Horticultural Practices and High-Throughput Sequencing Studies in Dwarfed Citrus using TsnRNAs

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Introduction

In the early 2000s, **TsnRNA-IIIb** found to significantly **reduce canopy volume** (up to 50%) **of navel oranges on trifoliate rootstock** planted at high density [1-4]. TsnRNA-IIIb treatment also found to increase yield per canopy volume and concentrate fruit in the optimal canopy area for harvest without affecting fruit quality. These observations led to the hypothesis that TsnRNA-IIIb treatment may offer an economic advantage to citrus growers. In 2017, almost 20 years after planting, TsnRNA-IIIb-treated trees in a research block in Exeter, CA, were visually observed to be still significantly smaller than the control trees (Fig. 1). A survey was initiated to **assess the potential savings offered by** the employment of the **TsnRNA technology for the main horticultural practices**.

In addition, to gain insight into the molecular mechanisms modulated by TsnRNA-IIIb, we performed microRNA and transcriptome analyses in dwarfed citrus trees treated with TsnRNA-IIIb and compared them to the non-treated controls.

Fig 1: Navel on trifoliate; control (left) vs treated with TsnRNA-IIIb (right)

Method

UC ANR Lindcove REC Field Trials

- **Parent Navel** (*Citrus sinensis* L. Osbeck) **on** ***Poncirus trifoliata*** **L. Raf. trees with TsnRNA-IIIb** (n=29) **and without** (control, n=8), 20-years old, TsnRNA-IIIb-trees planted at 10 x 22 ft, control trees spaced at 20 x 22 ft[1].
- **Navel on Flying Dragon** (n=16), 34-years old, spaced at 20 x 22 ft.

Field Data Collection and Analysis

- **Canopy volume** was calculated using the formula CV= $2/3p\ ab^2$, where $a = H$ and b = Sp/2 [2]. **Yield data** was obtained from LREC's packline Compac® software programs Sizer® and InVision®. **Horticultural operations** were timed for statistical analysis.
- **For statistical analysis**, data were analyzed using SigmaPlot14.0 (SPSS Science, Chicago, IL).

Molecular Studies

- Stem and root samples collected from Parent Navel with TsnRNA-IIIb (n=3) and without (control, n=3), and from Navel on Flying Dragon (n=2). Total RNA isolated with the Invitrogen™ TRIzol™ (Thermo Fisher Scientific, Waltham, MA, United States) reagent.
- For **Small RNA Profiling**, sRNA libraries were prepared with the Illumina TruSeq Small RNA Kit, and sequenced using an Illumina HiSeq™ 2500 instrument with single-end 50 bp reads.
- For **Transcriptome Profiling**, cDNA libraries were prepared with the Illumina TruSeq Stranded mRNA Kit, and sequenced with an Illumina HiSeq™ 4000 instrument with paired-end 100 bp reads (SeqMatic, Fremont, CA, United States).
- Clean reads were filtered using various online sequencing analysis tools [5-6, reference therein] and the OmicsBox software suite v. 1.4.11 (Cambridge, MA, United States).

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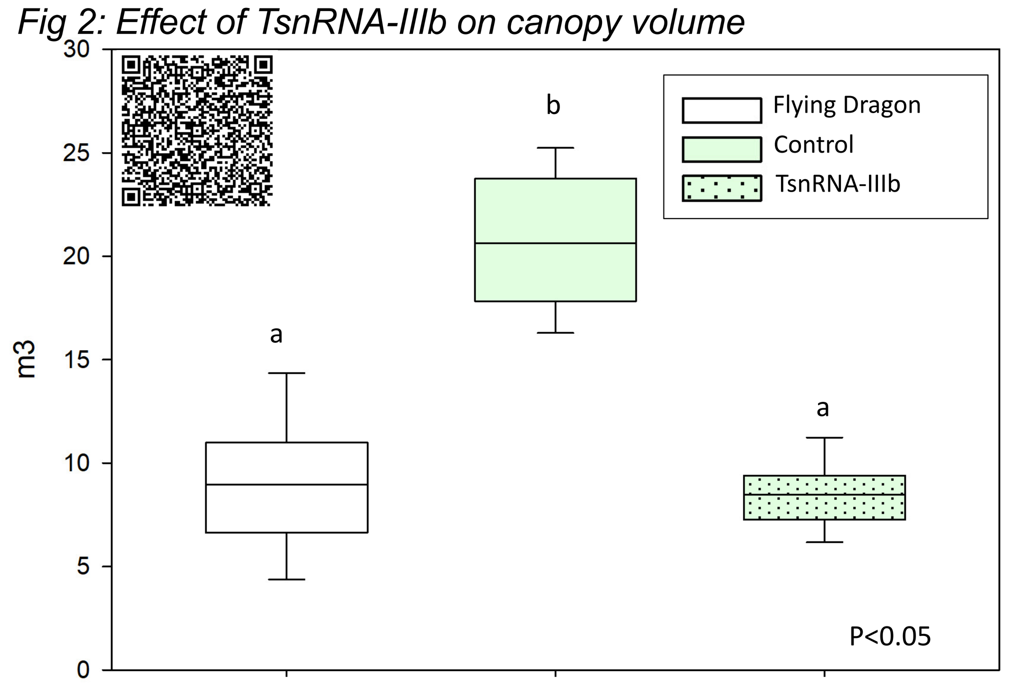
Results

