Dev Genes Evol (2007) 217:241–251
DOI 10.1007/s00427-007-0132-9

TECHNICAL NOTE

Gene silencing in the spider mite *Tetranychus urticae*: dsRNA and siRNA parental silencing of the *Distal-less* gene

Abderrahman Khila · Miodrag Grbić

Received: 17 August 2006 / Accepted: 9 January 2007 / Published online: 30 January 2007

Abstract A major prerequisite to understanding the evolution of developmental programs includes an appreciation of gene function in a comparative context. RNA interference (RNAi) represents a powerful method for reverse genetics analysis of gene function. However, RNAi protocols exist for only a handful of arthropod species. To extend functional analysis in basal arthropods, we developed a RNAi protocol for the two-spotted spider mite *Tetranychus urticae* focusing on *Distal-less* (*Dll*), a conserved gene involved in appendage specification in metazoans. First, we describe limb morphogenesis in *T. urticae* using confocal and scanning electron microscopy. Second, we examine *T. urticae Dll* (*Tu-Dll*) mRNA expression patterns and correlate its expression with appendage development. We then show that fluorescently labeled double-stranded RNA (dsRNA) and short interfering RNA (siRNA) molecules injected into the abdomen of adult females are incorporated into the oviposited eggs, suggesting that dsRNA reagents can be systemically distributed in spider mites. Injection of longer dsRNA as well as siRNA induced canonical limb truncation phenotypes as well as the fusion of leg segments. Our data suggest that *Dll* plays a conserved role in appendage formation in arthropods and that such conserved genes can serve as reliable starting points for the development of functional protocols in nonmodel organisms.

Communicated by S. Roth

A. Khila · M. Grbić (✉)
Department of Biology, University of Western Ontario,
London N6A 5B7, Canada
e-mail: mgrbic@uwo.ca

A. Khila
Department of Biology, McGill University,
Montreal, Quebec H3A 1B1, Canada

Keywords Chelicerata · Embryo development · RNAi · Evolution of development · Limb development · *Distal-less*

Introduction

After insects, the Chelicerata are the second largest group of arthropods. Chelicerates (horseshoe crabs, scorpions, spiders, ticks, and mites) branch first from the root of the arthropod phylum (Boore et al. 1995; Friedrich and Tautz 1995), and because of the basal position of Chelicerates in the arthropod clade (Schoppmeier and Damen 2001), they are an invaluable comparative group for understanding the evolutionary processes underlying the divergence of arthropods.

Apart from their utility in understanding how developmental patterning has evolved in metazoans, chelicerates comprise many species that are important for agriculture and medicine such as spider mites and ticks. Both of these groups belong to the order Acari, the most species-rich chelicerate order. Spider mites represent major pests in agriculture, while ticks are vectors of human diseases, including Lyme disease and hemorrhagic fever. Unfortunately, the developmental genetics of chelicerates is poorly understood. A major obstacle for progress in many aspects of chelicerate biology is the lack of a model organism in this group.

To date, the development of a chelicerate model system has been hampered by their life history, ontogeny, long development time, and large genomes. Most chelicerates are predatory, complicating laboratory rearing by the requirement for the production of both the chelicerate predator of interest and its prey. In addition, many species have a complex ontogeny. For example, ticks require multiple hosts to complete a life cycle, and their development can take up to 21 years (James and Harwood 1969).

Spiders are similarly long-lived. The development of the predatory spider *Cupiennius salei*, currently the best studied chelicerate, approaches 9 months. In addition, genome sizes of predatory chelicerates are moderate to large, ranging from 723 Mbp in *Tetragnatha elongata* to 7.1 Gbp in the tick *Boophilus microplus* (Gregory and Shorthouse 2003; Ullmann et al. 2005). Besides the aforementioned technical impediments, early developmental stages are often not accessible for developmental analysis in many chelicerate species (Telford 2000; Damen et al. 2005).

In contrast with their predatory relatives (the spiders and scorpions), the two-spotted spider mite, *Tetranychus urticae,* belongs to an assemblage of web-spinning species that feed on plants. Furthermore, unlike spiders and scorpions, which have long developmental times, *T. urticae* has a short generation time, completing its embryonic development in only 39 h with full maturation to the reproductive adult in less than 7 days (Rao et al. 1996). This rapid generation time and simple rearing protocol makes *T. urticae* a potential organism of choice for genetic studies. Moreover, *T. urticae* also has small eggs (150 μm) that are surrounded by a transparent chorion. In this species, sex determination is haplo-diploid, meaning that unfertilized (haploid) eggs will give rise to males and fertilized (diploid) eggs will develop into females. This provides another entrée to study haplo-diploid genetics, similar to that provided by the haplo-diploid wasp, *Nasonia* (Pultz et al. 2000). Finally, *T. urticae* has only three chromosomes (Oliver 1971) and possesses the smallest genome determined thus far within the arthopods—only 75 Mbp, or 0.08 pg per haploid genome. This is roughly equivalent to 60% of the *Drosophila* genome (Dearden et al. 2002) and makes *T. urticae* a potential candidate for a model chelicerate. Nevertheless, a prerequisite for developing *T. urticae* as a chelicerate model is the development of tools for functional analysis.

A promising approach to knockout (silence) genes in various plants and animals includes RNA interference (RNAi)-mediated gene silencing (Meister and Tuschl 2004). Although the RNAi mode of action is conserved, delivery protocols of experimental RNAi vary depending upon the model system. They range from direct delivery to a specific developmental stage (transfection of cell lines or injections into individual embryos) to the introduction of double-stranded RNA (dsRNA) into earlier reproductive stages, thereby allowing gene silencing in the progeny of injected females. Such administration suggests some species will be sensitive to systemic RNAi effects as has been recorded so far in plants, planarians, *Caenorhabditis. elegans,* and insects *Tribolium* and *Oncopeltus* (May and Plasterk 2005). For example, injection of dsRNA in pupal stages of *Tribolium* elicits phenotypes in embryos of injected females, increasing by several orders of magnitude the efficiency of interference compared to injection of single eggs (Bucher et al. 2002). It is believed that dsRNA is sequestered into the developing eggs, ultimately causing specific gene silencing.

To develop efficient protocols for gene silencing in *T. urticae*, we focused on *Dll*, a gene that plays a conserved role in appendage development in metazoans (Panganiban et al. 1997). A cross-reacting antibody developed against conserved domains of the Dll protein (Panganiban et al. 1995) has allowed extensive analysis of its expression pattern in various arthropod lineages. The expression of Dll protein has been studied in chelicerates (spider, horseshoe crab, and two-spotted spider mite; Schoppmeier and Damen 2001; Mittmann and Scholtz 2001; Dearden et al. 2002), myriapods (Popadic et al. 1996), uniramous and biramous crustaceans (Popadic et al. 1998; Hejnol and Scholtz 2004; Williams et al. 2002), and insects (Mittmann and Scholtz 2001; Panganiban et al. 1994; Jockusch et al. 2004). These studies have broadly revealed that Dll is expressed in the distal region of forming arthropod appendages, suggesting a conserved role for Dll in the specification of distal limb segments as assessed by functional studies in *Drosophila* (Cohen et al. 1989). The only functional study in arthropods outside of insects involved RNAi silencing of *Dll* in the spider *Cupiennius* and corroborated the role of Dll in distal appendage patterning (Schoppmeier and Damen 2001). Thus, a practical advantage of Dll silencing in the development of RNAi protocols is the expectation of an easily scored and interpretable phenotype.

