



## Silencing Genes in *Diaphorina citri* to Combat Huanglongbing Disease in Citrus

Dr. Ellen Jorgensen, PhD<sup>1</sup>, Tarjani Agrawal<sup>2</sup>, Alex Cho<sup>3</sup>, Yujin Cho<sup>4</sup>, Junho Huh<sup>5</sup>, Wonjoon Jeong<sup>6</sup>, Wonsook Jeong<sup>7</sup>, Jeehwan Kim<sup>8</sup>, Minuk Kim<sup>9</sup>, Timothy Kim<sup>10</sup>, Caroline Lee<sup>11</sup>, Lucas Lee<sup>12</sup>, Yongho Lee<sup>13</sup>, Jeany Park<sup>14</sup>, David Shon<sup>15</sup>, Erica Sugimura<sup>16</sup>, Hedy Tung<sup>17</sup>, Suyoung Yun<sup>18</sup>

<sup>1</sup>Ellen Jorgensen PhD, Biotech Without Borders, New York NY

<sup>2</sup>Tarjani Agrawal, PhD Candidate, New York University, New York NY

<sup>3</sup>Alex Cho, Stuyvesant High School, New York NY

<sup>4</sup>Yujin Cho, Korea International School, Jeju, South Korea

<sup>5</sup>Junho Huh – Seoul International School, Seoul South Korea

<sup>6</sup>Wonjoon Jeong, Fulton Science Academy, Alpharetta, GA

<sup>7</sup>Wonsook Jeong– Fulton Science Academy, Alpharetta GA

<sup>8</sup>Jeehwan Kim- Choate Rosemary Hall, Wallingford, CT

<sup>9</sup>Minuk Kim– Stuyvesant High School, New York NY

<sup>10</sup>Timothy Kim – Greenwich High School, Greenwich CT

<sup>11</sup>Caroline Lee – Syosset High School, NY

<sup>12</sup>Lucas Lee, St. Mary's High School, Manhasset

<sup>13</sup>Yongho Lee, Stuyvesant High School, New York NY

<sup>14</sup>Jeany Park – Kent School, Kent CT.

<sup>15</sup>David Shon – Portsmouth Abbey School, Portsmouth, RI

<sup>16</sup>Erica Sugimura – Stuyvesant High School, New York NY

<sup>17</sup>Hedy Tung – Stuyvesant High School, New York NY

<sup>18</sup>Suyoung Yun– Shattuck St. Mary's School, Faribault MN

**Abstract:** Huanglongbing is a disease that infects and damages citrus trees, causing them to produce green, inedible fruit. It originated in China and spread to the US through international trade. The disease is caused by the bacteria *Candidatus Liberibacter Asiaticus* (otherwise known as CLAs) and spread through an insect vector, the citrus psyllid. Though insects began showing up in the late 90's and early 2000's, they were not viewed as a serious threat until 2006 when the spread of HLB rapidly accelerated. The bacteria cause a localized infection rather than a systemic one, which makes the infection even harder to fight. The organisms slowly spread throughout the entire tree. By the time the disease is caught, there is nothing that can be done except to chop the tree down. The disease has destroyed over 60% of the citrus groves in Florida and has now begun to spread to California. This poses a serious economic threat: without the citrus industry, the US would lose billions of dollars each year. Using pesticides and antibiotics is not a good long-term solution, as they are not environmentally friendly and can ultimately lead to problems, such as antibiotic resistant bacteria. Furthermore, the antibiotics kill not only the bad bacteria, but also the good ones that are critical for the trees' survival. The solution that we are proposing is more effective, as it is very narrow and selective, allowing us to directly target the root of the problem. We are developing a DNA-based device that can produce double-stranded siRNA molecules capable of silencing essential genes in the psyllid organism, thereby meaningfully reducing their numbers in the citrus plants' environment.

[Ellen Jorgensen, Tarjani Agrawal, Alex Cho, Yujin Cho, Junho Huh, Wonjoon Jeong, Wonsook Jeong, Jeehwan Kim, Minuk Kim, Timothy Kim, Caroline Lee, Lucas Lee, Yongho Lee, Jeany Park, David Shon, Erica Sugimura, Hedy Tung, Suyoung Yun. **Silencing Genes in *Diaphorina citri* to Combat Huanglongbing Disease in Citrus.** *N Y Sci J* 2020;13(3):80-85]. ISSN 1554-0200 (print); ISSN 2375-723X (online). <http://www.sciencepub.net/newyork>. 8. doi: [10.7537/marsnys130320.08](https://doi.org/10.7537/marsnys130320.08).

