Chapter 8

RNAi Feeding Bioassay: A Protocol for dsRNA Screening Against Asian Citrus Psyllid and Related Hemipteran Insects

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Abstract

RNA interference (RNAi) comprises a natural mechanism of gene regulation and antiviral defense system in eukaryotic cells, and results in sequence-specific degradation of RNAs. Recent scientific studies demonstrate the feasibility of use RNAi-based strategies to control pest and pathogens in plants. A key step in developing RNAi-based products is a reliable method to appropriated screening of selected dsRNAs.

Herein presented are a bioassay for screening dsRNAs to control the Asian citrus psyllid (ACP), *Diaphorina citri*, vector of citrus Huanglongbing (HLB) and other hemipterans. The RNAi feeding bioassay, called in plant system (iPS), uses vegetative new growth citrus flush to deliver double-strand RNA (dsRNA) to ACP during natural feeding.

Key words RNA interference, Gene suppression, RNA silencing, Huanglongbing, Pest control

1 Introduction

Hemipteran pests in citrus (psyllids, leafhoppers, aphids, and whitefly) are known to transmit pathogens. The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Psyllidae), is the vector of the bacteria associated with citrus Huanglongbing (HLB) [1]. HLB is the major threat for the survival of citrus industry in all affected countries, especially the Brazilian and American ones. Since the first detection of HLB in Brazil, more than 55 million trees were eradicated in affected regions [2]. Economic studies from the University of Florida's Institute of Food and Agricultural Sciences, have shown that since 2006, HLB has caused losses to Florida growers estimated in $1.36 billion in revenues and 2125 permanent jobs were lost [3].

As traditional chemical control of ACP populations has led to massive and frequent use of insecticide, new strategies for psyllid

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management has been pursued as a cost effective, long-term solution for psyllid control. A promising genetic control strategy that are being tested today is based on mechanism called RNA interference (RNAi), a natural gene regulation and antiviral defense system of eukaryotic cells. RNAi is a mechanism that functions as a "gene silencer", by targeting specific RNA sequences for degradation, and in some situations, for translation inhibition, resulting in a reduction or complete elimination of the expression of a target RNA [4].

RNAi technologies open the possibility to design highly specific dsRNAs to be used as tailor-made pesticide [5]. Increased efficiency in developing highly specific products for pest targeting reduces negative impacts on crop ecosystems, potentially increasing pollination, and biological control efficacy [6, 7]. RNAi has been showing potential to reduce psyllid and other insect vector populations in citrus and other crops [8–10].

A key step in development of RNAi-based control strategies of a particular insect is the identification of suitable target genes, which can be significantly affected by RNAi (i.e., with high mortality). Another critical step to achieve an effective RNAi response is the development of a suitable and reliable method of dsRNA delivery [11, 12]. Also, the development of an appropriate delivery method for a RNAi-based product should take in consideration the cropping system, the pest, and/or the pathogen that is targeted [13]. Although genetically modified plants will be the main delivery approach for some crops, especially commodities, for the majority of the remaining crops, topically applied RNAi could be an interesting alternative. In this scenario, a bioassay should be designed to allow screening and validation of dsRNA triggers for functionality, specificity, and stability, toward the specific RNAi target of interest. For this purpose, bioassays should allow the evaluation of potential dsRNA as close as possible to how it should work in the field (regarding application and ingestion by the pest).

The development of an RNAi control strategy against ACP relies on effective delivery of dsRNA specifically through the vascular tissues. Here we describe an effective and simple bioassay which enables the screening of dsRNA against ACP and other hemipterans in a manner that closely mimics the natural feeding process on host plant. This bioassay enables screening dsRNA molecules targeting ACP and other hemipterans which may feed upon the same host plant, to evaluate species specificity and off-target effects.

2 Materials

1. Plant material. For this protocol we used the citrus cultivar 'Carrizo' citrange (*Citrus sinensis* X *Poncirus trifoliata*, Rutaceae), but any citrus specie can be used. The plants were maintained in 1.2 l containers, in a greenhouse under natural light

and temperature. The plants were constantly pruned to promote growth of new foliar shoots, referred to as "flush"—flush is the preferred site for ACP to feed and oviposit.