Previously, we isolated the *Dll* gene in an (expressed sequence tag) EST screen (Grbic, unpublished) and then confirmed its protein expression pattern using a cross-reacting anti-Dll antibody (Dearden et al. 2002). This study served as a starting point for developing a protocol for RNAi in spider mites. Previously established dsRNA gene silencing protocols in the spider *C. salei* include dsRNA injection into the perivitelline space of individual *C. salei* eggs (Schoppmeier and Damen 2001). However, because of differences in development and embryo size (spider mites have eggs ten times smaller than for *C. salei*) this protocol cannot be transferred to *T. urticae*.

Here, we report a protocol for gene silencing in *T. urticae*. We describe the development of appendages in *T. urticae*, and we developed a maternal injection protocol to deliver interfering RNAs into the maternal abdomen. To trace the localization of interfering RNAs into prospective F1 embryos, we used fluorescently labeled RNAs. We showed that both dsRNA and short interfering RNA (siRNA) cause depletion of Dll protein and generate appendage truncation phenotypes in the offspring. In addition, we describe an additional phenotype, which results in an appendage fusion in *T. urticae*. This phenotype is not described in the spider *C. salei*, suggesting a variation of leg specification programs in spider mite relative to

spiders. Finally, we found that siRNA-mediated silencing of Dll function was more efficient than dsRNA-mediated interference. This protocol opens new avenues for the functional analysis of development in chelicerates.

Materials and methods

T. urticae Distal-less

Two clones with overlapping sequences were identified using the BioEdit local blast program, comparing *Drosophila* Dll against the *T. urticae* EST database generated by Dearden et al. (2002). Sequence analysis and in situ hybridization further confirmed that these clones represent *T. urticae Dll* (*Tu-Dll*). *Tu-Dll* sequence is available in GenBank under the accession number DQ442864.

The design of siRNA

Three 21 bp siRNA oligonucleotides with a 3′ dTdT overhang were designed as described in Elbashir et al. (2001). siDll2 sense 5′-UACUCUGAAUCACCACUUAdTdT- 3′, antisense 5′-UAAGUGGUGAUUCAGAGUAdTdT-3′; siDll3 sense 5′-ACCAAGUGAAAGCCAUACAdTdT-3′, antisense 5′-UGUAUGGCUUUCACUUGGUdTdT-3′ and siDll4 sense 5′-GAGUUGGUCUAUCCUCGGGdTdT-3′, antisense 5′-CCCGAGGAUAGACCAACUC dTdT-3′ were purchased from Sigma. siDll2 is located on the 3′-UTR whereas siDll3 and siDll4 are located in the C-terminal region of the *Tu-Dll* protein. Each sense oligo was annealed with its antisense counterpart to form siRNA duplexes. siDll2 was labeled with fluorescein to follow its penetration into the embryos.

dsRNA preparation

A 130 bp fragment covering the C-terminal region of *Tu-Dll* was PCR amplified using the following modified primers: forward-*Eco*RI 5′-AACCAGGAATTCGTCTAT CCTCGGG-3′ and reverse-*Xho*I 5′-GGAACCTCGAGTT GGACCAAGTGATG-3′. This fragment was cloned using *Eco*RI and *Xho*I restriction sites in the T7-polylinker-T7 promoter containing vector (Litmus 2.8, Biolabs) allowing synthesis of the sense and antisense transcripts simultaneously. Sense and antisense *Tu-Dll* RNA were prepared as described in the pLitmus user manual and annealed overnight. The resulting dsRNA was purified using an RNeasy Kit (Qiagen) and eluted in 1X Spradling injection buffer supplemented with 5% glycerol. For fluorescein-labeled dsRNA, 250 nmol of Fluorescein-12-UTP (Boehinger Mannheim GMBH) was added to the reaction mix.

Injected females and their progeny were screened for the presence of fluorescence under a Leica MZFLIII dissecting Microscope using a GFP2 filter to trace the penetration of siRNA or dsRNA into the embryos. Images were captured using a Sony DXC 390P camera.

Injections of *T. urticae* females and processing of offspring embryos

Adult spider mite females were aligned on 1% agar slices on a slide and injected in the ventral abdomen near the gonad using a manual Eppendorf micro-injector. Injected females were transferred to a Petri dish with a bean leaf placed on wet cotton and allowed to lay eggs for 24 h at 26°C and 60% humidity. After 24 h, females were transferred to new leaves, and the remaining embryos were incubated for an extra 48 h to permit further development. Embryos were collected in PTW (1X PBS, 0.05% Tween-20), boiled for 1 min and placed on ice for 5 min. After this treatment, embryos were dissected manually in PBS on double-stick tape with a tungsten needle to remove the chorion and the vitelline membrane, then fixed 30 min at room temperature in 12% Formaldehyde/PTW. After fixation, the embryos were washed several times in PTW and processed for subsequent experiments.

Immuno-histochemistry and microscopy

Preparations of DIG-labeled probes and in situ hybridization were carried out as described in (Dearden and Akam 2000). Immuno-staining was performed using rabbit polyclonal antibody raised against *Drosophila* Distal-lesss (Panganiban et al. 1995). After several washes in PTW, embryos were blocked in PAT (0.2% (w/v) BSA in PTW) for 1 h and incubated at 4°C overnight with the primary antibody. The incubation was followed by five washes (10 min each) in PAT. Embryos were incubated at room temperature in PAT with peroxidase-conjugated secondary antibody for 2 h followed by washes in PAT, PBT (1X PBS, 0.3% Triton-100), and PTW. The peroxidase reaction was performed in a solution composed of 1 mg/ml DAB and 0.01% H2O2 in PTW. Images were collected using a Sony DXC-390P camera mounted on a Zeiss Axioplan II microscope. Nuclear double staining was performed using a monoclonal antibody against nuclear pore complex protein (BAbCO), as described above, followed by a 1 h incubation in 1/1,000 dilution of TOTO-3 (Molecular Probes) in PTW. Finally, the embryos were washed several times in PTW, mounted in 80% glycerol, and observed in a Zeiss 510 LSM confocal microscope. Sample preparation for scanning electron microscopy (SEM) was performed as described in Thomas and Telford (1999). SEM images were captured using a Hitachi 570 Scanning Electron Microscope.

Results and discussion

Appendage development in *T. urticae*

The development of limbs in Chelicerata has been previously described in the mite *Archeogozetes longisetosus* (Thomas and Telford 1999) and the spider *C. salei* (Schoppmeier and Damen 2001). However, embryos of these species are accessible for developmental analysis only at late stages (germ band stage onwards), and a description of early limb development in chelicerates has been lacking. To understand the development of *T. urticae* appendages at a morphological and cellular level, we described limb ontogenesis using SEM and confocal microscopy. Freshly oviposited eggs (120–150 μm) exhibit radial symmetry without recognizable animal/vegetal or anterior/posterior polarity (Dearden et al. 2002). Such eggs undergo a series of total cleavages to generate a blastoderm with a homogeneous layer of cells on the surface surrounding a central mass of yolk (Fig. 1a,b). The first indication of limb formation is the appearance of the limb buds on the surface of the embryo (Fig. 1c). Furrows outlining pedipalps and walking legs are pronounced while formation of the cheliceral primordium at this early stage is marked by bulging of cells lacking furrows characteristic for more posterior limb primordia (Fig. 1c). The initiation of limb-bud formation is marked by thickenings in the previously unicellular blastoderm in the areas that delimits future limbs. This process includes the formation of a multi-layered epithelium that forms protrusions from the surface of the embryo towards the center of the blastocoel (arrows in Fig. 1d). At this point, the ventral epidermis is still composed of a single cell layer.

