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Difference in Efficiency of RNA Interference during Embryonic Development in the Sawfly, *Athalia rosae ruficornis*: Embryonic RNAi versus Parental RNAi*

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Gene knockdown by introducing double-stranded RNA (dsRNA), known as RNA interference (RNAi), has been widely used for gene functional analysis in a variety of insects. The RNAi in insects is categorized as embryonic RNAi, larval RNAi and parental RNAi based on the timing of the introduction of dsRNA (Tomoyasu *et al.*, 2008; Huvenne and Smagghe, 2010; Terenius *et al.*, 2011). Embryonic RNAi, in which dsRNA is injected into eggs or early embryos, targets genes expressed during embryogenesis, and larval RNAi, in which dsRNA is injected into larvae or nymphs, targets genes expressed in larval and/or larval-adult transition stages. Parental RNAi induces gene knockdown in the next generation by injecting dsRNA into mothers. These RNAi methods are convenient and applicable to non-model species with limited genetic and genomic background; however, the efficiency of RNAi effects are largely dependent on the species, targeting genes and stages of dsRNA introduction (Terenius *et al.*, 2011). Embryonic RNAi is effective in a wide variety of insects and has been shown to be the most versatile method. On the other hand, larval RNAi and parental RNAi although are effective in several species, they do not work well in such model species as the fruit fly, *Drosophila melanogaster* and the silkworm, *Bombyx mori* (Miller *et al.*, 2008; Tomoyasu *et al.*, 2008; Terenius *et al.*, 2011). Coleoptera and Hymenoptera are susceptible to RNAi at various developmental stages (Beye *et al.*, 2002; Bucher *et al.*, 2002; Tomoyasu and Denell, 2004; Niimi *et al.*, 2005; Kuwayama *et al.*, 2006; Lynch and Desplan, 2006; Miller *et al.*, 2008; Werren *et al.*, 2009). We have reported that the sawfly, *Athalia rosae ruficornis*, belonging to the lower suborder of Hymenoptera shows RNAi responses in most developmental stages (Sumitani *et al.*, 2005; Yoshiyama *et al.*, 2010). Embryonic RNAi and parental RNAi are both applicable to functional analysis of genes expressed during embryonic development, although it is not known if there is difference in the efficiency of knockdown effects between these two methods. Here we examined the effectiveness of embryonic RNAi and parental RNAi by targeting an integrated constitutively active *green fluorescent protein* (*GFP*) gene and an endogenous *Distal-less* (*Dll*) gene.

We first compared the effects of embryonic RNAi and parental RNAi targeting the *GFP* transgene. For embryonic RNAi a 298-bp-long dsRNA targeting the *GFP* transgene was injected into mature eggs taken from females homozygous for the *GFP* transgene. The *GFP* dsRNA-injected eggs were allowed to undergo parthenogenetic development. The same dsRNA was injected into the hemocoel of female pupae homozygous for the *GFP* transgene for parental RNAi. Eclosed adults were aged for a week and mature eggs were artificially activated to induce parthenogenetic development. Table 1 summarizes the results of knockdown effects as evaluated based on the phenotype (absence of GFP fluorescence). Although both embryonic RNAi and parental RNAi knocked down the targeting *GFP* transgene during embryonic development, about one fourth of survived embryos subjected to embryonic RNAi exhibited GFP fluorescence at 96 h of embryogenesis. In contrast, GFP fluorescence was not detected in all embryos until the first instar larval stage in parental RNAi. Most eggs taken from *GFP* dsRNA-injected mothers developed normally, while about a half of *GFP* dsRNA-injected eggs did not survive apparently due to the damage caused in the injection procedure.

Similar results were obtained when the *Dll* gene was targeted (Table 2). When a 350-bp-long dsRNA corresponding to the 5' region including the *Dll* open reading frame (ORF) was injected into eggs taken from wild-type females (embryonic RNAi), about 60% of the *Dll* dsRNA-injected eggs survived and hatched. Nearly 90% of hatched larvae had defects in presumed *Dll*-expressing structures, such as the distal region (telopodite) of appendages.

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Table 1 Comparison of knockdown effects targeting the *GFP* transgene

	No. of eggs activated	No. embryos normally developed at 48 h (%)	Percent of individuals that lost GFP fluorescence			
			48 h embryos	96 h embryos	1st instar larvae	3rd instar larvae
Embryonic RNAi						
GFP dsRNA	355	200 (56.3)	100 (n=200)	73.4 (n=199)	40.3 (n=159)	0 (n=116)
D.W.	193	105 (54.4)	0 (n=105)	0 (n=102)	0 (n=97)	0 (n=85)
Parental RNAi						
GFP dsRNA	361	312 (86.4)	100 (n=312)	100 (n=311)	100 (n=302)	0 (n=278)
D.W.	338	305 (90.2)	0 (n=305)	0 (n=305)	0 (n=290)	0 (n=264)

Table 2 Comparison of knockdown effects targeting endogenous *Dll* gene

	No. of eggs activated	No. of larvae hatched and examined (%)	Percent of individuals showing knockdown phenotype		
			Severe (complete loss)	Mild (partial loss)	None (normal)
Embryonic RNAi					
Dll dsRNA	393	239 (60.8)	46.9	41.4	11.7
Parental RNAi					
Dll dsRNA	344	305 (88.7)	100	0	0
GFP dsRNA	174	162 (93.1)	0	0	100

Nevertheless, the phenotype varied in degree and only half of these defective larvae completely lost *Dll*-expressing structures. Contrastingly, in parental RNAi, most eggs taken from *Dll* dsRNA-injected mothers survived and all larvae showed a severe phenotype with complete loss in *Dll*-expressing structures.

Taking these results together, both embryonic RNAi and parental RNAi are effective for functional analysis of genes expressed during embryonic development. Parental RNAi is more efficient and practical for gene analyses in the sawfly considering the survival rate, less fluctuating effects and intensity of the knockdown phenotype.

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