**Keywords:** Silencing; Genes; *Diaphorina citri*; Combat; Huanglongbing; Disease; Citrus

### Introduction

Huanglongbing, or citrus greening, is a disease that is devastating the citrus industry worldwide. Presumably caused by a bacterial agent *Candidatus*

*Liberibacter*, the disease affects the tree health as well as fruit quality and yield. Fruits affected by the disease are small, asymmetrical, greener, and have higher acidity and lower sugar levels (Fan et al., 2010;

Liao and Burns, 2012; Chin et al., 2014; Zheng et al., 2018) than the healthy fruits (Dala-Paula et al., 2019). The disease affects practically all citrus species and cultivars.

Although the origin of the disease is difficult to determine, the first case of Huanglongbing was reported in India in the 18<sup>th</sup> century (Capoor, 1963; da Graca, 2008). Reports of HLB started appearing in China in 1919 (Reinking, 1919), Africa in 1937 (Van der Merwe and Andersen, 1937) and Philippines in 1960 (Fraser et al., 1966; McClean and Schwarz, 1970). From a small region, the disease has now spread worldwide through international trade and is affecting citrus trees in more than 50 countries throughout Africa, Asia, Oceania and the Americas (CABI, 2017; EPPO, 2017). The first case of *Diaphorina citri* in America was reported in Florida in 1998. Today, the insect has spread throughout the state. A major producer of oranges, Florida has suffered a great loss in orange production. It was reported that HLB infection reduced the production of oranges in the United States by 72.2% between the year 2008 and 2018 (USDA-NASS, 2018).

It is scientifically established that the causative agent for HLB disease is gram-negative bacterium *Candidatus Liberibacter*. Depending upon the area of origin, the bacterial species is divided into Asian (CLas), American (CLam) and African (CLaf) species. Previous studies have shown that the CLas and CLam species are transmitted from one plant to another by an insect vector, *Diaphorina citri*, commonly known as Asian citrus psyllid. The psyllids are attracted to the growing ends of the citrus branches and acquire the bacterium during the process of feeding. When psyllid moves to a different plant for feeding, it transmits the bacterium and infects the healthy plant. The infection is initially localized to a certain part of the phloem but slowly spreads throughout the entire tree, blocking the normal flow of nutrients and killing the tree in the process.

Once the bacterium is acquired, the insect will retain and transmit the bacterium throughout the psyllid's life. Therefore, it is important to control the insect population to keep HLB from spreading further. In the regions where the rate of infestation by the insect is low, the most effective strategy to control the disease is removal of symptomatic trees, protecting grove edges through intensive monitoring, foliar nutritional treatments and biological control of the vector ACP. Currently, pesticides and antibiotics are the best methods to control the insect population, but as they are harmful to the environment and can cause the development of antibiotic resistance in the bacteria. Also, antibiotics can harm bacteria that are beneficial to the tree, thereby impeding its growth.

With advancements in gene editing technology, it is possible to control insect populations without any side effects. Emerging technologies, such as RNA interference, could provide a new sustainable and environmentally friendly strategies for the management of psyllid populations. Here we present a strategy to control the psyllid population by silencing genes important for ROS quenching and exoskeleton production. Both these genes, superoxide dismutase (SOD) and chitin synthase, are critical for the survival of psyllid. Absence of either of these genes would subject the insect to cell damage by excess reactive oxygen or would expose the insect body system to harmful factors in the environment.

## Materials and Methods

### 1. siRNA production

siRNA were designed from the DNA sequence of chitin synthase and superoxide dismutase gene and were commercially synthesized as separate pieces of single stranded DNA (ssDNA). The length of each of the single strands is 50bp. The single strands were joined together by simultaneous heating and cooling to form a duplex structure. The design of the siRNA is such that it leaves 4 bases 'GATC' on the 3' side, following annealing with the complementary strand so that it can ligate with the plasmid. siRNAs were designed using the free program, Block It from Invitrogen.

### 2. Modification of previous cassette

In 2017, TecCEM, one of the participating teams from Mexico, developed a universal cassette (BBa\_k2246000) that was used for the production of siRNA *in vivo*. The universal cassette is made up of two important genetic elements. The first element is the sequence for AmilCP gene that synthesizes a blue/purple chromoprotein. The second element is the DNA space where the siRNA sequence can be cloned. The original cassette contained a siRNA sequence for the AWD psyllid gene. We modified the cassette for our purposes by removing the siRNA sequence from the empty cassette and inserting a spacer DNA sequence cloning of other siRNA sequences that we plan to use for our experiment. The original universal cassette was ordered as a G block fragment.

