RNAI-MEDIATED KNOCKDOWN OF SUGAR METABOLISM AND TRANSPORT GENES IN *BACTERICERA COCKERELLI* (SULC)

by

Neda Arad

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As members of the Master's Committee, we certify that we have read the thesis prepared by Neda Arad, titled RNAi-mediated knockdown of sugar metabolism and transport genes in *Bactericera cockerelli* (Sulc) and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

Professor Judith K. Brown — Date: 28 April 2022

Professor Michael A. Riehle — Date: 28 April 2022

Dr. David A. Baltrus — Date: 28 April 2022

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

Professor Judith K. Brown — Date: 28 April 2022
Master's Thesis Committee Chair
The School of Plant Sciences

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DEDICATION

To my beloved husband, Ali Kamali.

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ABSTRACT

The citrus greening disease is among the most damaging disease of citrus known to be caused by an insect-transmitted, fastidious bacterial pathogen. In this study, five genes involved in sugar metabolism and transport in the gut of potato psyllid (PoP), *Bactericera cockerelli* (Sulc) (Psylloidea: Triozidae), a surrogate study system for the Asian citrus psyllid (ACP), were evaluated for knockdown using RNA-interference (RNAi). Knockdown was quantified by real-time quantitative PCR (qPCR) expressed as fold-change in gene expression relative to water and a non-target sequence, luciferase. Silencing *AQP2* and *TRET1* resulted in 20-30% PoP mortality by 9 days post-inoculation (dpi), either individually, or combined and delivered as a group, or 'stacked', with other dsRNA targets. Among the five targets tested for dsRNA-mediated knockdown, silencing of *AGLU1* resulted in the earliest and the greatest mortality in potato psyllid, both individually and stacked. Knockdown with different combinations of the other four targets revealed that the greatest PoP mortality was achieved from knockdown with all five dsRNAs, at 65.11%. Different combinations of dsRNAs were evaluated to determine if knockdown could be attributed to additive or synergistic effects of knockdown of two or more targets. Among the dsRNAs evaluated Trehalase and Alpha-glucosidase showed synergistic effects based on the qPCR analysis and by increased mortality. Also, RNAi penetrance and persistence of knockdown were enhanced over time, by stacking certain dsRNAs. Results showed that dsRNA screening by oral ingestion of dsRNAs delivered in a 20% sucrose solution was informative for evaluating the potentially deleterious effects of either single or 'stacked' dsRNAs on mortality and other phenotypes.

Key words: biopesticide, citrus greening, sugar metabolism, sugar transport, potato psyllid, RNA

1- INTRODUCTION

1-1 Citrus Greening Disease

Citrus greening disease is a primary cause of citrus crop and tree loss in numerous parts of Asia, Africa, and North and South America. Citrus greening was become known by different names before it was identified as one disease. These names include yellow shoot or huanglongbing in China; dieback in India; likubin or decline in Taiwan; vein phloem degeneration in Indonesia; leaf mottle in the Philippines; and yellow branch, blotchy-mottle, or greening in South Africa. However, "greening" was widely adopted (Graca 1991). It is thought that citrus greening disease originates in China as a yellow shot disease in the 1980s (Graca 1991). Citrus greening in the United States was first identified in Florida in 2005 (Chung and Briansky 2005). Now, citrus greening is established in the majority of the subtropical and tropical citrus-producing areas of the world (Iwanami 2022).

1-2 Symptoms

Symptoms of the disease in citrus include blotchy mottling of leaves, chlorosis of new shoots, and tree dieback followed by death. Fruits are discolored, pithy, and low in sugar content, becoming unmarketable as the disease progresses (Graca 1991; Bové 2006). Earlier infection at or soon after propagation results in symptoms throughout the tree, but the symptoms and the causal organism are often somewhat confined if the infection occurs later (McClean 1970).

Infected trees or branches suffer heavy leaf drops followed by out-of-season flushing and blossoming, with dieback occurring in severe cases (Martinez 1972). Leaf symptoms are generally

of two types: Primary symptoms are characterized by yellowing normal-sized leaves along the veins or developing a blotchy mottle (Scheneider 1968). Secondary symptoms include small upright leaves that show various chlorotic patterns resembling those induced by zinc and iron deficiencies. Analysis of symptomatic leaves shows a higher content of potassium and lower content of calcium, magnesium, and zinc (Keon and Langenegger 1970). Infected fruits are small and lopsided, with a bitter taste, maybe due to higher acidity and lower sugars. Many fruits fall prematurely, and those on the shady side of the tree do not color properly and remain green. Any seeds in severely affected fruit are often aborted. The disease was reported to destroy young trees in one to two years in China (Zhao 1982). Root systems are inadequately developed with few fibrous roots, perhaps due to root starvation. New root growth is repressed, and the roots often start decaying from the rootlets (Graca 1991).

1-3 Nature of the Causal Agent

Since the disease is a graft- and insect-transmissible disease, it was first concluded that a virus is a causal agent. Some researchers thought the tristeza virus to cause (Graca 1991) in China. However, in South Africa, it was shown that tristeza and greening could readily be differentiated since the aphid *Toxoptera citricidus* transmitted tristeza but not the greening and psylla vice versa (McClean 1965). In 1970, Lafleche & Bove reported the existence of mycoplasma-like organisms (MLOs) in citrus phloem tissue infected with African and Indian greening. The phloem hindrance of the greening pathogen was advocated by the mention that girdling prevented spread within a plant (Graca 1991). Comparative electron microscope studies on the structures of the greening organism and several other types of prokaryotes revealed that it was not an MLO (Moll and Martin 1974). Gamier & Bove suggested that the organism was a bacterium belonging to Division,

Gracilicute. The remission of symptoms of and disappearance of organisms from the phloem was observed following the treatment of infected plants with penicillin G, which inhibits a late step in peptidoglycan synthesis (Bové et al. 1980).

