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A survey of the effectiveness of non-cell autonomous RNAi throughout development in the sawfly, *Athalia rosae* (Hymenoptera)

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ABSTRACT

RNA interference (RNAi) is a powerful and convenient tool not only for functional analysis of specific genes, but also for large-scale screening of gene function in insects; however, reports on its efficiency throughout development in a single species are limited. We demonstrate here that non-cell autonomous RNAi by injection of double-stranded RNA (dsRNA) knocks down targeting genes in most developmental stages in the sawfly, *Athalia rosae*. Injection of dsRNA targeting the *green fluorescence protein* (*gfp*) gene into eggs of a transgenic strain carrying the constitutively expressing *gfp* gene resulted in the absence of GFP fluorescence during embryogenesis, while a portion of the *gfp* dsRNA-injected embryos began exhibiting GFP fluorescence at late embryogenesis. When *gfp* dsRNA was injected into parental female pupae, the RNAi effect was carried over to all embryos of the next generation and the effect lasted until mid-larval stages. Parental injection of dsRNA was more efficient than embryonic injection in terms of penetrance of the effect and the survival rate. After injection of *gfp* dsRNA into last instar larvae, the RNAi effect was sustained during prepupal and pupal stages and in adults. The *gfp* gene transcript markedly decreased in these knockdown phenotypes. It was revealed by employing fluorescence-labeled dsRNA that injected dsRNA was taken up in internal organs. Knockdown of an endogenous gene, *Distal-less* (*Dll*), resulted in typical phenotypes represented by the lack and malformation of *Dll*-expressing organs, such as distal parts of the appendages and wing edges without showing off-target effects. In contrast, RNAi by dsRNA injection seems to be hardly effective in mid- to late-larval stages.

1. Introduction

An effective approach for gene functional analysis is to induce gene misexpression. One of the most convenient tools to knock down gene transcripts is RNA interference (RNAi), which achieves post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA) introduced into cells in a sequence-specific manner (Fire et al., 1998; Montgomery, 2004). Since injection of dsRNA is a rapid and easy approach, RNAi has been utilized in a variety of insects, especially in non-model species with less genetic and genomic background (Belles, 2010; Mito et al., 2011; Tomoyasu et al., 2008). Non-cell autonomous RNAi, such as systemic RNAi and environmental RNAi, is defined as the gene-silencing effects occurring in cells and tissues different from the location of the application or production of dsRNA (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). Non-cell autonomous RNAi has been reported in several species belonging to various insect orders; however, the RNAi responses seem to be conditional in such model species as *Drosophila melanogaster* and *Bombyx mori* (Belles, 2010; Huvenne and Smagghe, 2010; Miller et al., 2008; Terenius et al., 2011; Tomoyasu et al., 2008).

Insect RNAi by dsRNA injection is generally categorized into embryonic, larval and parental RNAi based on the stages of recipients (Belles, 2010; Mito et al., 2011). Embryonic RNAi, in which dsRNA is injected into eggs or early embryos, has been applied for many insects since the first success of this method in *D. melanogaster* (Kennerdell and Carthew, 1998). The silencing effects of this method, however, seem not to be durable to allow analysis in post-embryonic stages. Alternative methods for post-embryonic gene knockdown to be practicable are larval and parental RNAi, both of which were innovated in the coleopteran *Tribolium castaneum*. Function of the targeted gene is interfered with in the entire body of pupae and adults by larval RNAi, in which dsRNA is injected into the larval hemocoel (Tomoyasu and Denell, 2004). In parental RNAi, which introduces dsRNA into the hemocoel of parental females, gene silencing effects are detected in the offspring (Bucher et al., 2002).

Successful gene knockdown by introduction of dsRNA has been reported in some hymenopteran species (Amdam et al., 2003; Beye et al., 2002; Liu et al., 2009; Lynch and Desplan, 2006; Sumitani et al., 2005; Werren et al., 2009), and hence this order is thought

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to be susceptible to non-cell autonomous RNAi. Nevertheless, in a species showing susceptibility to RNAi responses, knockdown effects have been examined in a certain organ at a specific stage depending on the characteristics of the genes to be investigated (Belles, 2010; Terenius et al., 2011; Yang et al., 2011). Practical gene knockdown in a variety of organs at various developmental stages is an expedient method for functional analysis of a variety of genes and functional genomics by genome-wide screening (Perrimon et al., 2010). Effectiveness of RNAi in several developmental stages in a single species was demonstrated in *T. castaneum* utilizing a strain carrying the *green fluorescence protein* (*gfp*) gene (Tomoyasu et al., 2008). A transgene, such as that constitutively expressed, and its product is easily detected without affecting viability, is very useful to examine the RNAi effect throughout development. In this study we examined gene knockdown effects throughout development in the sawfly, *Athalia rosae*, targeting the constitutively active *gfp* transgene and the endogenous *Distal-less* (*Dll*) gene.

2. Materials and methods

2.1. Sawfly

Laboratory stocks of the sawfly, *A. rosae*, were maintained at 25 °C under a 16 h light:8 h dark condition as described previously (Sawa et al., 1989). The wild type and a transgenic strain in which the *green fluorescence protein* (*gfp*) gene is expressed constitutively (Sumitani et al., 2003) were employed. General biology, artificial induction of the parthenogenetic development of unfertilized eggs (egg activation) and staging of development were described elsewhere (Hatakeyama et al., 1990; Oishi et al., 1993; Sawa and Oishi, 1989). Briefly, embryogenesis takes 5 days (120 h) and larval development takes about 8 days (5 instars) in males and about 9 days (6 instars) in females. The last instar larvae wander around and dig into soil for cocoon formation. Prepupae and pupae are staged as PCF (post-cocoon formation) 1–9 (each stage lasts about 24 h), and pupal molt takes place at PCF 5. Eclosion occurs at PCF 10, and adults stay in cocoons for about a day (PCF 11) and then come out (1-day-old adults).