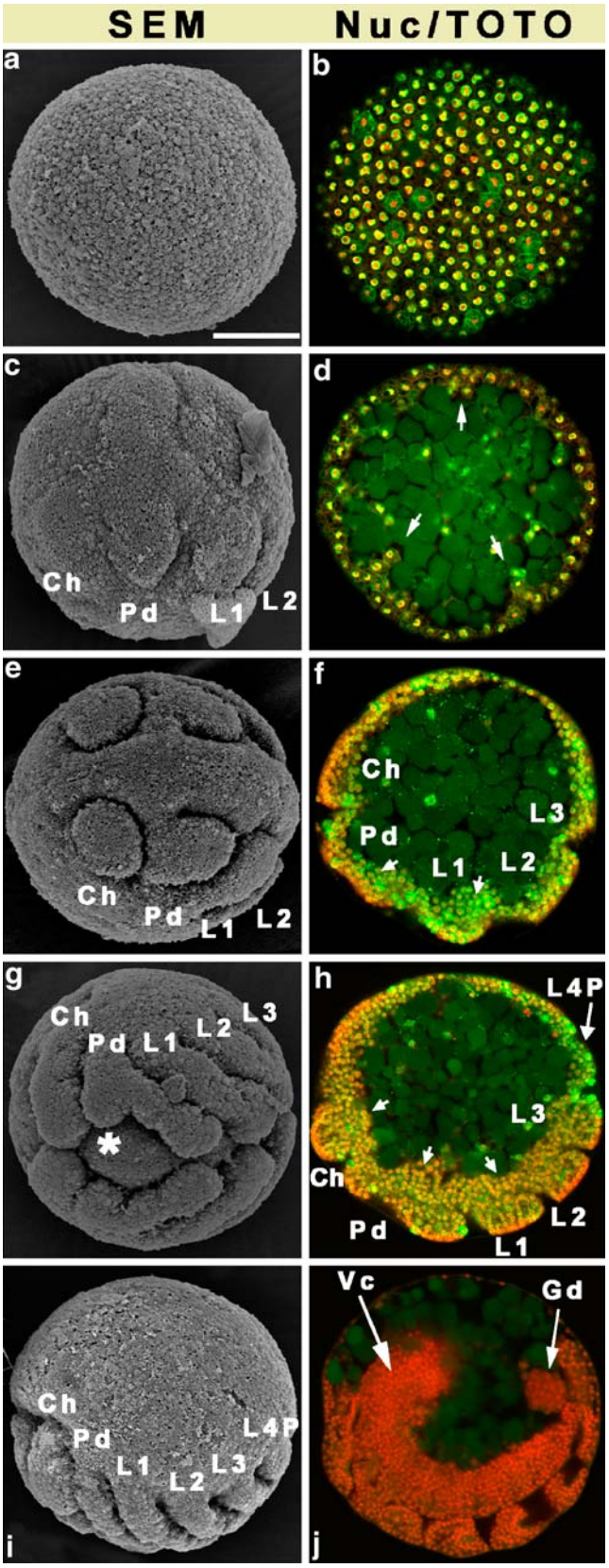

Fig. 1 Development of *T. urticae* appendages. **a**, **b** Blastoderm stage ▶ embryo showing a peripheral layer of cells and a central yolk mass. **c**, **d** Embryos with extended germ band. The limb buds of the chelicerae and the three first pairs of walking legs are visible in the ventral side of the embryos (**c**). Note changes in cell orientation from the surface of the embryo towards the center (*arrows* in **d**). **e**, **f** All appendages are now separated by furrows from the ventral epidermis; the fourth pair of walking legs has not formed yet at this stage. The layer of epidermal cells is thicker; these cells are dividing towards the center (*arrows* in **f**). **g**, **h** The chelicerae, the pedipalps, and the three first pairs of walking legs are now extended. The pedipalpal lobes are formed (*asterisk* in **g**) as well as the buds of the fourth pair of walking legs (*long arrow* in **h**). The layer of epidermal cells is thicker (*arrows* in **h**). **i**, **j** The appendages complete their extension while the ventral cord is fully condensed. *Ch*: chelicerae; *Pd*: pedipalps; *L1–3*: first, second and third pair of walking legs; *L4P*: primordia of the fourth pair of walking legs; *Gd*: gonads; *Vc*: ventral cord. *Scale bar*: 40 μm. In **a**, **b**, and **d**, the orientation of embryos is not known, in all other panels embryos are oriented with anterior to the left

Later in development, the chelicerae, the pedipalps, and the first three pairs of walking legs become separated from the ventral epidermis by invaginating cells that form deep furrows around each limb primordium (Fig. 1e). At this stage, epidermal cells forming furrows undergo a series of divisions to form a multilayer epithelium that invaginates towards the center of the embryo (arrows in Fig. 1f). Cells in the ventral area continue their growth towards the embryo interior (arrows in Fig. 1h), segregate from the epidermis, and form a compact ventral cord at later stages of embryogenesis (Fig. 1j). During the process of the limb-bud extension, the pedipalpal lobes (asterisk in Fig. 1g and all upcoming figures) bud off from the pedipalps. The mouth (stomodeum) appears first as a frontal opening (arrow in insert of Fig. 2a), followed by the extension of the labrum (Fig. 2a). These two structures rotate posteriorly toward the ventral side of the embryo and become covered by the flattened chelicerae (Fig. 2c). The chelicerae and the pedipalps undergo a series of movements and changes in shape (compare appendages in Figs. 1e,g, and 2c,d). The chelicerae rotate from their postoral position to become preoral while the pedipalpal lobes are transformed into gnathostoma in spider mite larvae (Fig. 2c,d), an event described in oribatid mites (Thomas and Telford 1999). After the deposition of cuticle at the end of *T. urticae* embryogenesis (39 h at 26°C; Dearden et al. 2002), six-legged larvae hatch (Fig. 2d) and undergo two molts to become nymphs. The limb buds of the fourth pair of walking legs lag in development relative to other appendages (arrow in Figs. 1h and 2b). This fourth pair evaginates during postlarval nymphal stages, after which, young adults become reproductively active.