The recalcitrance to culturing on artificial media has made studying this organism difficult. The presence of other bacteria in greening-free and greening-affected citrus vascular tissue and psyllids have also confounded isolation of a single suspect organism. Ultimately, a bacterial-like organism was consistently associated with citrus greening disease and tentative causality was established by Lafleche and Bove in 1970 (Laflèche and Bové 1970), and in 1984 the isolation of a long rod-shaped, gram-negative organism from African greening-infected citrus leaf mid-ribs was reported (Graca 1991).

1-4 Liberibacter and Psyllids

Greening disease is associated with three phloem-limited alpha-proteobacteria, including '*Candidatus Liberibacter asiaticus*' (CLas), '*Ca. L. americanus*', and '*Ca. L. africanus*', with origins in Asia, Brazil, and Africa, respectively (do Carmo Teixeira et al. 2005; Bové 2006). The most prevalent and widespread species is CLas, and the disease is associated with the bacteria found in Asia and the Americas (Hall and Gottwald 2011). The Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae), is an important pest of citrus because it transmits phloem-limited bacteria [*Candidatus Liberibacter* spp., notably *Ca. L.* asiaticus (LAS)] is associated with citrus greening disease, currently considered the world's most serious disease of citrus (Bové 2006; Hall et al. 2013). ACP persistently transmits LAS and, although the rate of LAS transmission by ACP individuals usually is low, citrus greening can spread rapidly in a citrus grove, and the geographic range of the disease is expanding threatening citrus industries in new

areas. ACP invaded many countries in Central and North America starting in the 1990s, and citrus greening subsequently has been detected in the USA, eastern and western Mexico, Belize, Puerto Rico, and Cuba (Hall et al. 2013).

A counterpart of Liberibacter has emerged as a new pathogen of herbaceous, solanaceous hosts is associated with Zebra chip (ZC) disease of potato (*Solanum tuberosum* L.) and vein-greening disease of tomato and pepper (Brown et al. 2010; Reyes-Corral et al. 2021), both caused by '*Ca. Liberibacter solanacearum*' (Munyaneza et al. 2007; Rehman et al. 2010) and transmitted by the potato/tomato psyllid (PoP), *Bactericera cockerelli* (Sulc) (Psylloidea: Triozidae) (Hansen et al. 2008).

Transmission of CLas by ACP and CLso by PoP adults occurs in a persistent, propagative manner (Ghanim et al. 2017). The CLso cells can be observed throughout the ACP midgut, hemocoel, fat body, salivary glands, and ovaries of CLas-infected psyllids (Xu et al. 1988; Ammar et al. 2011). Psyllid-mediated transmission of CLas is most efficient when early-instar nymphs ingest the bacterium from citrus leaves on which eggs are oviposited during female feeding. Once acquired, Liberibacter spp. is transmitted for the life of the vector (Xu et al. 1988; Hung et al. 2004). After ingestion, CLas accumulates to increasingly more significant numbers throughout the infection cycle, with the highest relative titer detectable in the fourth immature instar and young (teneral) adults (Inoue et al. 2009; Ammar et al. 2011). Acquisition of CLas by adult ACP does not typically result in competent transmission (Xu et al. 1988; Inoue et al. 2009; Pelz-Stelinski et al. 2010). Adult psyllids harboring CLas can transmit the bacterium to citrus or other susceptible hosts after feeding for a few hours or less (Munyaneza et al. 2007; Buchman, Sengoda, and Munyaneza 2011; Buchman et al. 2012).

1-5 Economic Importance

In addition to providing fresh fruit and juice, processed citrus is used to produce by-products. Citrus fruits are notable for their fragrance. They not only contain large amounts of vitamin C but are also rich sources of antioxidants. Citrus is a substantial fruit crop giving an average annual yield of 124246 thousand tons. It is cultivated in more than 140 countries globally (Bist and Bista 2020). Citrus greening is a highly damaging and incurable disease of citrus and is causing a significant threat to the world's citrus industry (Singerman and Rogers 2020). Assessment of losses due to greening is not easy. Sometimes only parts of a tree are affected, so losses are negligible. When the entire tree is infected, the crop loss is total (McClean 1970). Citrus greening is believed the most destructive of citrus diseases wherever present. Any citrus growing area where the disease or its vectors are detected must quickly adapt all production and management systems to avoid the rapid collapse of the industry (Farnsworth et al. 2014). The medium-term consequences of citrus greening are well represented in a study that assessed the economic influence on the Florida citrus industry during the first five years of coexistence with the disease, including an estimation of a 23% yield reduction from 2006 to 2011, revenue losses of $1.71 billion, and the loss of 8,257 jobs (Hodges and Spreen 2012).

In the United States, ACP and citrus greening were first determined in Florida in 1998 and 2005, respectively (Halbert 2005). The psyllid is now well demonstrated in citrus-producing regions of the state and is also identified in Texas, Louisiana, Mississippi, Georgia, South Carolina, Hawaii, and southern California (French et al. 2001; FDQO[1] 2008). According to the USDA, Florida has lost 72% of its citrus production since 2006 and 175,000 acres of citrus trees since the rise of ACP.

