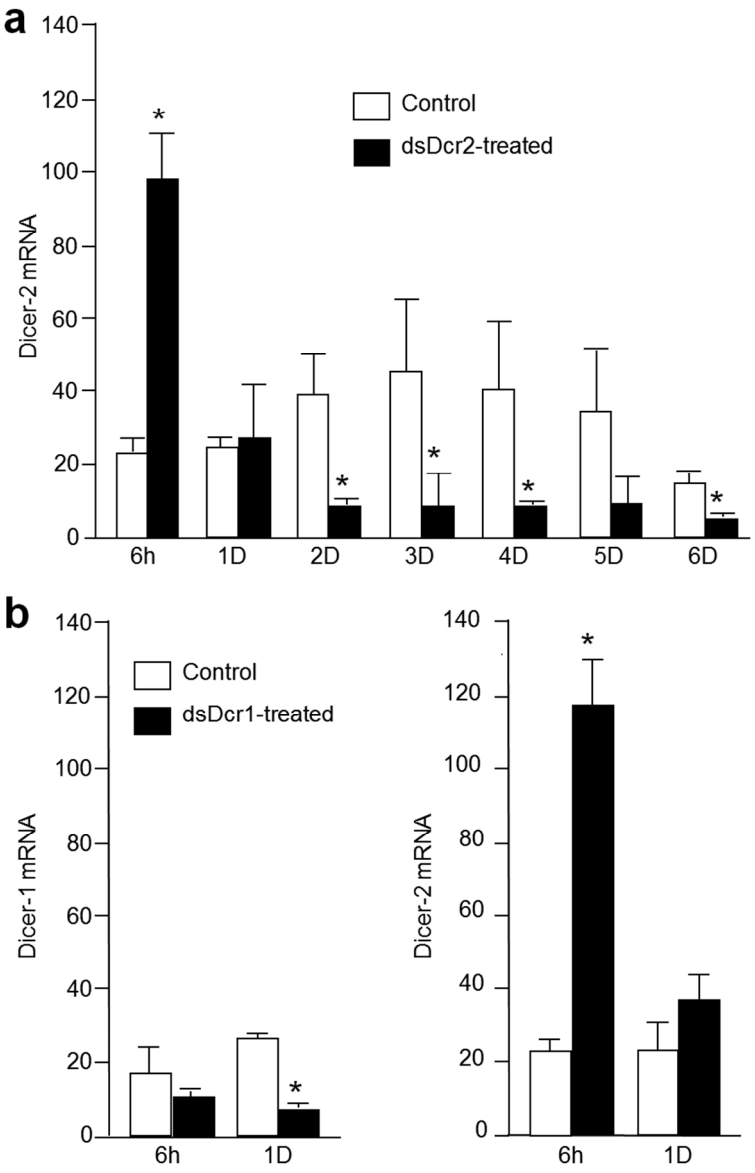

Fig. 2. Expression of Dicer-2 after exogenous application of dsDcr in Blattella germanica. (a) Expression of Dicer-2 in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 µg of dsDcr2; they were treated as freshly emerged and examined 6 h later, and then every day (D) during the instar. (b) Expression of Dicer-1 (left) and of Dicer-2 (right) in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 µg of dsDcr1; they were treated as freshly emerged and examined 6 h and 1 D later. Results represent the mean ± SEM (n = 5-9) and are expressed as copies of Dicer-1 (left) or Dicer-2 (left) mRNA per 1000 copies of BgActin-5c mRNA; the asterisk indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant ($p < 0.05$), according to the REST software tool (Pfaffl et al. 2002).
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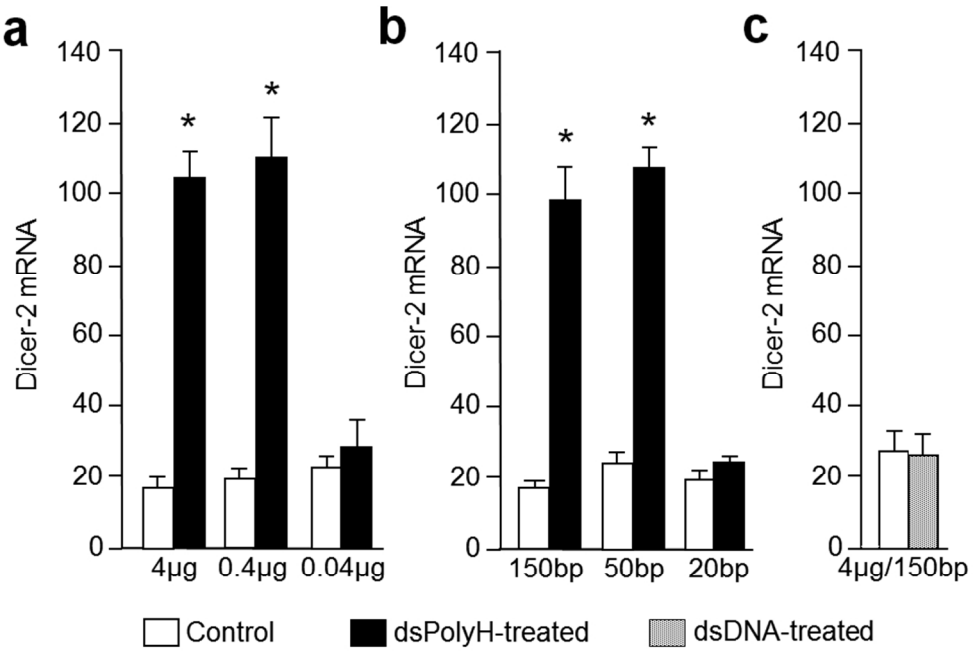

Fig. 3. Expression of Dicer-2 in specimens of Blattella germanica treated with dsPolyH or dsDNA. (a) Effect of the dose of dsPolyH. (b) Effect of the length of the dsPolyH. (c) Effect of a dsDNA whose sequence corresponds to the 150 bp dsPolyH. Specimens were treated as freshly emerged fifth instar female nymphs and examined 6 h later; results represent the mean ± SEM (n = 8-12) and are expressed as copies of Dicer-2 mRNA per 1000 copies of BgActin-5c mRNA; the asterisk indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant ($p < 0.05$), according to the REST software tool (Pfaffl et al. 2002).
83x58mm (300 x 300 DPI)