### 3. Cloning of empty cassette in pSB1C3 vector

The universal cassette with the placeholder or spacer DNA sequence was ligated to a backbone containing chloramphenicol resistance gene for easy selection in the cloning process. Both the universal cassette and backbone were digested with EcoR1 and Pst1. Following this, they were ligated using DNA ligase enzyme.

### 4. Transformation of pSB1C3 plasmid with the universal cassette

Bacterial transformation was carried out following the cloning of the universal cassette in the pSB1C3 vector. The vector was transformed in a special strain of *E. coli*, HT115 (DE3) that is devoid of RNase enzyme.

### 5. Characterization of BBa\_K608008 plasmid

BBa\_K608008 is a plasmid with a GFP and chloramphenicol resistance gene. This plasmid was obtained from the iGEM distribution kit for the purpose of a characterization experiment. Upstream to the GFP sequence is a strong promoter and a medium ribosome-binding site that allows for strong GFP expression. Three other plasmid constructs with different RBS and promoter strengths were also obtained from the iGEM distribution kit. BBa\_K608010 is a medium promoter/strong RBS plasmid, BBa\_K608011 is a medium promoter/medium RBS plasmid, and BBa\_K608012 is a medium promoter with weak RBS.

### 6. Fluorescence measurement

#### a. Measuring the concentration of bacterial cells

For measuring the concentration of cells, a standard curve was generated using the beads provided in the iGEM kit. Beads are used for this purpose because they absorb light at 600nm. Different solutions of beads were made with varying concentration and absorbance of each of these solutions, which were measured at 600nm using a spectrophotometer. Following this, absorbance of four bacterial cultures with different plasmid constructs was measured at 600nm and a standard curve was used to determine concentration of bacterial cells in each of the four cultures.

#### b. Measurement of fluorescence values

Each of the four cultures were subjected to absorbance measurements at 520nm, the wavelength at which GFP emits light.

#### c. Normalization of GFP values

In order to determine fluorescence values/concentration of cells, values of absorbance at 520nm for each of the cultures were divided by their respective concentration values at 600nm.

### Safety practices

All of our experiments followed strict safety protocol. We avoided working with the Clas infected materials in the lab to avoid any spread of the pathogen. We designed our genetic material (siRNA) in such a manner that it wouldn't affect any other animal species besides psyllids, if accidentally ingested. Since we are not experienced in working with psyllids, we decided to collaborate with Dr. Michelle Heck, a molecular biologist at USDA Agricultural Research Service in Ithaca, for all experiments that required us to handle the insect. We secured our environment by developing traps for any escaping psyllids that fed on engineered *E. coli*.

## Results

### A. Literature survey

We performed an extensive literature survey with the help of Dr. Georgios Vidalakis at UCR to identify the target genes against which siRNA can be developed. We identified two targets, superoxide dismutase and chitin synthase, both of which are essential for the survival of the insect. Superoxide dismutase or SOD, is a peroxisomal enzyme that is required for regulating the levels of harmful reactive oxygen species in the cells. Absence of the same can cause toxicity in the cell by oxidizing the membranes of the cell. This enzyme was shown to be important for psyllid survival as silencing the gene induced mortality in the insects (Taning et al., 2016). Chitin synthase, on the other hand, is important for defining the structure of the insect. It is a critical enzyme that catalyses the conversion of N-acetylglucosamine to chitin. Recently, this gene was identified and characterized in the psyllids (Lu et al., 2019) and it was found to be important for survival of the insect (Figure 1).

### Figure 1: siRNA sequence for SOD and chitin synthase gene

### B. Cloning of universal cassette in pSB1C3 vector

The idea behind the experiment was to knockdown chitin synthase and superoxide dismutase gene in psyllids by overexpressing siRNA in the *E. coli* and feeding the bacteria to the psyllids. Previously, this method had been shown to work effectively for knocking down genes in other organisms like *C. elegans*; therefore, we continued developing this method. For this purpose, we ordered a DNA cassette that can transcribe siRNA. The DNA cassette has amiCP gene under a promoter sequence that synthesizes blue/purple chromoprotein and a spacer DNA where any siRNA sequence can be inserted. The blue chromoprotein is a reporter which, when synthesized, made us sure that the transcription machinery was working fine. It also gave us a confidence that the siRNA sequence, downstream of the blue protein is getting transcribed (Figure 2).