[1] Federal Domestic Quarantine Order

In the united stated, 314 million dollars and 5.6 million metric tons of citrus were lost due to ACP. A downward production trend is still retained in that area. NASS[2] statistics show how the production of oranges in Florida dropped from 6.94 to 3.33 billion tons (67.4% decrease) over the 8-year interval from 2007–08 to the 2015–16 seasons (USDA[3] 2016). Other than plant movement by people, ACP is the primary cause of the spread of the citrus greening disease (Halbert and Manjunath 2004). Therefore, practical pest and disease management approaches are demanded to decrease their population.

1-6 Control

Different management strategies are required to avoid the possible threat of citrus greening. They include legislative control, thermotherapy, chemotherapy, eradication and replacement, breeding for resistance, and vector control (Bist and Bista 2020). Legislative control by establishing methods like regulatory plans avoids pathogen transmission (Kawano 1988). Thermotherapy through hot air treatment of grafted wood is an effective way to eliminate yellow shoot disease (Lin 1969). In chemotherapy, leaf symptoms of citrus greening were controlled using tetracycline hydrochloride and penicillin carbendazide to complete control citrus greening (Cheema, Kapur, and Sharma. 1986). The best result was gained significantly in the spring season through the tetracycline hydrochloride injection (Schwarz and Van Vuuren 1971; Martinez, Nora, and Armedilla 1970; Graca 1991). Trees younger than four years of age, nonbearing trees, and the trees that display up to 50-70% of the disease symptoms should be eliminated (Baniqued 1998).

[2] National Agricultural Statistics Service
[3] United States Department of Agriculture

Different hybrids of sweet oranges and Tahiti lime have been produced in South Africa and are being examined for possible resistance (De Lange et al. 1985).

Many endeavors have been made to control the vector through different cultural, chemical, and biological approaches. Releases of the parasite, *Tamarixia radiate,* was effective against citrus greening disease (Baniqued 1998). The primary parasite *Tetrastichus erytrea* in South Africa is species of parasitoid *Tetrastichus* that oviposit in the psylla nymph and seems to restrict the psylla population (Samways and Grech 1986). Another way to biologically control the disease is using Fungi. *Tetrastichus erytrea* is attacked by *Cladosporium oxysporum* and *Capnodium citri*, which are population dependent and are threatened by dryness (Samways and Grech 1986). ACP is parasitized by *Beauveria* and *Cephalosporium lecanii*, which is practical at high density (Gavarra and Mercado 1988; Bist and Bista 2020). Also, the use of the systemic insecticides, dimethoate and monocrotophos, have provided effective controlled of ACP (Graca 1991).

Managing the psyllid vector to reduce the rate of CLas transmission relies primarily on insecticide applications, augmented by antibiotics and small molecule chemistries that have been shown to offer some promise (Grafton-Cardwell, Stelinski, and Stansly 2013). The ectoparasitoid, *Tamarixia radiata* (Hymenoptera: Eulophidae) (Hoddle and Pandey 2014), is a natural predator of ACP and has been reared and released in several locations in the U.S. as a biological control measure (Michaud and Olsen 2004). However, routine use of chemical and other insecticidal products has increased costs for citrus production, particularly for the juice market, beyond sustainable levels (Yamamoto et al. 2009).

Biopesticides are well known for their lower environmental effects than conventional synthetic pesticides and have been investigated to devise environmentally friendly, cost-effective, and

integrated management strategies (Olson 2015). The U.S. EPA[4] categorizes three types of biopesticides, including microbial, biochemical, and plant-incorporated-protectants or PIPs. In microbial biopesticides, the whole, live organisms are used for biocontrol. Biochemical pesticides based on peptides such as Vismax (https://griffinfertilizer.com/details-on-citrus-peptide-field-trials/) and small oligonucleotides (Hunter et al. 2021) show promise for controlling pests by non-toxic mechanisms. Both transgenic and non-transgenic approaches have been used to deliver PIPs through treated seed or genomic targets can be modified by genome editing and the seed treatment-based method pioneered by Morflora (Olson 2015).

1-7 RNA interference

RNA interference (RNAi) as an environmentally friendly control has become a potentially effective strategy for managing insects highly susceptible to gene knockdown strategies (Jewkes et al. 2011; Hajeri et al. 2014; Niu et al. 2019; Borah and Konakalla 2021; Sharma, Christiaens, et al. 2021; Sharma, Taning, et al. 2021). The RNAi is a naturally occurring mechanism in eukaryotes that involves knocking down gene expression through targeting and binding to the messenger RNA in a specific manner. The discovery of dsRNA-mediated gene-specific silencing in animals was first demonstrated for Caenorhabditis elegans (Fire et al. 1998) and has become a powerful method for functional genomic studies in eukaryotes (Price and Gatehouse 2008).

RNAi is a cellular mechanism that causes gene knockdown a process that researchers call silencing (Haiyong 2018). In eukaryotic organisms, RNAi is triggered by double-stranded RNA (dsRNA)(Clark and Pazdernik 2016). During RNAi, long dsRNA is diced into small fragments

[4] Environmental Protection Agency

~21 nucleotides long by an enzyme called "Dicer." These tiny fragments referred to as small interfering RNAs (siRNA), bind to proteins from a particular family: the Argonaute proteins. After binding to an Argonaute protein, one strand of the dsRNA is removed, leaving the remaining strand available to bind to messenger RNA target sequences according to base-pairing rules: A binds U, G binds C, and vice versa. Once attached, the Argonaute protein can either cleave the messenger RNA, destroy it, or recruit accessory factors to regulate the target sequence in other ways (Song and Rossi 2017).