2.2. Double-stranded RNA (dsRNA)

A 298-bp-long fragment of the *gfp* cDNA was PCR-amplified using gene-specific primers (5′-CGTGACCACCCTGACCTAC-3′ and 5′-GTTCTTCTGCTTGTCGGCCA-3′) and the pPIGA3GFP/hspGFP-S65T transformation vector plasmid (Sumitani et al., 2003) as a template. A 350-bp-long *A. rosae Distal-less (Dll)* cDNA fragment corresponding to the 5′ terminal region of its open reading frame was PCR-amplified using the gene-specific primers (5′-CGACATG GAGCAGCATCTTCACC-3′ and 5′-CGTAGGTGTTGTGCTGATGCATGG-3′) and the plasmid containing *A. rosae Dll* cDNA (Oka et al., 2010, GenBank ID: AB378321) as a template. The PCR products were cloned into a pCRII-TOPO dual promoter plasmid carrying T7 and SP6 promoter sequences using a TOPO TA cloning kit (Invitrogen). The cDNA fragments containing the T7 and SP6 promoter sequences and the 298-bp-long *gfp* or 350-bp-long *A. rosae Dll* were then amplified using M13 (−20) forward and M13 reverse primers. Sense and anti-sense strands of each fragment were transcribed using a MEGAscript kit (Ambion) and were annealed in water to form dsRNAs. Synthesized dsRNAs were stored at −20 °C as a concentrated solution (5 μg/μl) until used. Fluorescent labeling of synthesized *gfp* dsRNA and a DNA fragment corresponding to the same region of the *gfp* dsRNA was performed using a Label IT Cy3 labeling kit (Mirus).

2.3. Injection

Microjection of dsRNA into mature unfertilized eggs taken from females was performed according to the methods described previously (Sumitani et al., 2005). The larvae and pupae to be injected were anesthetized by chilling for 30 min in a plastic container placed in an ice bath. Individuals receiving injection were left on an ice pack during the injection procedure. Injection was performed using a handmade injection apparatus (Hatakeyama et al., 1990): a fine glass needle, made by pulling a 25 μl Microcaps (Drummond) and cutting the tip, was connected to a 1 ml plastic syringe. dsRNA was injected into the dorsal hemocoel in the second abdominal segment of a larva, or at the suture between third and fourth abdominal segments of a pupa. The average volume of ten some pico-liters of dsRNA at a concentration of 3 μg/μl was injected into eggs (600 ng/mg equivalent), and 3 μg per individual of dsRNA was injected into last instar larvae (60 ng/mg equivalent) and pupae (100 ng/mg equivalent).

2.4. Reverse transcription PCR (RT-PCR)

Fifty nanograms of total RNA extracted from each individual using an RNeasy Mini kit or RNeasy Plus Micro kit (Qiagen) were used as templates. The primer sets were designed to amplify a 739-bp-long fragment corresponding to the entire ORF of the *gfp* gene (5′-CGGTCGCCACCATGGTGAGCAAGGG-3′ and 5′-CGGCCGCTT TACTTGTACAGCTC-3′) and to amplify a 339-bp-long fragment of *A. rosae elongation factor-1α* gene (5′-CTTCACTCTTGGTGTCAAC-CAGCTC-3′ and 5′-ACATCCTGAAGAGGAAGACGGAGAG-3′, GenBank ID: AB253792) as a positive control. Reverse transcription and subsequent PCR were carried out using a One-Step RT-PCR kit (Qiagen) according to the supplier's protocol.

2.5. Whole-mount in situ hybridization

The RNA probe to detect *A. rosae Dll* transcript was prepared as described in Oka et al. (2010). A 304-bp-long fragment corresponding to the 3′ region of *A. rosae Dll* cDNA cloned into a pCRII-TOPO dual promoter plasmid was labeled using a DIG RNA Labeling (T7/SP6) kit (Roche). Forty-eight-hour-old embryos, from which chorions, vitelline membranes, embryonic membranes and yolks had been removed, and primordia of thoracic legs and wings dissected out of PCF 4 prepupae, were fixed with 4% paraformaldehyde in PBS (phosphate-buffered saline). They were transferred to PBT (0.1% Tween-20 in PBS), dehydrated, re-hydrated and used for hybridization. Pre-hybridization, hybridization, washing and detection of signals followed the procedure described in Oka et al. (2010) and Yamamoto et al. (2004).

2.6. Microscopy

The GFP and Cy3 fluorescence and morphology were observed and photographed under a binocular microscope (MZ16F; Leica) equipped with fluorescent optics with GFP2 and DsRed filters. Individuals sacrificed for scanning electron microscopy (SEM) were prepared as described in Oka et al. (2010). Briefly, samples were fixed with alcoholic Bouin's fixative, cleaned in an ultrasonic cleaner, dehydrated in an ethanol series, immersed in *t*-butyl alcohol, freeze dried, coated with gold, and examined under a scanning electron microscope (TM-1000; Hitachi).

3. Results

3.1. Knockdown of constitutively expressing gfp transgene

Double-stranded RNA (dsRNA) of the *gfp* gene was injected into eggs, larvae and pupae of a transgenic strain carrying *gfp* genes to