Horticultural Assessments

To determine whether using TsnRNA-IIIb-dwarfed trees can offer citrus growers potential advantages in the standard horticultural practices, we analyzed the time efficiency of harvesting; and conducting pest inspections in TsnRNA-IIIb dwarfed navel trees compared to the non-treated controls. First, we verified the statistically significant canopy volume reduction in TsnRNA-IIIb-treated trees persisted over time in the field (Fig.2).

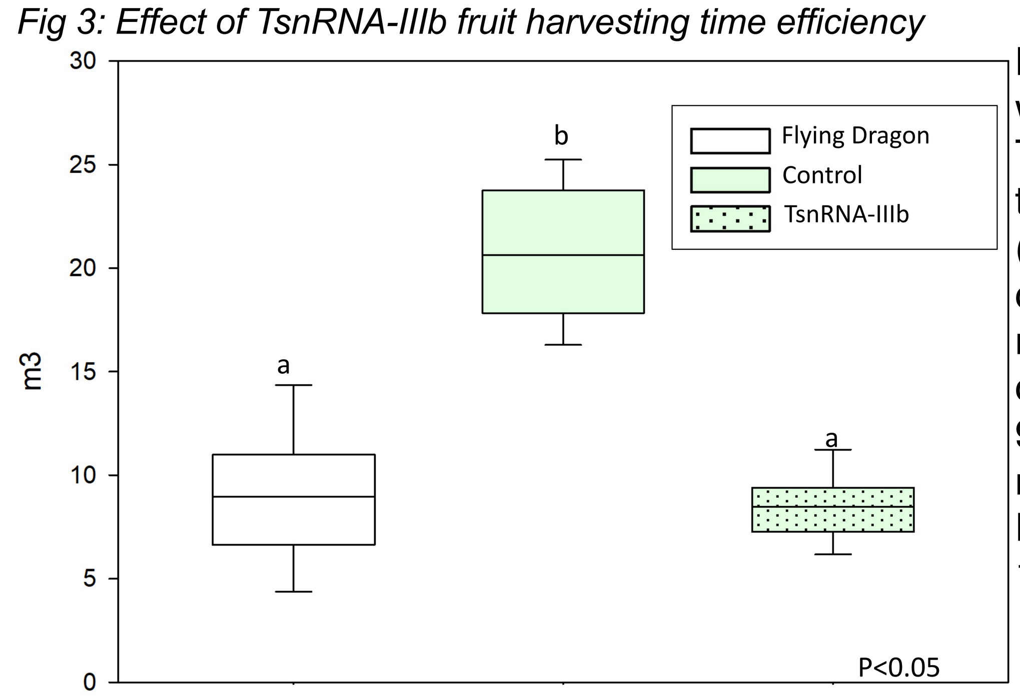
The **canopy volume** of TsnRNA-IIIb-treated trees (mean= 8.48m^3) was significantly reduced compared to the non-treated controls (mean= 20.47m^3) and Flying Dragon navel trees (mean= 9.08m^3) (Fig. 2).