Researchers widely use RNAi to silence genes and study their function. siRNAs can be designed, cheaply synthesized, and readily administered to cells to bind any gene. siRNAs are commercially available to silence virtually any gene in a living eukaryotic cell, increasing biomedical knowledge. Furthermore, the ability to decrease the expression of a single gene makes RNAi an attractive therapeutic approach for treating infectious diseases or genetic disorders. There are currently several clinical trials testing the safety and effectiveness of siRNA drugs.

Genome and transcriptome sequences of ACP (Hunter, Reese, and International Psyllid Genome Consortium 2014; Reese et al. 2014; Vyas et al. 2015; Taning et al. 2016; Saha et al. 2017) and PoP (Nachappa, Levy, and Tamborindeguy 2012; Fisher et al. 2014) are promising for finding new disease management solutions. These approaches rely on knowledge of the functional genomic discovery of protein and DNA targets of the psyllid host that may be exploited to undermine Liberibacter invasion and its circulative transmission by psyllid vector. Also, they can be used for understanding the Liberibacter-psyllid interactions crucial for invasion and subsequent infection (Hunter et al. 2021). Genome sequencing and transcriptome studies of the whitefly *Bemisia tabaci* (Genn.) (Aleyrodidae) (Chen et al. 2016; Xie et al. 2017) and cryptic species (de Moya et al. 2019)

have facilitated the selection of suitable genes for RNA interfering and gene-editing technology for the effective management of whiteflies and plant viruses they transmit (Vyas et al. 2017; Suhag et al. 2020).

Effective RNAi in insects requires a suitable method for dsRNA delivery to trigger the silencing of the target gene. Several approaches have been evaluated, including microinjection, soaking, and uptake or ingestion during feeding (Yu et al. 2013; Kunte et al. 2020). Delivery of exogenous dsRNA, which contributes to homologous endogenous mRNA degradation and post-transcriptional gene silencing (Huvenne and Smagghe 2010), is successful for agricultural pest control (Baum and Roberts 2014). Target gene(s) knockdown post-ingestion dsRNA, for agricultural pest control, has been reported in ACP (El-Shesheny et al. 2013; Galdeano et al. 2017; Santos-Ortega and Killiny 2018; Yu and Killiny 2020; Yan, Ren, and Shen 2021), PoP (Wuriyanghan and Falk 2013), whiteflies (Upadhyay et al. 2011; Ghanim et al. 2017; Vyas et al. 2017; Sahu and Mishra 2021), southern green stinkbug (Sharma, Christiaens, et al. 2021), western corn rootworm (Bolognesi et al. 2012), pea aphid (Christiaens, Swevers, and Smagghe 2014), cotton bollworm (Jing and Han 2014), Oriental fruit fly (Liu et al. 2015), beet armyworm (Hafeez et al. 2020), brown planthopper (Fang et al. 2020; Shen, Chen, and Zhang 2021), and the grape mealybug (Arora et al. 2020).

1-8 Gene Identification

In this study, PoP was used as a surrogate study system of ACP was selected. Most states where citrus can be grown outdoors, including Arizona, have an ACP or citrus greening quarantine. The PoP is more trackable than ACP and easier to work with particularly given it colonizes herbaceous hosts such as tomato, in contrast to ACP which colonies primarily woody hosts such as citrus and

requires flush growth for reproduction. Comparisons of genome and transcriptome sequences have shown that in general, ACP and PoP encode orthologs of same core genes. Consequently, it is possible to hypothesize that knockdown of target genes in PoP should result in gene knockdown in ACP. To this aim, the dsRNAs were designed to specifically knockdown the expression of sugar metabolism and transport genes, including Aquaporin2 (*AQP2*), alpha-glucosidase1 (*AGLU1*), Trehalase1 (*TRE1*), Trehalase2 (*TRE2*), and the facilitated trehalose transporter (*TRET1*), which are gut-expressed genes in PoP (Ibanez, Hancock, and Tamborindeguy 2014; Vyas et al. 2017; J. Chen et al. 2010; Terra and Ferreira 1994). These genes are known to be essential for sugar metabolism and transport in PoP, and so, were selected as potentially lucrative RNAi targets (Fig.1).