#### a. Cloning of G-block with pSB1C3 backbone

After we received the synthesized cassette from IDT, we tried to ligate our cassette using EcoR1 and Pst1 restriction sites into linearized pSB1C3 backbone that was digested with the same enzyme. Although, when we screened transformed colonies we got blue colored colonies indicative of positive ligation, we failed to detect the insert of the correct size by colony PCR. We got a much larger insert than the expected size.

#### b. Cloning of siRNA in the vector

We tried ligating siRNA for chitin synthase and superoxide dismutase into the G-block ligated to pSB1C3 backbone. We tried three different ways. In the first way, we digested the vector with BamHI and HincII enzymes and removed the spacer DNA. We then attempted to ligate our siRNA with the vector. We were able to cut our clone with BamHI restriction enzyme, which shouldn't have been possible in the case of a positive clone, as insert would have destroyed the BamHI restriction site. In the second method, which was adopted from golden gate assembly, we kept the restriction enzymes active in the ligation solution, a strategy we thought would prevent the self-ligation of the vector and would promote ligation of insert with the vector. However, this strategy also failed. In the third method, we started ligation from scratch. We used two different backbones for ligation of our G-blocks. One was from the J04450-pSB1A3 plasmid, which had the ampicillin resistance gene, and the other backbone was the original that we started with, which had chloramphenicol resistance gene. Neither of these vectors were successfully ligated to the G-block.

### Figure 2: Modified Universal Cassette.

The universal cassette contains the amilCP gene that synthesizes blue chromoprotein, a short spacer DNA where siRNA can be cloned and the K2246001 terminator sequence. Upstream and downstream of short spacer DNA is the BamHI and HincII restriction enzyme site.

### C. Bronze Requirement Experiment

One of the requirements for the Bronze medal is to characterize an existing part in the registry of standard biological parts. We chose to compare the strengths of three different ribosome-binding sites with each other, and to an existing part, which has a strong promoter by determining the expression of GFP protein. For this purpose, we chose an existing part, BBa\_K608008 ([http://parts.igem.org/Part:BBa\\_K608008](http://parts.igem.org/Part:BBa_K608008)), which contains the GFP gene under a strong promoter and medium ribosome-binding site. We compared BBa\_K608008 with three other plasmids, which have different strengths of ribosome binding sites in combination with a medium strength promoter. These three plasmids either have strong RBS, medium RBS or weak RBS. Following transformation of these plasmids in *E. coli*, we measured the absorbance of the cells to determine the concentration of cells in the culture. We simultaneously measured GFP fluorescence and normalized it to the concentration of cells. We observed that strong promoter-medium RBS combination has a similar strength as medium promoter-weak RBS combination (Figure 3). We also observed that medium promoter-medium RBS had the lowest expression of GFP whereas medium promoter-strong RBS had the highest GFP expression, suggesting that it is the strongest combination (Figure 3). However, these results were not consistent with the data generated by the Freiburg 2011 team and will need further validation.

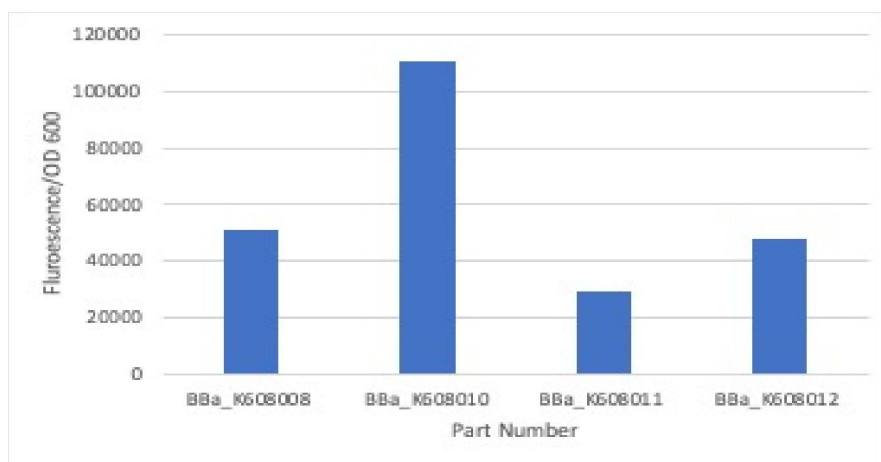


Figure 3/Table 1: Normalized fluorescence intensity values of various GFP constructs.