Fig 2: Effect of TsnRNA-IIIb on canopy volume



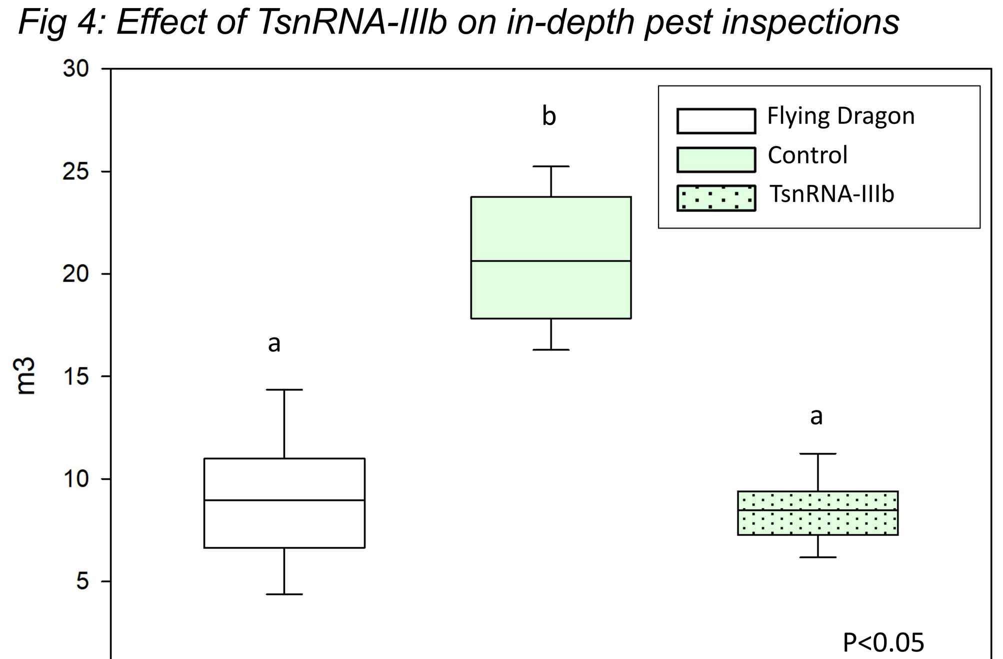
Next, we timed fruit harvesting operations for three consecutive years (2020-2022) for each tree included in this study. Recorded times were normalized to 100lb of fruit weight for comparisons among different trees (Fig.3).

Fig 3: Effect of TsnRNA-IIIb fruit harvesting time efficiency



Fruit harvesting was faster in TsnRNA-IIIb-treated trees (mean= 8.4min) compared to the non-treated controls (mean= 9.8min) and navels on Flying Dragon (mean= 12.2min) (Fig.3).

Fig 4: Effect of TsnRNA-IIIb on in-depth pest inspections

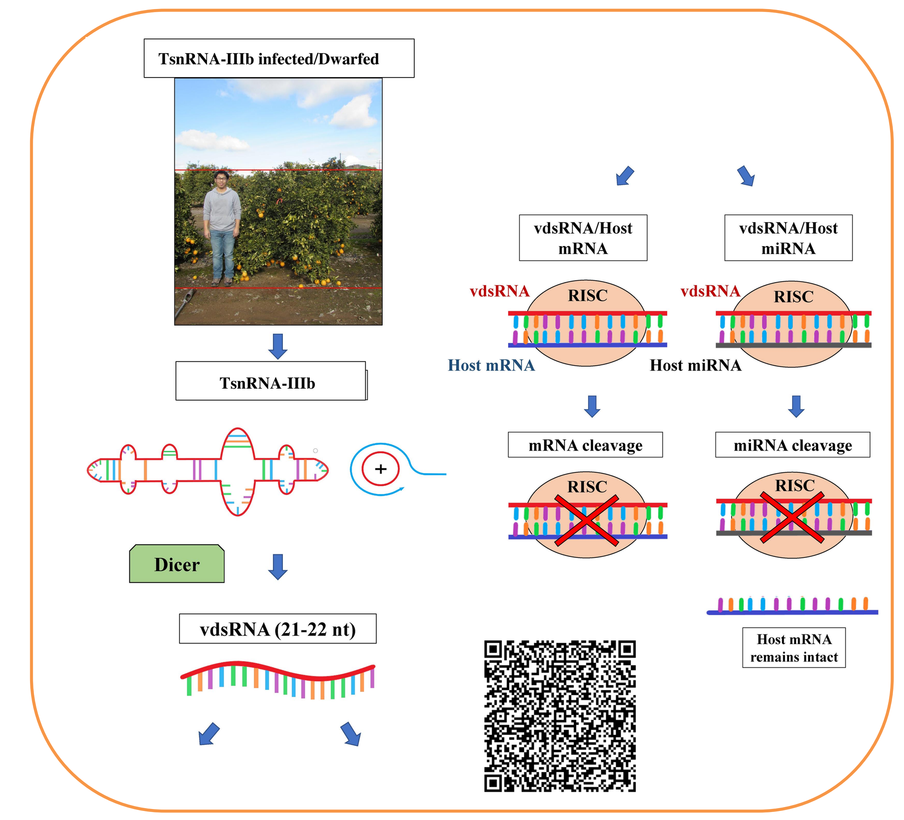


In-depth Pest inspections, where all new flushes are inspected, were conducted on a subset of tree for each group (n=4) in Spring 2022. Here, a significant difference was found while performing the inspections on TsnRNA-IIIb-treated trees (mean=15.5min) in comparison with the controls (mean=19.5min) but not with the navels on Flying Dragon (mean=16min) (Fig.4).