2. Asian Citrus Psyllid (ACP). The ACP colony was reared on *Citrus macrophylla* in a greenhouse at 22 °C and natural light. Adult psyllids of ca. 5 days post eclosion were used for the bioassays.
3. Garden scissors.
4. 0.2% bleach solution (v/v).
5. Razor blade.
6. Microtubes, 1.5 ml.
7. Water, with the pH adjusted to 5.0–5.50.
8. dsRNA solution. DsRNA could be synthesized using any commercially available kit. DsRNA solution could be adjusted to 100 ng/μl to facilitates dosage adjustments.
9. Parafilm.
10. Syringe with a 0.46 mm needle.
11. Cage made with two magenta boxes, with the upper box having screen on the sides.
12. Growth chamber, set at 22 °C and L16:D8 h photoperiod.

3 Methods

1. Collect citrus flushes of about 10–15 cm long from potted plants using a garden scissor, keeping then on a container with water (*see* **Note 1**).
2. Wash the flushes by immersion on a 0.2% (v/v) bleach solution for 10 min. Rinse with filtered water to remove any bleach residue.
3. Using a razor blade, remove the leaves, leaving 3–4 leaves (the youngest leaves).
4. Using a razor blade, cut the base of the stem at a 45° angle while submerged in filtered water, standardizing flushes to approximately 10 cm in length.
5. Transfer the flush to a 1.5 ml microtube containing 0.5 ml of water and wrap the tube top with Parafilm to avoid the insects to fall on the water and to reduce water evaporation (Fig. 1a) (*see* **Note 2**).
6. Set the tubes on a rack and place the flushes in a growth chamber for at around 4 h to certify there was no damage on the flush that compromise its capacity of absorb water.