Plasmid constructs containing a promoter of medium strength and ribosome-binding site of varied strengths (weak, medium and strong) were tested for expression of GFP. GFP fluorescence values were normalized to the concentration of cells and plotted on the graph. BBa\_K608008 has strong promoter and

medium RBS, BBa\_K608010 has medium promoter and strong RBS, BBa\_K608011 has medium promoter and medium RBS and BBa\_K608012 has medium promoter and weak RBS.

The Y-axis shows fluorescence intensity normalized to cell concentration.



#### D. Outreach activities

After we learned about the urgency of HLB situation in the United States, we immediately started brainstorming how we may raise more awareness to combat the disease. We decided to turn to educational institutions for getting more young people involved, which will raise more awareness among their parents and other adults in their communities. We drafted a lesson plan for a lab protocol that allows students to learn about the detection, identification, and quantification of plant pathogens like HLB. By testing DNA samples from the psyllids, students can observe that the gene sequences match that of HLB-causing bacteria. To make our lessons match the current educational standards, we consulted high school teacher, Maegan Wallner. We incorporated her suggestions to make the lesson plan more teacher-friendly with notes and extra information for the instructor. We also designed a siRNA psyllid trap after consulting Dr. Vidalakis and citrus farmer Kris Sutton. The purpose of this trap was to stop psyllids that fed on *E. coli* expressing siRNA from escaping the lab setting.

#### Discussion

Huanglongbing is a citrus disease that has vastly affected American citrus production in the past and still continues to create havoc in various regions of the United States and around the world. The disease is caused by a bacterial species, *Candidatus Liberibacter*, which spreads from one tree to another by virtue of an insect vector, the psyllid. Previous attempts to control the vector using insecticides have shown some success but have also presented significant risk to the environment (Monzo and Stansly, 2017). In an attempt to reduce the bacterial burden on citrus population, we, the Biotech Without borders iGEM team, decided to develop a method that can reduce insect population without harming the environment. We decided to approach this problem from a molecular angle by using RNA interference to silence the genes essential for survival of the insect. In doing so, we attempted to prevent the insect population from growing as well as protect the environment from harmful chemicals.

As a first step in the project, we decided to develop tools that can be used by us or others in the future for synthesizing siRNA. We developed a universal DNA cassette by modifying an existing universal cassette, BBa\_K3277000, developed by the tecCEM team in 2017. The change that we made replaced the AWD siRNA sequence in the existing cassette with a spacer DNA, 18bp in length. This spacer DNA has no DNA element, it is just a sequence that acts as a placeholder for incoming DNA. In doing this, we killed two birds with one

stone. First of all, this tool removed the need to purify the plasmid every time we digested it. Since spacer DNA is a tiny DNA fragment of 18bp, it will pass through the column unnoticed. Secondly, it made our clone screening simpler. If we were to use the existing cassette, BBa\_K3277000, for our siRNA cloning, it would have been difficult to screen positive clones with our sequence and negative clones with the AWD sequence by restriction digestion, because AWD siRNA and our siRNA sequences are similar in length. By replacing the AWD siRNA sequence with the spacer DNA sequence, which is just 18bp in length, we reduced the screening time and required steps drastically.

After developing the first part of our tool, we began joining this cassette with the backbone that harbored a chloramphenicol resistance gene. This was done so that we can select positive clones generated in the process by growing *E. coli* on a medium supplemented with an antibiotic. Although, we observed positive blue colonies, none of them carried the insert of expected size. The reason for this failure could be incomplete digestion, self-ligation, imbalance in the ratio of insert and vector, inactivity of the ligase enzyme or improper reaction conditions. This result also affected our subsequent steps where we planned to ligate siRNA to the vector. Since our starter vector was not correct, we never visualized any positive ligation of siRNA with our vector.

As part of Bronze medal requirement and to determine the accuracy of the results of Freiburg 2011 team, we decided to determine the optimal strength of ribosome binding sites for the highest expression of a protein. We used an existing part from the iGEM distribution kit, which had a GFP gene downstream of a promoter and a ribosome-binding site and compared it to three other vectors with variable ribosome binding site strength. Although we observed that medium promoter and strong ribosome binding site made the best combination, this wasn't consistent with the data observed by Freiburg team in 2011. Some of the reasons, which we believe could have resulted in this inaccuracy, are inaccurate measurements of cell concentration, faulty fluorescence values, cell death, and or experimental errors.