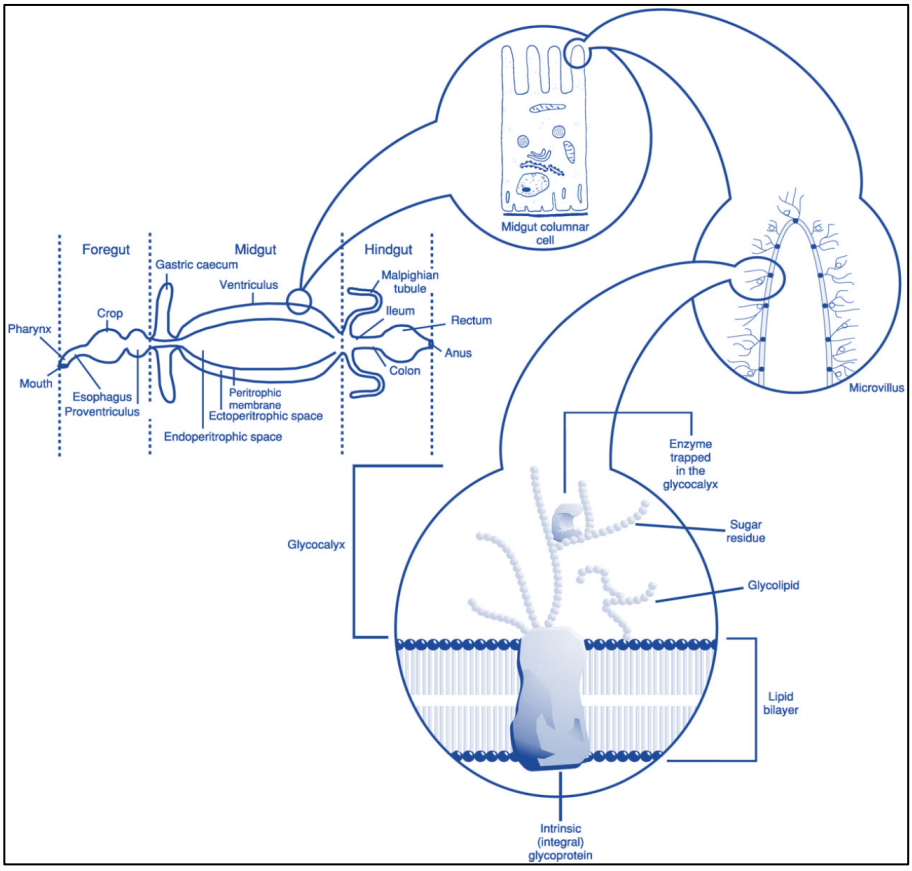

Fig. 1. Diagrammatic representation of insect gut compartments (Terra and Ferreira 2012).

1-8-1 Aquaporin

Aquaporins are intrinsic proteins found in all living organisms that have a vital role in channeling water through cellular membranes. The shuttling of water through the epithelium foregut and hindgut liners is believed to be achieved by aquaporins. These water channels have many tasks within insects, including a complete adaptation of the protein to an active function of water movement. Hemipteran insects are intrinsically plant phloem-xylem feeders. Phloem feeders encounter two obstacles to feeding: The first is a low supply of critical amino acids in the phloem. The second obstacle is intense osmotic pressure resulting from the sugar-rich diet. The osmotic pressure is two to five times lower in the insect tissues than in the phloem. Due to being unable to control water ingestion from the phloem, insects dehydrate and die without adaptation to decrease that osmotic pressure (Douglas 2006).

Presently, four AQPs have been identified in PoP, including BcAQP2-like (AQP2), BcAQP4-like, BcAQP5-like, and BcAQP9-like (Ibanez, Hancock, and Tamborindeguy 2014). In phylogenetic analysis, only AQP2, clustered with PRIP proteins, an AQP group shown to transport water or water and urea. Quantitative real-time PCR (qPCR) analyses revealed the expression of these aquaporins in the PoP gut, where the AQP2 transcript showed a higher level of expression than the others (Ibanez, Hancock, and Tamborindeguy 2014).

1-8-2 Alpha-glucosidase

Alpha-glucosidases (AGLUs) are a large class of enzymes in the large family of glycoside hydrolases, whose primary role is the hydrolysis of oligosaccharides and release of alpha-D-glucose from the non-reducing end of alpha-linked substrates (Price and Gatehouse 2008). These

enzymes are essential for glucose metabolism in organisms (Lu et al. 2021). After sucrose hydrolysis in the midgut lumen by alpha-glucosidase, fructose and glucose are released (Pompon et al. 2011). Glucose is converted to trehalose in the fat body and maintains a concentration gradient or is stored as glycogen in the fat body (Price and Gatehouse 2008). *AGLU1* was previously considered a candidate gene for RNAi control of *Bemisia tabaci* MED (Malik et al. 2016; Vyas et al. 2017; Mondal, Brown, and Flynt 2020). Insects' AGLU as soluble forms are in the midgut lumen or bound to microvillar membranes. They are also trapped in the midgut cell glycocalyx (Terra and Ferreira 1994).

It has been shown that purified midgut AGLU of the pea aphid, *Acyrthosiphon pisum*, catalyzes transglycosylation reactions in the excess sucrose condition. So, glucose is released from sucrose without increasing the osmolarity of the medium (Cristofoletti et al. 2003). This phenomenon, which is associated with a quick fructose absorption, defines why the midgut luminal osmolarity declines as the ingested sucrose-containing phloem sap passes along the aphid midgut (Ashford, Smith, and Douglas 2000). In another study, the application of AGLU dsRNA induced 50% knockdown in mature adults in ACP (Santos-Ortega and Killiny 2018).

1-8-3 Trehalase

Trehalase is an enzyme gaining interest in insect physiology as it regulates energy metabolism and glucose generation through trehalose catabolism. Trehalase has a crucial role in carbon metabolism in insects. The stored energy reserve in the form of trehalose is hydrolyzed by trehalase and used to meet the energy demands for flight and development. Trehalose contents in different tissues are necessary for reproductive development in insects (Mariano et al. 2009; Huang and Lee 2011; Lu et al. 2019).

Insect trehalase has two forms, TRE1 is the soluble form, while TRE2 is the membrane-bound form. TRE1 has been purified from hemolymph, the cavity of goblet cells in the midgut, and egg homogenates of some insects. TRE2 has been described in flight muscles, follicle cells, ovary cells, spermatophore, midgut, brain, and thoracic ganglia to face the blood side and degrade the extracellular trehalose. So, the glucose is produced from this reaction, then enters the cell and is utilized for physiological activities. Trehalose is an essential hemolymph sugar for insects since it is the primary energy and carbon source and acts as a protectant against harsh environmental conditions. The apical trehalase (TRE1) may be soluble (glycocalyx associated or secreted into the midgut lumen) or microvillar. In contrast, the midgut basal trehalase (TRE2) is an integral protein of the basal plasma membrane (Terra and Ferreira 1994). Insect trehalase classification and a generalized diagram of the utilization of trehalose in insects is presented in Fig. 2 and 3, respectively.