Cont. Results

Molecular Studies

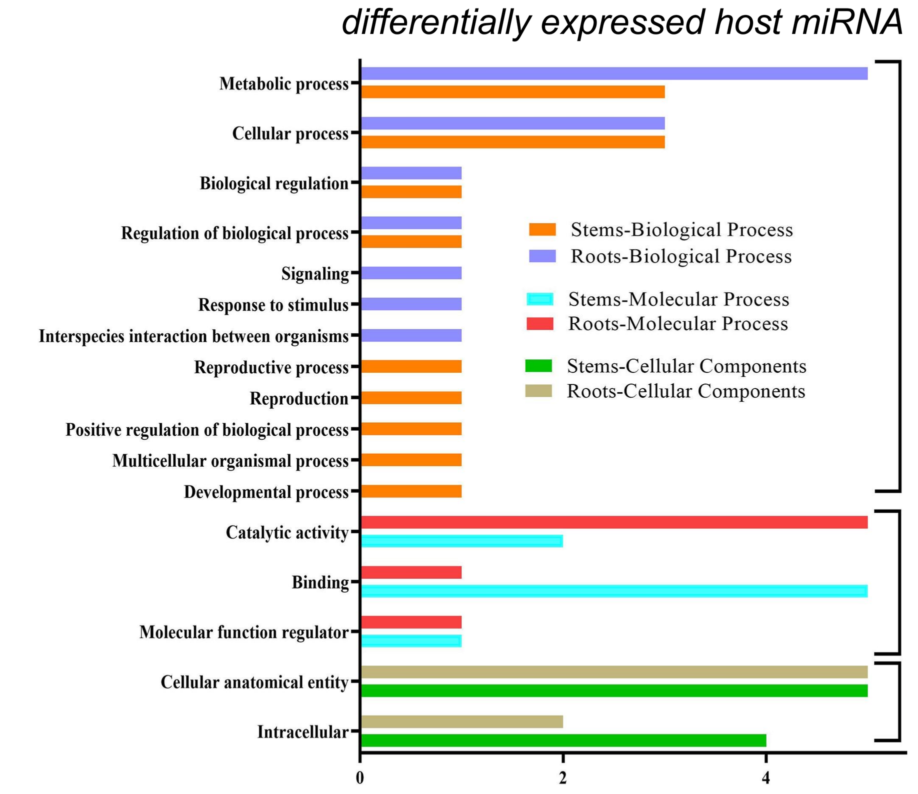
Fig 5: Model of TsnRNA-IIIb effect on host mRNA and miRNA



To investigate how TsnRNA-IIIb affects canopy volume at the molecular level, we hypothesized TsnRNA-IIIb could affect the expression of the host transcriptome via miRNAs either directly or by silencing the host miRNAs (Fig. 5).

Our small RNA profiling study revealed that TsnRNA-IIIb appears to regulate the expression of the host transcriptome by interfering with the expression of the host miRNAs (Fig. 6).

Fig 6: Functional classification of predicted host targets of differentially expressed host miRNA