Our description of *T. urticae* limb development together with the description of limb morphogenesis in the spider *C. salei* and the mite *A. longisethosus* suggests that chelicerates display a range of limb development programs. In both spiders and mites, most of the appendages are both specified and extended during embryonic stages. The only

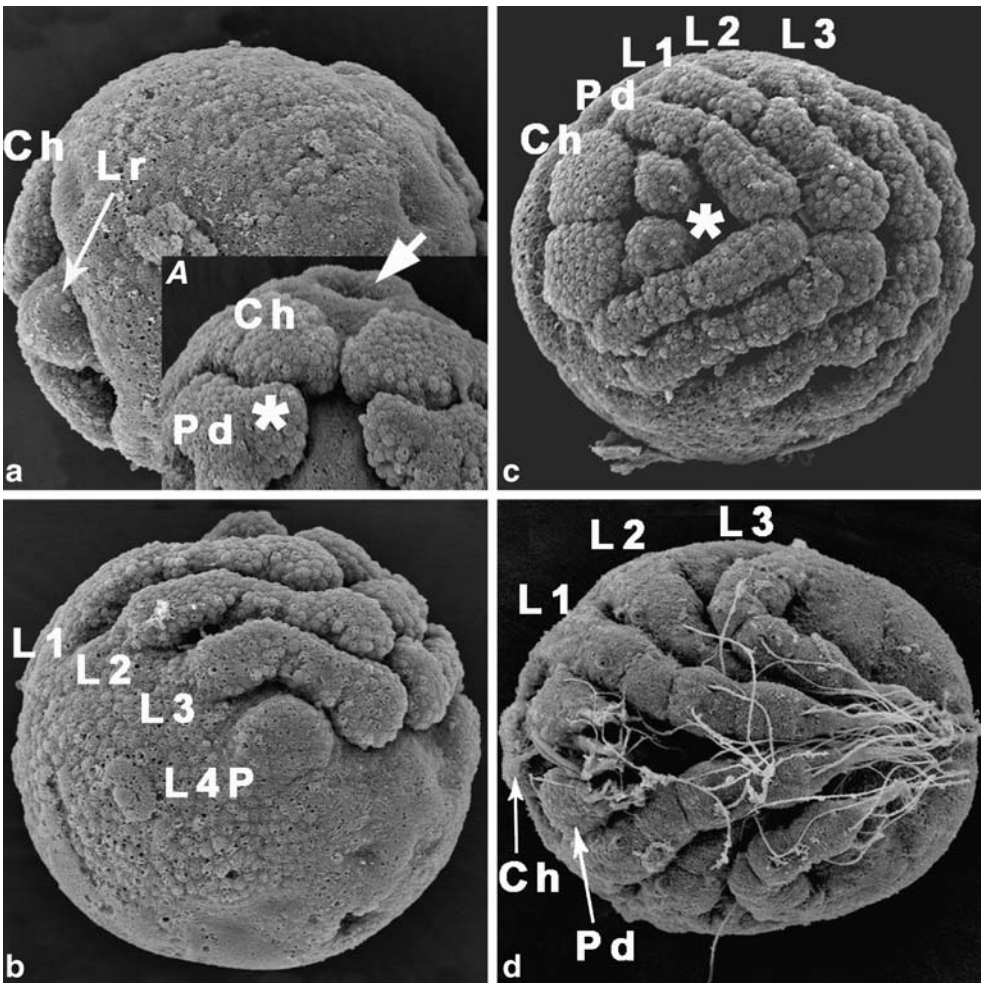

Fig. 2 Development of the gnathal structures and the fourth pair of walking legs in *T. urticae*. *A* (*insert*) The stomodeum (future mouth) forms at a frontal position anterior to the chelicerae (*arrow*). **a** After the formation of the stomodeum, the labrum extends and rotates towards the ventral posterior side of the embryo (*long arrow*, dorsal view). **b** All the appendages are already extended whereas the fourth pair of walking legs is still in its primordial form (*L4P*). **c** The chelicerae and the pedipalpal lobs (*asterisk*) have undergone movements and shape changes and now cover the labrum and the stomodeum. **d** The appendages have completed their development in the hexapod larva; note the presence of sensory structures (setae). Except where the anterior is to the top, all embryos are oriented anterior to the left

clear exception is the fourth pair of walking legs. In the spider *Cupiennius*, the fourth pair of walking legs is defined and extended synchronously with the other appendages (Schoppmeier and Damen 2001). The limb buds of the fourth pair of walking legs in *T. urticae* form as distinct cellular bulges after the extension of the other appendages (arrow in Figs. 1h and 2b), but they extend only at postlarval stages. Interestingly, in the oribatid mite *A. longisetosus*, the limb primordia of the fourth pair of walking legs are barely visible in the embryonic stages, they do not express Dll protein but they also extend later during postlarval stages (Thomas and Telford 1999). Observed variation in the formation of the posterior walking legs in mites shows developmental potential for the selection process that could lead to more dramatic modification of the walking legs in Acari including eriophyd mites, which posses only to pairs of walking legs (Frost and Ridland 1996).

Tu-Dll gene and expression pattern of *Tu-Dll* mRNA

The expression pattern of *Tu-Dll* was visualized by both in situ hybridization using an antisense probe to identify mRNA (Fig. 3) and by immuno-staining (Fig. 5d) using a cross-reactive rabbit polyclonal antibody generated against *Drosophila* Distal-less (Panganiban et al. 1995). *Tu-Dll* mRNA is first expressed at the early germ band stage in a bilaterally symmetrical cell clusters corresponding to future limb-bud primordia at each side of the ventral midline in the prosomal segments. The expression of Dll mRNA and protein corresponding to the primordium of the fourth walking leg was delayed (Fig. 3a,b). Additional domains of *Tu-Dll* expression were observed in a group of cells anterior to the chelicerae that constitute the future labrum (Fig. 3c,d) and two lateral spots corresponding to the head region.

This early expression of *Tu-Dll* mRNA in the limb buds, together with its previously described protein expression