The injection of TRE1 and TRE2 dsRNA into *Spodoptera exigua* resulted in efficiency rates up to 83% at 72h post-injection (Chen et al. 2010). The relative expression of TRE1 and TRE2 decreased after dsRNA injection in rice brown planthopper (Zhao et al. 2016). Inhibition of ACP trehalase using RNAi and validamycin downregulated the synthesis of glucose and downstream chitin metabolism-related genes, and the trehalose content was upregulated (Yu et al. 2021).

1-8-4 Trehalose transporter

Trehalose transport protein (TRET1) is a high capacity, facilitated trehalose carrier that transports freshly synthesized trehalose from the fat body through the hemolymph (Kikawada et al. 2007; Kolosov et al. 2019). Trehalose is then cleaved into glucose by membrane-bound or soluble trehalase, and glucose is taken up by cells in the vicinity for varied functions. These exchanges

between cells suggest that the hemolymph trehalose levels in insects are regulated through a specific transmembrane trehalose transporter (Shukla et al. 2015).

Studies indicate that anhydrobiotic insect larvae of the African chironomid, *Polypedilum vanderplanki*, have unique trehalose transporters active during desiccation stress (Kikawada et al. 2007; Mitsumasu et al. 2010; Uchida et al. 2017). Infection of ACP by CLas resulted in the TRET1 being among the most highly expressed genes in the ACP gut transcriptome, indicating a significant change in ACP gut trehalose metabolism (Kruse et al. 2017). A reduced TRET1 expression by RNAi post-dsRNA ingestion by *Bemisia tabaci* (Genn.) through RNAi has been reported (Vyas et al. 2017).

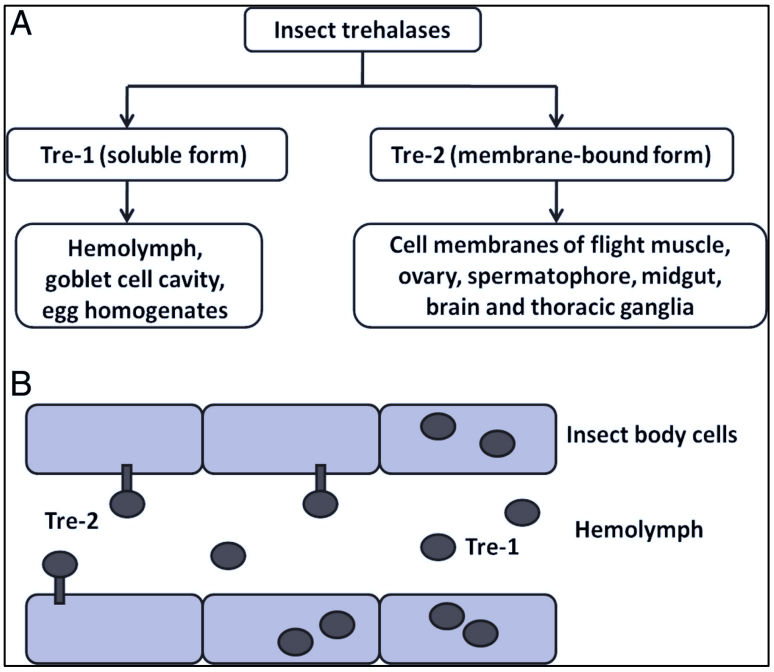

Fig. 2. Insect trehalase. Soluble and membrane bound forms of insect trehalase and their occurrence in the insect body (Shukla et al. 2015).

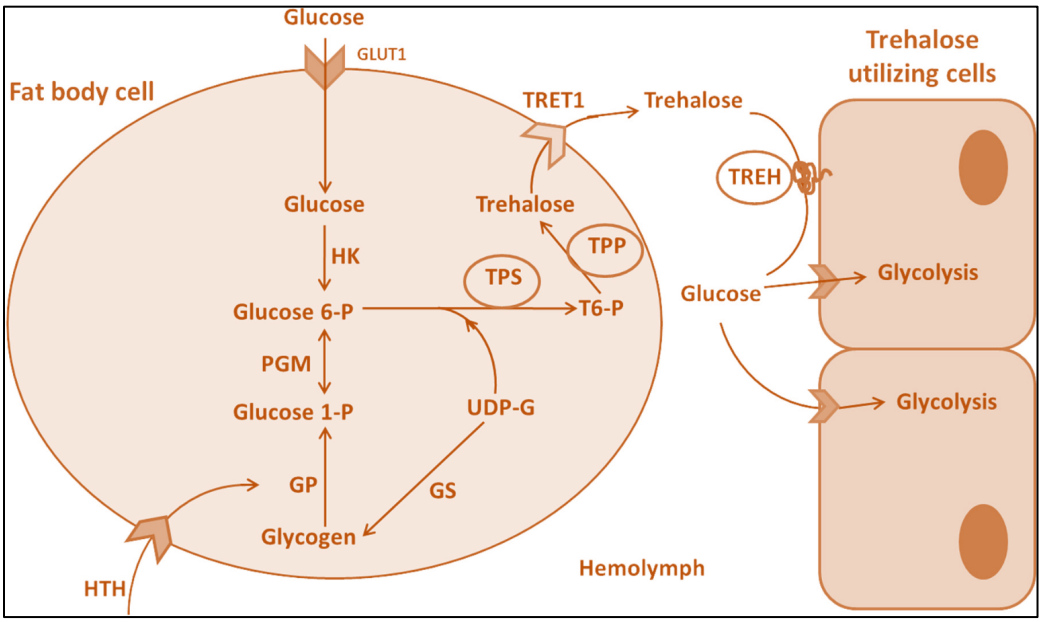

Fig. 3. Schematic depicting the biosynthesis and utilization of trehalose in insects. HTH drives the synthesis of trehalose in fat body cells which is exported via TRET1 transporter. Trehalose is then cleaved into glucose by membrane bound (shown here) or soluble trehalase and glucose is taken up by cells in vicinity for varied functions. HK, hexokinase; PGM, phosphoglucomutase; GP, glycogen phosphorylase; HTH, hypertrehalosemic hormone (Shukla et al. 2015).