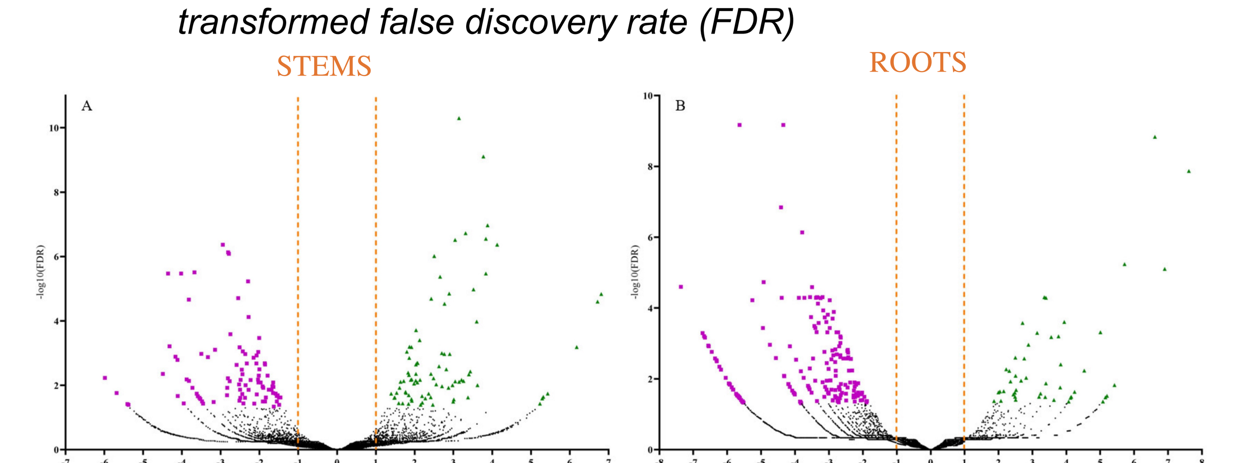


TsnRNA-IIIb affects a wide range of biological functions

Gene Ontology (GO) annotation of the clusters of orthologous groups (COG) functional classification of the predicted miRNA targets **differentially expressed in response to TsnRNA-IIIb** revealed TsnRNA-IIIb ultimately affects numerous and diverse biological functions.

In another approach to investigate the TsnRNA—IIb responsive host genes that are responsible for the dwarfing phenotype, we performed a transcriptome study of differentially expressed genes (DEGs) (Fig. 7) and identified several DEGs in both the stems and the roots.

Fig 7: Volcano plots; fold change vs neg log 10-transformed false discovery rate (FDR)

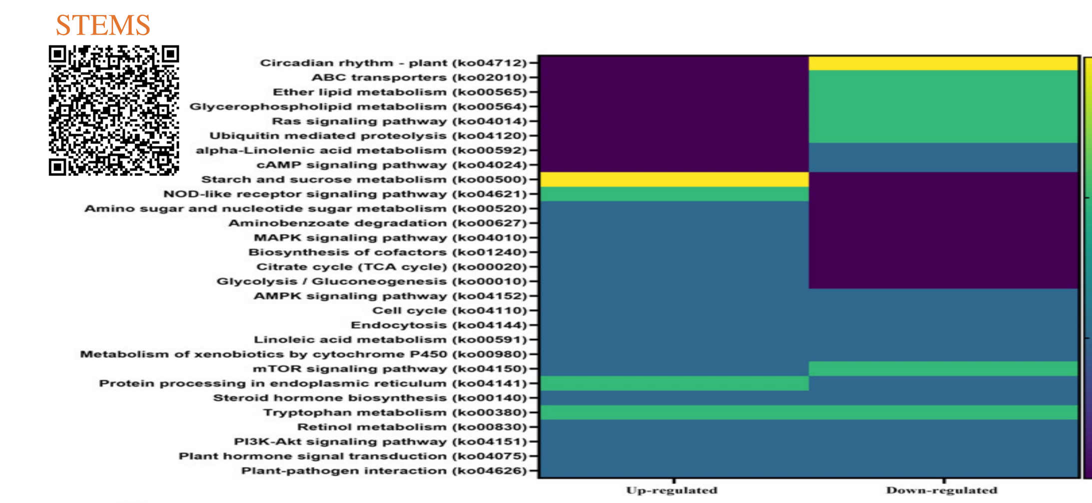


▲ Positive FC= Upregulated DEGs
■ Negative FC = Downregulated DEGs

Cont. Results

Kyoto Encyclopedia of Genes and Genomes analysis (KEGG) analysis on DEGs showed that TsnRNA-IIIb responsive DEGs belong to different biological functions (Fig. 8).

Fig 8: Kyoto Encyclopedia of Genes and Genomes analysis



Conclusions

The citrus industry has been constantly moving towards planting orchards with an increased number of trees per acre. Dwarf trees are key to the successful development of high-density plantings, which will be critical to meet the challenges posed by water shortages, diseases (e.g. HLB), farmland reduction, and increasing **labor costs**.

In this study;

1. We confirmed that dwarfed **TsnRNA-IIIb-treated navel on trifoliate trees** displayed a long-term **persistent and significant canopy volume reduction in the field**.
2. We found that **fruit harvesting**, when normalized for 100lb of fruit, required less time compared to the controls, which may be related to the distribution of fruit in the canopy in TsnRNA-IIIb-treated trees [2].
3. for **in-depth pest inspections**, where all new flushes are inspected, the duration of the inspection of the dwarfed trees was **reduced compared to the full-sized controls**
4. At the molecular level, our data indicate that TsnRNA-IIIb-induced dwarfing phenotype results from the reprogramming of numerous and diverse biological pathways.

The importance of elucidating the molecular mechanism lies in the potential to develop commercial applications that do not require a transmissible agent. The data presented here are very encouraging and provide evidence-based information to discuss the possible advantages for horticultural practices offered by TsnRNA-IIIb.

References and Acknowledgments

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