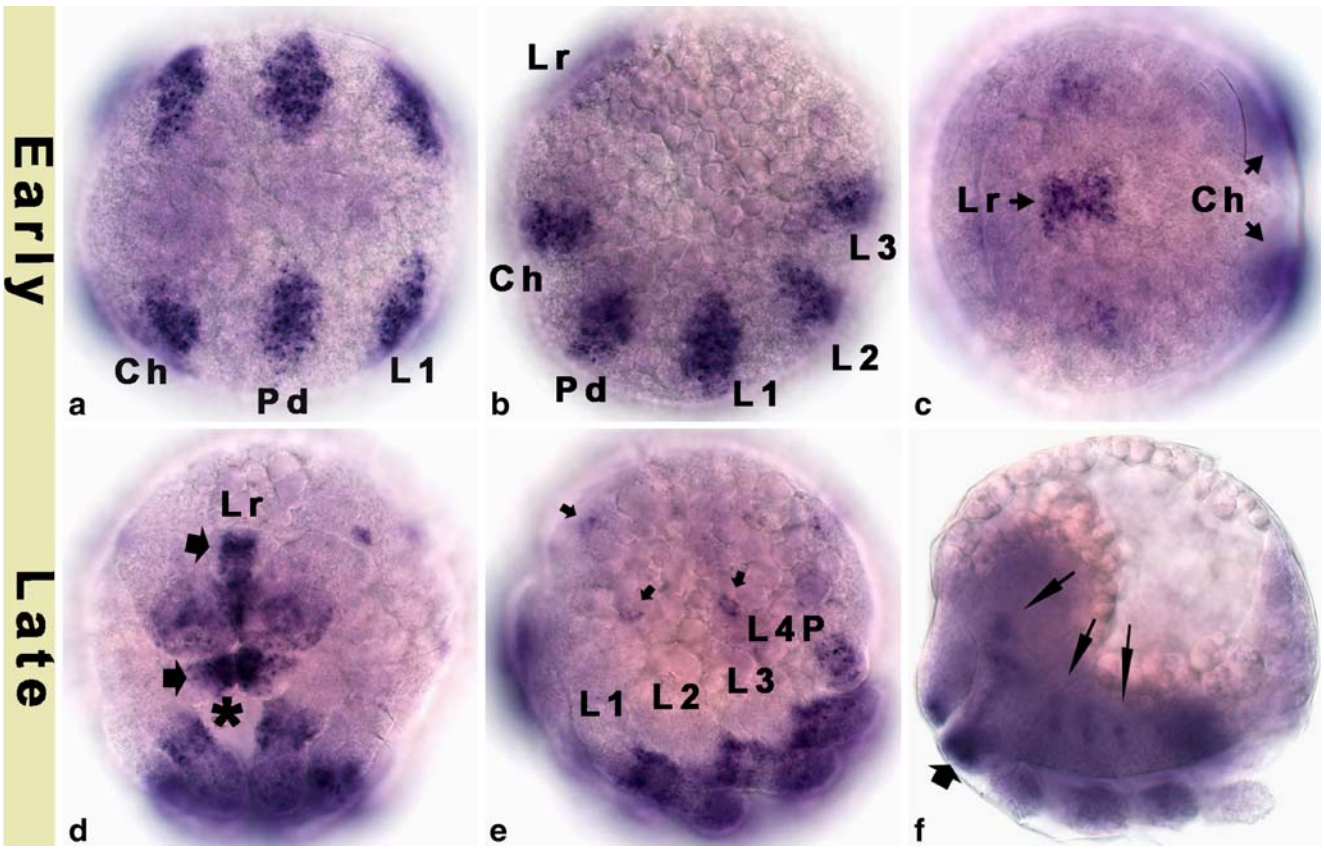

Fig. 3 *Dll* mRNA expression pattern in *T. urticae* embryos. **a**–**c** Same early germ band embryo showing *Dll* expression in the primordia of the chelicerae, the pedipalps, and the three first walking legs (**a**, **b**), as well as in the primordia of the labrum (**c**). Note the absence at this stage of *Tu-Dll* in the segment where the future fourth pair of walking legs forms. **d**, **e** This expression persists in the distal part of the extended appendages and appears now in the fourth pair of walking legs (*L4P* in **e**). Note *Dll* expression in clusters of epidermal cells, which probably correspond to sensory structures (*arrows*). The labrum and the pedipalpal lobs (*asterisk* in **d**) rotate towards the chelicerae and show higher levels of *Dll* mRNA (*thick arrows* in **d**). **f** By the end of embryogenesis, *Dll* mRNA is detected in clusters of neurons in the condensed ventral cord (*long arrows*). The level of *Tu-Dll* is still higher in the gnathal structures compared to the walking legs (*thick arrow*)

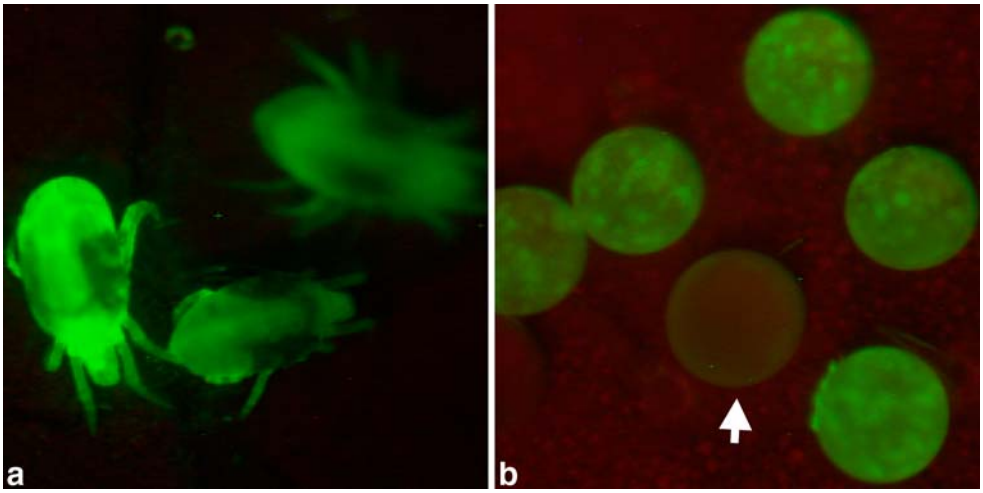

Fig. 4 *T. urticae* maternal RNAi injections. **a** Spider mite females after injection with either dsYFP or siGFP labeled with fluorescein. The injection was localized as close as possible to the gonads; the fluorescence diffuses throughout the entire female body. **b** Offspring of the injected females. The embryos which have taken the RNA show uniform fluorescence distribution, whereas those which did not absorb RNA do not show any fluorescence (*arrow*)

(Dearden et al. 2002), implicates *Tu-Dll* in distal limb specification of epidermal cells in *T. urticae*. As the appendages grow, *Tu-Dll* expression persists in the distal part of the chelicerae, the pedipalps and their lobes (endites of the pedipalp in spider *Cupiennius*, Schoppmeier and Damen 2001), the three first pairs of walking legs, and only then appears in the primordia of the fourth pair as they extend later in nymphal stages (Fig. 3d,e). Interestingly, later in development, *Tu-Dll* mRNA levels are clearly higher in the labrum and the pedipalpal lobes compared to the other limbs (Fig. 3, thick arrows in d and f). *Tu-Dll* mRNA is also detected in late embryos in several clusters of peripheral nervous system cells (Fig. 3, arrows in e) and in a subset of neurons in the ventral nerve cord (Fig. 3, arrow in f). This pattern was not described in the mite *A. longisetosus* (Thomas and Telford 1999). The mRNA and protein patterns observed appeared to be identical, suggesting that *Tu-Dll* is not subject to posttranscriptional regulation. Altogether, the conserved pattern of *Tu-Dll* expression indicates a function in limb specification and a later role in the developing peripheral and central nervous systems.

We detected heterochronic changes that included delayed *Tu-Dll* expression in the hind legs buds, which did not follow the pattern of simultaneous expression in all walking leg primordia of the spider *C. salei* (Schoppmeier and Damen 2001). In addition, the expression pattern of Dll mRNA in *T. urticae* fourth walking leg primordia contrasted to the lack of Dll expression in this pair in *A. longisethosus* (Thomas and Telford 1999), indicating a differential regulation of Dll in these two mite species.

In contrast to the spider, where *Dll* was expressed in opisthosomal limb buds and the posterior region, we did not detect an expression of *Tu-Dll* in the opisthosoma, suggesting that some patterns of *Dll* expression are not conserved in chelicerates. In addition, we observed various intensities of *Dll* expression in spider mite limbs. This observation, taken in combination with the differential phenotypes that resulted from intermediate RNAi effects, raises the possibility of dose-dependent Dll functions in *T. urticae* appendage development.