1-8-5 Luciferase

In RNAi-mediated gene knockdown assays, a non-target control dsRNA, such as Luciferase (LUC), together with a water control, are essential for quantitative analysis of knockdown (Paredes-Montero, Arif, and Brown 2022). A dsRNA target can experience two effects. The first is the RNAi effect initiated based on sequence complementarity between the applied RNA and endogenous mRNA. The second effect can be considered a non-specific or general effect resulting from dsRNA ingestion. If water only, and no non-target gene is included among the experimental controls, the potential effects of RNAi, could be over-estimated. In this study, a 328 bp fragment

of the firefly luciferase gene (AY535007.1:2532-4184) was utilized as a negative non-target control to measure gene(s) knockdown in each experiment.

1-8-6 Ribosomal protein large subunit

The selection of reference genes is a crucial step for qPCR analyses. These analyses are based on comparing the expression level of the target gene to a reference gene known to be stably expressed. In this study, the Ribosomal protein large subunit 5 (RPL5) (GenBank KT185023) was used as the reference gene for data normalization (Ibanez and Tamborindeguy 2016).

2- MATERIAL AND METHODS

2-1 PoP Colonies

The CLso-infected PoP laboratory colony was established using infected adult PoP obtained from a commercial greenhouse in Snowflake, AZ during 2004 (Brown et al. 2010). Psyllid colonies were reared on tomato plants 'Roma' *Solanum lycopersicum* (L.) in an insect-proof cage maintained in an otherwise insect-free growth room (BugDorms, BioQuip Products, Rancho Dominguez, CA, USA) maintained at 23-25˚C with an L12:D12 photoperiod at The University of Arizona, Tucson, AZ. Potato psyllids were identified as the 'Central' type based on the mtCOI fragment that differentiates among haplotypes in the U.S., according to Swisher et al., 2012.

The CLso type was determined as haplotype A based on a single nucleotide repeat (SSR) marker located within a genomic region that is amplified by Lso-SSR-1F/1R primers (Lin et al. 2011). Psyllids were analyzed approximately 3-4 times per year, for presence or absence of CLso, by PCR amplification with CLso-A-specific 16S rRNA gene primers and cycling parameters (Levy et al. 2011; Lin et al. 2011; Nachappa et al. 2012).

2-2 Gene Cloning

2-2-1 Gene sequence and primer design

Orthologs of the candidate genes were determined using BLASTn[5] with more than 98% homology in the PoP transcriptome databases[6] (Vyas et al. 2015). The annotations were then confirmed by

[5] Basic Local Alignment Search Tool
[6] http://sohomoptera.arizona.edu/

BLASTn analysis through the UniProtKB/Swissport database (The UniProt Consortium 2019). Subsequently, the sequences were studied phylogenetically and comparative amino acid sequence analysis through the reference sequences of several closely related insect species available in the GenBank database. Coding sequences were translated to protein sequences and used for an ortholog search in the KEGG [7] database using BlastKOALA (Kanehisa and Goto 2000). The genome of the cotton aphid, Aphis gossypii, was used as a reference for the BLAST search using the acronym "api" taxonomic ID. The target orthologs were given to a functional pathway utilizing the KEGG mapper, which is the pathway reconstructions tool [8]. A pathway was generated based on searching in the literature and KEGG analysis to represent the sugar metabolism and transport in a hypothetical epithelial cell (Fig. 4). Maltose and sucrose are converted to glucose by AGLU. Trehalose is transferred by TRET and converted to glucose by TRE. Glucose is released and either enter the glycolysis cycle or is transferred to mitochondria to produce ATP.

Each gene's sequence was mapped to predicted transcripts using BLASTn against all predicted transcripts of the chosen organism to define which other genes are hit by the designed dsRNA and to identify predesigned dsRNAs that are available in public libraries. Then, the sequence was cut into 21 nucleotide long siRNAs with a one nucleotide shifting window, and the specificity and efficiency of predicted siRNAs were calculated for these in silico diced sequences. The specificity of a dsRNA was predicted by performing BLASTn searches of *in silico* diced siRNAs analogous to the selected transcript, with a penalty for nucleotide mismatch of −2. No mismatches were observed, indicating no predicted off-target effects of the dsRNA.

[7] Kyoto Encyclopedia of Genes and Genomes
[8] https://www.genome.jp/kegg/mapper.html

The dsRNA primers (Table 1) and qRT-PCR TaqMan primers and probe sets (Table 2) were designed using Geneious Prime software version 8 (https://www.geneious.com/prime/).