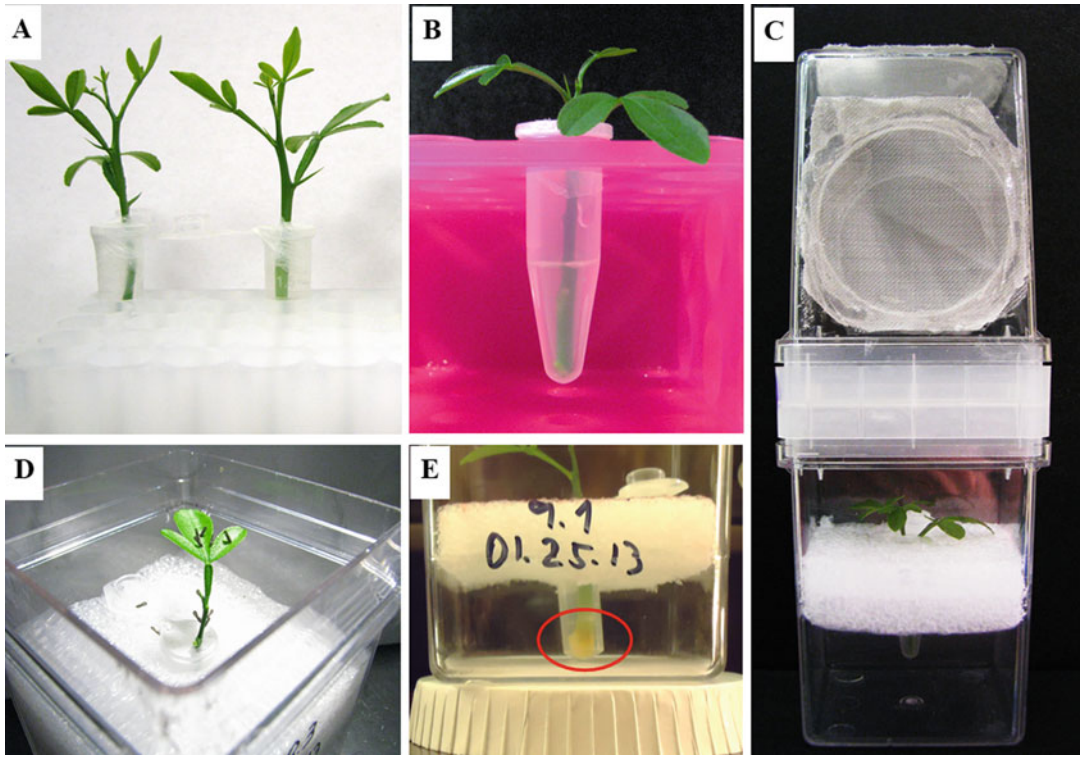

Fig. 1 RNA feeding bioassay. (**a**) Citrus flushes with approximately 10 cm long and 3–4 leaves is placed in a 1.5 ml microtube and the tube top wrapped with Parafilm. (**b**) A dsRNA dose is diluted in water and add to the microtube, and the flush is kept under light to absorb at least 90% of the dsRNA solution. (**c**) The treated flush is placed into a cage made with two magenta boxes with the upper box having screen on the sides. (**d**) Adult ACP were feed on the treated flush and mortality is monitored for 10–15 days. (**e**) Flush bottom showing rotting due to bacterial growth (red circle)

7. Using a syringe and a needle, remove the excess of water, leaving only the amount necessary to cover the flush base. Then add the dsRNA solution, the total amount of dsRNA should be determined for each gene (Fig. 1b) (*see* **Note 3**).
8. Place the flushes in a growth chamber, and after 90% of the dsRNA solution being absorbed, fill the tube with water (*see* **Notes 4** and **5**).
9. Transfer each flush to an individual cage, add 10–20 adult psyllids (or aphids, whiteflies), keeping the cages in a growth chamber (Fig. 1c, d) (*see* **Note 6**).
10. Monitor insect mortality for a 10–15-day period. Insects could be collected at determined intervals using mouth operated aspirator for mRNA quantification to evaluate the level of gene suppression (*see* **Note 7**).

4 Notes

1. Select tender flushes, those that are easily foldable with your fingertip. These flushes are appropriated for ACP or aphid feeding. For leafhoppers feeding it is necessary to use longer and larger flushes, with at least 15 cm tall and stem diameter of at least 0.5 cm.
2. Take care of the quality of the water to be used to avoid rotting of the flush base due to the presence of bacteria (Fig. 1e). An alternative is to adjust the water pH to 5.0–5.50 to reduce bacterial growth.
3. The dosage of dsRNA could be provided at one time or maintained as a constant supply of the dsRNA in a particular concentration for the entire curse of the experiment. Both options can be tested in comparative insect mortality trials (either for rate or overall mortality). In previous reports [10] a one-time dose was efficient to cause ACP mortality; however, a constant supply of dsRNA was not tested.
4. If is necessary to confirm dsRNA delivery to insect through the flush, use a reporter dsRNA, designed so that the sequence does not match with any known mRNA transcript in your insect (a green fluorescent protein, GFP, for example), so it can be detected on insect by regular RT-PCR. This dsRNA could also be used as a negative control treatment. Furthermore, it can be used to estimate dsRNA absorption and accumulation on insect's body by quantitative RT-PCR. When conducting a RT-qPCR of reporter dsRNA after insect feeding, it is important to sample a tissue other than the gut such as the hemolymph, fat body, or ovary. Careful collection of tissues which are not in direct contact with the gut provides evidence that the dsRNA was truly absorbed by the cells, avoiding detect the dsRNA present in the digestive tract. Another option is to let the insects feed on the source of the dsRNA (plant, diet, etc.) for a period of 24–48 h, then transfer them to an untreated food source. After feeding for 36 h, or more, the insect should excrete any food residue from the treated food source, which contains dsRNA. After this period, proceed with sample collection for dsRNA detection.
5. The translocation and accumulation of the dsRNA in the flush tissues can be track by labelling the dsRNA with fluorescence (Cy3 or other fluorescence) and visualizing plant tissue sections using a fluorescence microscope.
6. Collect in separate tubes each group of 10–20 psyllids that will be added in each cage. To prevent insects from escaping from the tube before being transferred to the cages, place the tubes with the insects at 4 ° C for 10–15 min to reduce insect movement.

7. Do not account for insect mortality in the first 48 h as they may have been caused by injuries caused by insects handling during colony collection and transfer to the cage.

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