Tracing of dsRNA and siRNA distribution in *T. urticae* embryos

One of the major problems in generating dsRNA-mediated gene silencing is the delivery of the dsRNA to the target site. Often, the distribution of the injected dsRNA is not visualized and phenotypes are analyzed in all injected eggs (or progeny of the injected mother in parental RNAi) under the assumption that dsRNA are uniformly distributed in the embryos. To determine whether our dsRNAs become incorporated in embryos laid by the injected mothers, we used fluorescein-labeled yellow fluorescent protein (YFP) dsRNA (dsYFP) and green fluorescent protein (GFP) siRNA (siGFP). Our use of fluorescein-labeled RNAs allowed monitoring of the uptake of these molecules by the developing oocytes of *T. urticae* adult females. Although injections were performed in the ventral side of the abdomen, the fluorescence diffused throughout the entire body (Fig. 4a). This ubiquitous distribution of the fluorescently labeled RNAs suggests that both dsRNA and siRNA are efficiently transmitted across cell membranes in various spider mite tissues. The fluorescence was also detected in the oviposited embryos at various intensity levels (Fig. 4b). This indicates that molecules of dsRNA and siRNA injected into adult *T. urticae* females can be incorporated into the oocytes and persist in the oviposited eggs until the end of embryogenesis. To assess the efficiency of dsRNA and siRNA incorporation into embryos and also subsequent persistence, we examined the percentage of embryos with fluorescence at daily intervals for 3 days after injection. During the first day, high numbers of embryos show efficient RNA uptake (50% for dsRNA and 60% for siRNA). However, this efficiency drops dramatically both in the number and fluorescence intensity for embryos laid during the second and the third

days after injection (data not shown). This result suggests that RNAi delivery is efficient only during the first day after injection in *T. urticae*.

RNAi-silencing of *Tu-Dll*

The hallmark of *Dll* phenotypes in various arthropods is the truncation of the distal appendages (Cohen et al. 1989; Beermann et al. 2001; Schoppmeier and Damen 2001), which is consistent with the expression pattern of both mRNA and protein (Mittmann and Scholtz 2001; Panganiban et al. 1994; Jockusch et al. 2004; Popadic et al. 1998; Hejnol and Scholtz 2004; Williams et al. 2002). However, the genetic analysis of Dll in *Tribolium* using various alleles of the *Dll* gene showed, in addition to the leg truncation, that lesion in the *Dll* gene can also cause proximo-distal fusion of

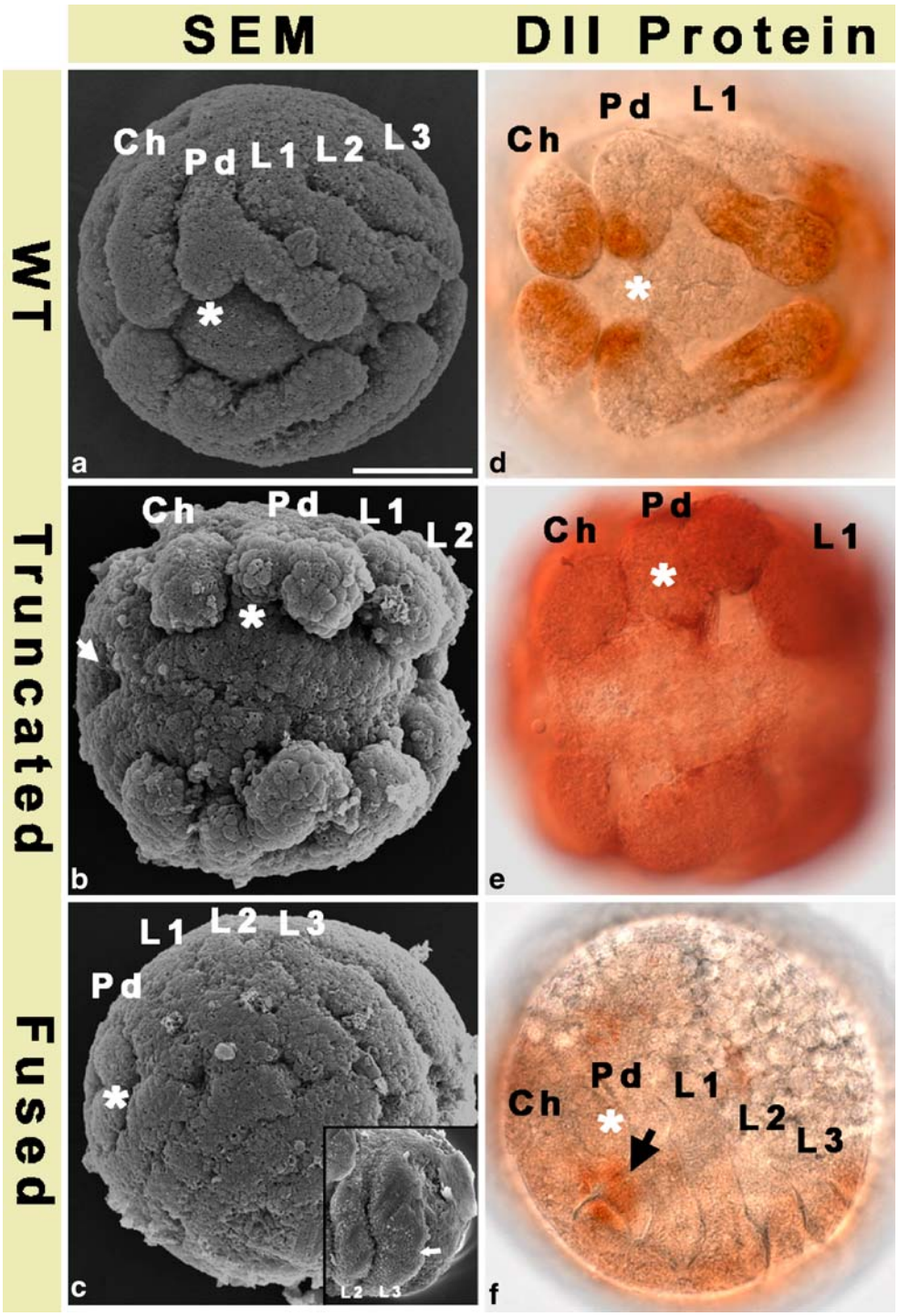

Fig. 5 Scanning Electron Microscopy and anti-Dll antibody staining of *T. urticaeDll*–RNAi embryos. **a**, **d** Wild type embryos; **b**, **e** Truncated appendages phenotype; **c**, **f**: Fused appendages phenotype. Each appendage in **c** (intermediate phenotype) is touching its counterpart across the ventral midline (*insert* in **c**: severe phenotype [magnified walking legs 2 and 3] where appendage is fused with its counterpart to form a continuous limb across the ventral midline; *white arrow* marks midline). Abbreviations, *Ch*: Chelicerae; *Pd*: Pedipalps; *L1*, *L2*, *L3*: first, second, and third walking legs. Pedipalpal lobs are marked with an *asterisk*. *Scale bar*: 40 μm. The *white arrow* in **b** points to the stomodeum. The *black arrow* in **f** indicates residual Tu-Dll protein in the pedipalpal lobes

the leg and antennal segments. This fusion was milder in the heterozygous state further suggesting a quantitative requirement of *Dll* in *Tribolium* limb formation (Beermann et al. 2001).