Although, we didn't succeed in getting the results in time for the end of the competition, we hope to carry this project forward and develop a device that can control psyllid populations and help the farmers protect their citrus production.

#### Collaborators

Part of this work was done in collaboration with researchers at Florida State University, who helped us in obtaining psyllid specimens, and the NYU New

York 2019 iGEM team who helped us access a fluorometer for measuring GFP fluorescence.

#### References:

1. Fan, J., Chen, C., Blansky, R. H., Gmitter, F. G., and Li, Z.-G. (2010). Changes in carbohydrate metabolism in *Citrus sinensis* infected with 'Candidatus Liberibacter asiaticus'. *Plant Pathol.* 59, 1037–1043. doi: 10.1111/j.1365-3059.2010.02328.x.
2. Liao, H. K., and Burns, J. (2012). Gene expression in *Citrus sinensis* fruit tissues harvested from huanglongbing-infected trees: comparison with girdled fruit. *J. Exp. Bot.* 63, 3307–3319. doi: 10.1093/jxb/ers070.
3. Chin, E. L., Mishchuk, D. O., Breska, A. P., and Slupsky, C. M. (2014). Metabolite signature of Candidatus Liberibacter asiaticus infection in two citrus varieties. *J. Agric. Food Chem.* 62, 6585–6591. doi: 10.1021/jf5017434.
4. Zheng, Y. M., Kumar, N., Gonzalez, P., and Etxeberria, E. (2018). Strigolactones restore vegetative and reproductive developments in Huanglongbing (HLB) affected, greenhouse-grown citrus trees by modulating carbohydrate distribution. *Sci. Hortic.* 237, 89–95. doi: 10.1016/j.scienta.2018.04.017.
5. Dala-Paula, B. M., Plotto, A., Bai, J., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., & Gloria, M. B. A. (2019). Effect of Huanglongbing or greening disease on orange juice quality, a review. *Frontiers in plant science*, 9, 1976.
6. Capoor, S. P. (1963). Decline of citrus trees in India. *Bull. Natl. Institute Sci. India* 24, 48–64.
7. da Graça, J. V. (2008). "Biology, history and world status of Huanglongbing," in *I Taller Internacional sobre Huanglongbing de los cítricos (Candidatus Liberibacter spp.) y el psílido asiático de los cítricos (Diaphorina citri)* (Hermosillo), 1–7.
8. Reinking, O. A. (1919). Diseases of economic plants in southern China. *Philippine Agric.* 8, 109–135.
9. Van der Merwe, A. J., and Andersen, F. G. (1937). Chromium and manganese toxicity. It is important in Transvaal citrus greening? *Farm. South Africa* 12, 439–440.
10. Fraser, L. R., Singh, D., Capoor, S. P., and Nariani, T. K. (1966). Greening virus, the likely cause of citrus dieback in India. *FAO Plant Protect. Bull.* 14, 127–130.
11. McClean, A. P. D., and Schwarz, R. E. (1970). Greening or blotchy-mottle disease of citrus. *Phytophylactica* 2, 177–194.
12. CABI (2017). *Citrus Huanglongbing (Greening) Disease (Citrus Greening) – Datasheets. 2017.*
13. EPPO (2017). *PQR database. Paris, France: European and Mediterranean Plant Protection Organization.*
14. USDA-FAS Foreign Agricultural Service. *Global Agricultural Information Network.* (2018b).
15. Monzo, C., and Stansly, P. A. (2017). Economic injury levels for Asian citrus psyllid control in process oranges from mature trees with high incidence of Huanglongbing. *PLoS ONE* 12:4. doi: 10.1371/journal.pone.0175333.
16. Taning, C. N. T., Andrade, E. C., Hunter, W. B., Christiaens, O., & Smagghe, G. (2016). Asian citrus psyllid RNAi pathway–RNAi evidence. *Sci. Rep.* 6, 38082.
17. Lu, Z. J., Huang, Y. L., Yu, H. Z., Li, N. Y., Xie, Y. X., Zhang, Q.,... & Su, H. N. (2019). Silencing of the chitin synthase gene is lethal to the Asian citrus psyllid, *Diaphorina citri*. *International journal of molecular sciences*, 20(15), 3734.

3/7/2020