Oral delivery of double-stranded RNA and the effects of RNA interference on the green peach aphid, *Myzus persicae*

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The green peach aphid (GPA), *Myzus persicae*, is a polyphagous insect that feeds on a broad range of hosts. It also transmits over 100 plant viruses. As a result, feeding damage and the viral diseases the aphid transmits contribute to yield losses in several economically important crops worldwide including potato, canola and lupin. Genetic resistance and application of insecticides are currently the primary means of controlling GPA. However, insecticides are not always effective: for example, carbamate or neonicotinoids are chemicals to which GPA can readily develop resistance. An alternative approach is the use of the naturally occurring phenomenon of RNA interference (RNAi) to silence essential genes in the aphids. RNAi is the sequence-specific degradation of homologous RNA molecules guided by small RNAs, and can be triggered by the introduction of double-stranded RNA (dsRNA). This approach is potentially economical, environmentally-safe and has been shown to be an effective strategy against several different plant pathogens and pests including nematodes and some insects.

Several methods have been developed to deliver dsRNA to insects such as soaking cell lines, microinjection, oral feeding (*ad libitum*) and *via* transgenic plants. Recently, genes of insect pests of the orders Lepidoptera, Coleoptera and Hemiptera have been silenced. For example gene silencing in the western corn rootworm (*Diabrotica virgifera virgifera*), the cotton bollworm (*Helicoverpa armigera*), the pea aphid (*Acyrthosiphon pisum*), and GPA through injection, feeding artificial diets containing dsRNA (*in vitro*) or using transgenic plants expressing dsRNA (*in planta*) has been attempted. Microinjection of dsRNA into the abdomen or thorax involves the use of very fine glass needles to administer dsRNA into various nymphal stages of insects. This method requires use of appropriate size of needles and substantial skill to reduce undue stress, injury or even death as well as empirical injection of volumes and doses of dsRNA. Microinjection can be laborious and may not be suitable where a large number of insects are required in a large-scale functional analysis (of target genes) and for small nymphs of some insects, as they may be prone to injury and stress.

Oral delivery of dsRNA to insects (also called artificial feeding) involves administration of dsRNA suspended in sucrose solution in a feeding chamber: artificial feed that is sandwiched between thinly stretched parafilm layers is placed over the mouth of the container and aphids are allowed to feed. This method is convenient, non-invasive, allows the use of nymphs; *ad libitum* feeding is close to the natural feeding behaviour of aphids. Artificial feeding is also well suited for large-scale functional analyses and has also been used successfully to screen 290 dsRNA targets in the western corn rootworm. One of the limitations of oral delivery is that it is difficult to ascertain whether the insect has ingested dsRNA and the amount ingested. However, most published artificial feeding systems for RNAi in insects do not use markers to trace uptake, and thus all experimental insects are assumed to have fed, and the effects of gene silencing are averaged. This could result in underestimation of gene knockdown. However, if uptake of solutions can be traced, only insects that have taken up 'feed' containing dsRNA will be identified and used for accurate analysis of gene silencing. For example, fluorescent-labelled Cy-3 has been used to investigate uptake of dsRNA in Hemipterans such as the glassy-winged sharpshooter, the grain aphid and the potato/tomato psyllid. However, Cy-3 labelling is expensive when replicated experiments for testing many different target dsRNA are involved. The use of inexpensive vital dyes to trace uptake of dsRNA may allow effective assessment of aphids in such experiments.

The overall aim of the current study were to investigate the effects of *in vitro* RNAi in GPA through oral delivery of dsRNA. The objectives involved: identification of dyes that were non-toxic to GPA, did not affect the stability of dsRNA, and could be easily seen inside the aphid body as an indicator to uptake of dsRNA solutions. For this, suitability of 11 inexpensive dyes were studied. Effectiveness of this system was assessed by studying the effects of silencing a proton-translocation gene. To do this, GPA nymphs were fed with the target dsRNA for 24 hrs *in vitro*, after which phenotypic effects and percentage of active aphids with dyes were assessed as well as presence of dyes in the aphid body. Transcript abundance was measured through semi-quantitative PCRs after 24 hrs of feeding on dsRNA. To assess long-term effects of feeding on survival and fecundity of GPA, aphids fed on dsRNA with visible dye and those fed without dye were transferred onto tobacco and monitored daily for 12 days.

Of the 11 dyes tested, optimal concentrations of two were effective in tracing uptake of feed/dsRNA and allowed the efficient assessment of RNAi in GPA. The vital dyes neutral red (NR) and acridine orange (AO) were easily seen in the salivary glands and alimentary tract of aphids at concentrations of 0.02% and 0.0025% respectively. Based on spectrophotometric analysis and

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agarose gel electrophoresis, the quality and stability of dsRNA was not affected by NR or AO. Our results also indicated that 2 µg/uL of dsRNA of the target gene was effective in inducing RNAi in GPA. After feeding for 24 hrs, there was 22% reduction in survival of aphids fed on dsRNA with NR and 30% reduction in those fed on dsRNA without NR as compared to controls. In both cases, there was a significant reduction in transcript levels as compared to ds*GFP* and no-dsRNA controls (p<0.05) and expression of the target gene was not affected in aphids fed on dsGFP. Interestingly, transcript abundance of the target gene in the aphids fed on dsRNA with dye was much lower than in those fed without dye, indicating a more pronounced gene knockdown in the former. This demonstrates that assessing gene expression in only those aphids that have fed provides a better measure of transcript abundance than in aphids pooled together. There were also long-term effects on aphid survival; the treated aphids remained alive on tobacco for only five to six days as compared to controls indicating silencing this gene disrupted the normal life-cycle.

To our knowledge, this is the first study that makes use of vital dyes to trace the uptake of dsRNA through *in vitro* RNAi in GPA. This study suggests that the use of vital dyes as internal markers in feeding-based RNAi has great potential and allows assessment of only those individuals that have taken-up the feed. Both NR and AO are easy to use and inexpensive compared to the fluorescently labelled Cy-3 dye and will provide an economical option in RNAi studies. The optimised concentrations of NR and dsRNA are being employed successfully in RNAi of GPA genes *in vitro.*