Silencing of the *Tu-Dll* gene activity was performed using both dsRNA and siRNA directed against *Tu-Dll* sequences. Only high RNA concentrations (5 μg/μl) resulted in specific phenocopy of predicted *Tu-Dll* mutant phenotypes as compared to control embryos. To describe specific defects in limb development, we first analyzed the morphology of RNAi induced phenotypes using SEM as shown in Fig. 5. Two classes of RNAi induced phenotypes specific to the offspring of *Dll*–RNAi injected females and absent from the two negative controls (dsYFP and siGFP) were identified: those with truncated appendages and those with fused appendages (Fig. 5). The first class of truncated appendages is a conserved phenotype characteristic of the previously reported mutations in *Drosophila* and *Tribolium* (Cohen et al. 1989; Beermann et al. 2001) or RNAi induced silencing of *Dll* gene in spider (Schoppmeier and Damen 2001). This phenotype was consistently induced by the injection of four independent dsRNA targeted against different regions of *Tu-Dll* (see "Materials and methods"), suggesting that it represents a true loss-of-function phenotype for *Tu-Dll*. The development of all embryos showing truncated appendages seemed to be arrested earlier and at approximately similar stages compared to the embryos showing fused appendages. In the chelicerae, the pedipalps and their lobes were all truncated, as well as the three first pairs of walking legs (Fig. 5b), while the primordia of the delayed fourth pair are not visible. We do not have molecular evidence of which limb segments of the appendages are affected because of the lack of limb segment-specific markers. However, the *Tu-Dll* expression patterns and morphological markers, such as the presence of parts of the pedipalp lobes (asterisk in all figures) that derive from the proximal portion of the pedipalps, suggest that only the distal portion of the appendages is missing. In addition, we did not detect the presence of the labrum in any of these embryos. Cumulatively, analysis of RNAi-induced limb truncation phenotypes suggests that Dll is required for the formation of distal portions of the appendages in *T. urticae* embryos.

Unlike the truncation phenotype, embryos with fused appendages were found at various and more advanced developmental stages. In these embryos, the proximal–distal axis of the appendages seems to be defined, however, the morphological differentiation of segments is poor and limb pairs touch abnormally at the ventral midline (Fig. 5c). In the most severe cases, tips of limbs remained fused across the midline (Fig. 5c inset). Fused appendages did not show clear signs of limb segmentation exhibited by distinct lobe-like constrictions characteristic for limbs of normal embryos (compare Fig. 5a WT and C fused limb). This phenotype does not seem to extend to the adjacent segments as the furrow between each pair formed, suggesting that Dll function is segment-autonomous. Interestingly, the fourth pair of walking legs was also invariably missing in this phenotypic class.

The limb fusion phenotype, where each appendage merges with its counterpart across the midline, has not been described in the spider *Cupiennius*. The closest defect was described in some alleles of *Tribolium Dll* that cause the fusion of leg and antennal segments (Beermann et al. 2001). This phenotype in *Tribolium* was dependent on the dose of Dll, suggesting by analogy that various concentration of

Table 1 Maternal *Dll*–RNAi in *T. urticae* using dsRNA and siRNA

Quantitative results from *Dll*–RNAi experiments

	Fluorescent embryos (%)	Appendages fused (%)	Appendages truncated (%)	Unknown Percent	Total embryos
(a) RNAi through dsRNA injection					
dsDll[a]	39.2	6	2	4.5	199
dsYFP[b] (control)	29.4	0	0	5.1	391
(b) RNAi through siRNA injection					
siDll2[b]	26.2	4.8	6.7	4.3	210
siDll3	0	13.8	7.6	2.2	225
siDll4	0	22.5	10.3	5.2	213
siGFP[b] (Control)	28	0	0	5	275

Adult females were injected separately with three Dll siRNA (siDll2, siDll3 and siDll4) and a 130 bp Dll dsRNA. siGFP and dsYFP were used as non-specific negative controls, respectively, for siRNA and dsRNA mediated silencing. Non-fluorescein-labeled dsYFP[a] was co-injected with fluorescein-labeled dsYFP at 5/1 concentration. siGFP[b] and siDll2[b] are labeled with Fluoresein, while siDll3 and siDll4 are not labeled. For each experiment, the table shows the total number of embryos collected and the percentage of embryos with fluorescence, embryos with non specific defects, embryos with fused appendages and embryos with truncated appendages. Classified as unknown are those found in all experiments including negative controls; embryos either showing early cleavage defects or premature termination of development.

RNAi can induce dose-dependent phenotype. Thus, we suggest that the truncated limbs are a consequence of a strong Dll silencing whereas the fused limbs result from partial Dll silencing, suggesting a dose-dependent Dll function during *T. urticae* appendage development. Based on these results, we speculate that spider mite *Dll* may function by a dose-sensitive regulation of its targets and/or co-factors.

In both spiders and spider mites, silencing of *Dll* caused truncated limbs and deletion of the labrum. However, many spider embryos showed a mosaic phenotype that is believed to be created by the injection method where local injection of the individual eggs with RNAi under the periviteline membrane may result in an uneven distribution of the dsRNA in the embryo (Schoppmeier and Damen 2001). We have not detected any mosaic phenotypes in the spider mite. This is likely attributable to our maternal dsRNA injection method; maternal deposition is more likely to be uniform than injection of the individual eggs.

To further confirm that *Dll*-RNAi effects are specifically due to the absence and/or reduction in the amount of translated *Tu-Dll* protein, we stained *Dll*-RNAi embryos with a cross-reactive antibody directed against *Drosophila* Dll (Panganiban et al. 1995). By contrast with normal embryos (Fig. 5d), Dll protein was not detected in treated embryos containing truncated appendages, (Fig. 5e). In the case where appendages were present but fused, faint Dll staining was detected in the walking legs together with some residual expression in the pedipalpal lobes (arrows in Fig. 5f). The residual protein persisting in the pedipalpal lobes probably reflects the strong mRNA expression normally seen in these structures at later developmental stages (see Fig. 3d,f). These observations suggest that the truncation phenotype is a consequence of either strong or total loss of Dll, while the fusion phenotype is a result of decreased amounts of Dll protein. None of the embryos showed apparent segmentation or dorsal–ventral polarity defects associated with either the truncated or the fused appendages, suggesting that these phenotypes are not a result of nonspecific developmental defects caused by dsRNA.

We also quantified our RNAi treatments to establish whether differences exist between effects of longer *Dll* dsRNA molecule and various *Dll* siRNA fragments. All four RNAi treatments produced qualitatively the same phenotypes (truncated and merged appendages) but with distinct quantitative frequencies (Table 1). The most efficient silencing of *Tu-Dll* was achieved with siDll4 and siDll3 producing cumulatively 33 and 21% of phenotypes, whereas siDll2 induced only 11% of Dll-associated phenotypes. *Tu-Dll* dsRNA (130 bp) produced a significantly lower proportion of limb defects in treated embryos. In addition, the percentage of aberrant embryos scored as unknown phenotypes in all treatments was very similar. These embryos showed a nonspecific variable aberrations that did not include limb phenotype, further corroborating specificity of dsRNA treatments.

Our parental RNAi injections coupled with fluorescently labeled dsRNA shows that dsRNA can diffuse into various *T. urticae* tissues and that it is incorporated into embryos. However, a higher incorporation efficiency of labeled dsRNA was achieved during the first day of injection suggesting that fluorescent tracer is either evacuated or consumed from the maternal body. As we found fluorescence in spider mite excretions, it is possible that *T. urticae,* as a voracious sap feeder, simply flushes out large quantities of fluid from injections thereby eliminating dsRNA. The efficiency of our RNAi protocol was in the same range as the RNAi protocol that included individual egg injections in the spider *Cupiennius*. However, our efficiency is lower than the efficiency of RNAi reported for *Tribolium* and *Oncopeltus* (Bucher et al. 2002; Liu and Kaufman 2005).

Our RNAi experiments also showed that in the spider mite, siRNA injections induce phenotypes more efficiently than longer dsRNA. We identified in the spider mite EST screen two major components of the RNAi response, *Tu-dicer* and *Tu-argonaute* (data not shown), supporting the possibility that the mechanism of RNAi operates in *T. urticae*. In addition to *Tu-Dll* silencing, we also silenced the *eyes absent* gene causing eye deletion phenotype (Lee and Grbic, unpublished) using the same parental RNAi protocol. Thus, the development of parental RNAi protocols in another chelicerate will open new avenues for functional analysis in this group of arthropods.

Acknowledgements We thank Michael Crawford, David Cribbs, Michelle Crozatier and Alain Vincent for critical reading of this manuscript. We also thank Kwang-zin Lee and Anica Bjelica for support and helpful discussions, Vladimir Zhurov for help with SEM, and Lou Verdon and Jay Whistlecraft from Southern Crop Protection and Food Research Centre for rearing of spider mites. The anti-Dll antibody was kindly provided by Grace Panganiban. This work was supported by an NSERC Strategic Grant and Marie Curie Incoming Professorial Fellowship awarded to M.G.

References

Beermann A, Jay DG, Beeman RW, Hulskamp M, Tautz D, Jurgens G (2001) The short antennae gene of *Tribolium* is required for limb development and encodes the ortologue of the *Drosophila* Distalless protein. Development 128:287–297

Boore JL, Collins TM, Stanton D, Daehler LL, Brown WM (1995) Deducing arthropod phylogeny from mitochondrial DNA rearrangements. Nature 376:163–165

Bucher G, Scholten J, Klingler M (2002) Parental RNAi in *Tribolium* (Coleoptera). Curr Biol 12:R85–R86

Cohen SM, Bronner G, Kuttner F, Jurgens G, Jackle H (1989) *Distal-less* encodes a homoeodomain protein required for limb development in *Drosophila*. Nature 338:432–434

Damen WGM, Janssen R, Prpic NM (2005) Pair rule gene orthologs in spider segmentation. Evol Dev 7:618–628

Dearden P, Akam M (2000) A role for Fringe in segment morphogenesis but not segment formation in the grasshopper, *Schistocerca gregaria*. Dev Genes Evol 210:329–336

Dearden PK, Donly C, Grbic M (2002) Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite *Tetranychus urticae*. Development 129:5461–5472

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila* melanogaster embryo lysate. Embo J 20:6877–6888

Friedrich M, Tautz D (1995) Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. Nature 376(6536):165–167

Frost WE, Ridland PM (1996) Grasses. In: Lindquist EE, Sabelis MW, Bruin J (eds) Eriophyoid mites—their biology, natural enemies and control. Elsevier, Amsterdam, The Netherlands, pp 619–629

Gregory TR, Shorthouse DP (2003) Genome sizes of spiders. J Heredity 94:285–290

Hejnol A, Scholtz G (2004) Clonal analysis of *Distal-less* and *engrailed* expression patterns during early morphogenesis of uniramous biramous crustacean limbs. Dev Genes Evol 214:473–485

James MT, Harwood RF (1969) Ticks and tick-borne diseases: Herm's medical entomology, 6th edn. Collier–Macmillan, London, UK, pp 323–324

Jockusch EL, Williams TA, Nagy LM (2004) The evolution of patterning of serially homologous appendages in insects. Dev Genes Evol 214:324–338

Liu PZ, Kaufman TC (2005) *Even-skipped* is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect. Development 132:2081–2092

May RC, Plasterk RHA (2005) RNA interference spreading in *C. elegans*. Methods Enzymol 392:308–315

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349

Mittmann B, Scholtz G (2001) Distal-less expression in embryos of *Limnulus polyphemus* (Chelicerata, Xiphosura) and *Lepisma sacharina* (Insecta Zygentoma) suggests a role in the development of mechanoreceptors, chemoreceptors and the CNS. Dev Genes Evol 211:232–243

Oliver JH (1971) Parthenogenesis in mites and ticks (Arachnida-Acari). Am Zool 11:283–299

Panganiban G, Nagy L, Carroll SB (1994) The role of the *Distal-less* gene in the development and evolution of insect limbs. Curr Biol 4:671–675

Panganiban G, Sebring A, Nagy L, Carroll S (1995) The development ofcrustacean limbs and the evolution of arthropods. Science 270:1363–1366

Panganiban G, Irvine SM, Lowe C, Roehl H, Corley LS, Sherbon B, Grenier J, Fallon JF, Kimble J, Walker M, Wray GA, Swalla BJ, Martindale MQ, Carroll SB (1997) The origin and evolution of animal appendages. Proc Natl Acad Sci USA 94:5162–5166

Popadic A, Rusch D, Peterson M, Rogers BT, Kaufman TC (1996) The origins of arthropod mandible. Nature 380:395

Popadic A, Panganiban G, Rusch D, Shear WA, Kaufman TC (1998) Molecular evidence for the gnathobasic derivation of arthropod mandibles and for the appendicular origin of the labrum and other structures. Dev Genes Evol 208:142–150

Pultz MA, Zimmerman KK, Alto NM, Kaeberlein M, Lange SK, Pitt JN, Reeves NL, Zehrung DL (2000) A genetic screen for zygotic embryonic lethal mutations affecting cuticular morphology in the wasp *Nasonia vitripennis*. Genetics 154:1213–1229

Rao PP, Praslicka J, Sutakova G (1996) Effect of temperature and rearing method on development and fecundity of *Tetranychus urticae* (Acarina, Tetranychidae). Biologia 51:509–516

Schoppmeier M, Damen WG (2001) Double-stranded RNA interference in the spider *Cupiennius salei*: the role of Distal-less is evolutionarily conserved in arthropod appendage formation. Dev Genes Evol 211:76–82

Telford MJ (2000) Evidence for the derivation of of *Drosophila fushi-tarazu* gene from a Hox gene orthologous to lophotrochozoan Lox5. Curr Biol 10:349–352

Thomas RH, Telford MJ (1999) Appendage development in embryos of the oribatid mite *Archegozetes longisetosus* (Acari, Oribatei, Trhypochthoniidae). Acta Zool (Stockh) 80:193–200

Ullmann AJ, Lima CMR, Guerrero J, Piesman IV, Black WC (2005) Genome size and organization in the black-legged tick, *Ixodes scapularis* and the southern cattle tick, *Boophilus microplus*. Insect Mol Biol 14:217–222

Williams TA, Nulsen C, Nagy LM (2002) A complex role for Distal-less in chrustacean appendage development. Dev Biol 241:302–312