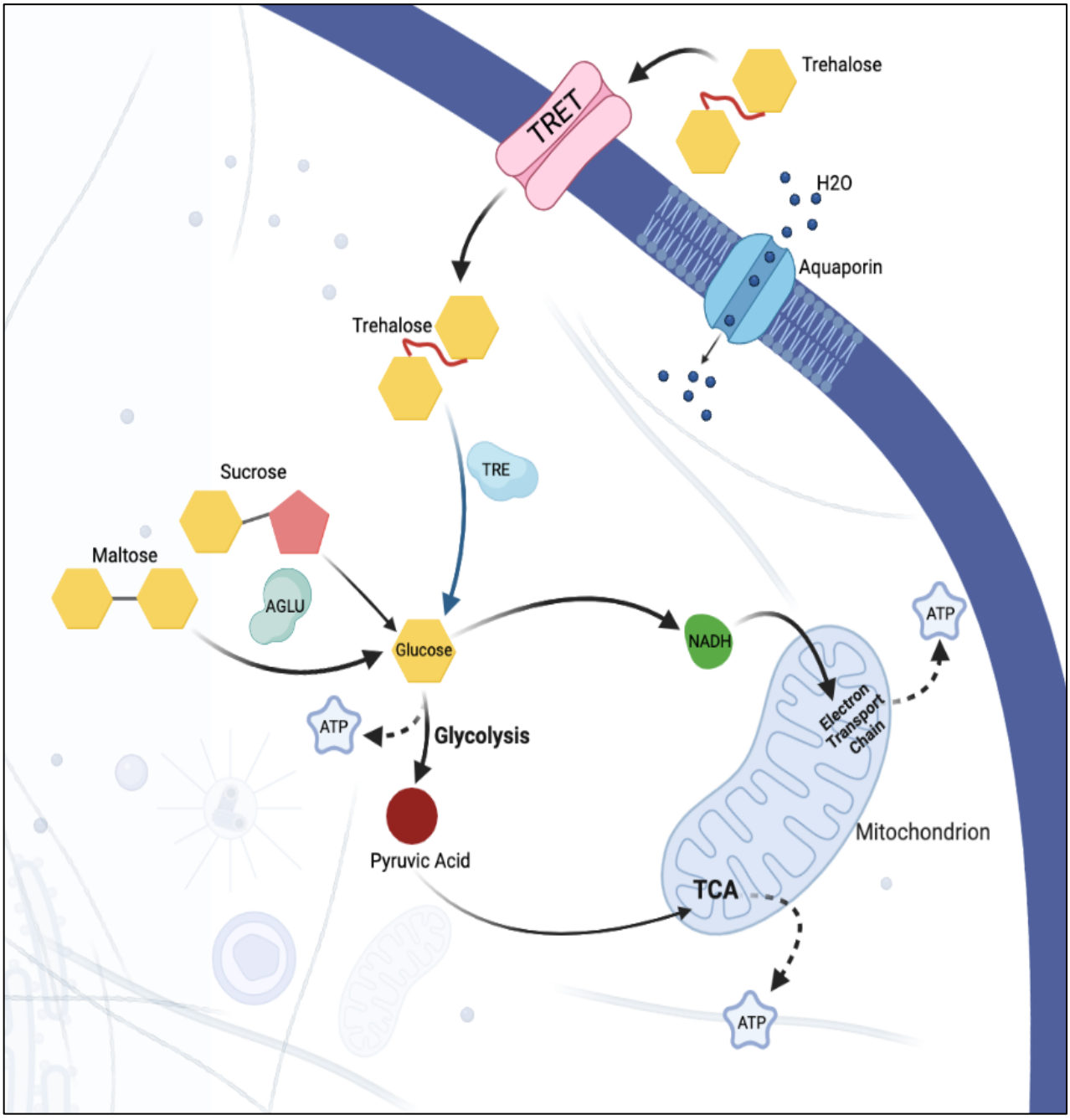

Fig. 4. Sugar metabolism and transport in a hypothetical epithelial cell

Table1. Oligonucleotide primer pairs for dsRNA synthesis

Gene	Sequence	Amplicon Size	Application
AGLU1	Forward (F): 5'GGTCACGGCTAAATACACACCTCTGG3' Reverse (R): 5'TGCCGAACACAGGTTCGATGTCC3'	245bp	dsRNA synthesis
AQP2	F: 5'TCTGAGTTGTGTCAGCCTGCTGG3' R: 5'TGCTTGTTGAGCGTGGTCATGCC3'	300bp	
TRET1	F: 5'GAAAGCGCTGCAATGGCTAAGAGG3' R: 5'GTCAATGGTACTGCCAGCGTCC3'	253bp	
TRE1	F: 5'AGAACTTTGAGGACGCCCAGGAG3' R: 5'AGTAGGTGTCCCAGTAGTACAGCTCC3'	258bp	
TRE2	F: 5'ATCCTGTCATCGTCCCAGGTGGTAG3' R: 5'ACTTCACCATCGGAATGAGCAACGG3'	219bp	
***LUC*-T7**[9]	F: 5'*AGACCACTTCAACGAGTACGACTTCGTGC3' R: 5'*AGACCACGGTACATCAGCACCACCCGAAAGC3'	328bp	

Table2. TaqMan primers and probe sequences

Gene	Sequence	Amplicon Size	Application
AGLU1	F: 5'AAGACGGTCAACGGTCAAAG3' R: 5'GGCGAAAGCGTGGTAGTAAT3' Probe: 5'TCTCATTCCAAGTCCATGCTGGCC3'	111bp	qPCR
AQP2	F: 5'TGCTGGTCCTGGTCATCTTC3' R: 5'TCCTGTGAAGTCAATGGCGG3' Probe: 5'CGCTGGCTATCGGACTGACA3'	122bp	
TRET1	F: 5'CACAGTTTCCCTCGGATCAA3' R: 5'TACACCTCTCCTGCTCTTCCGTCC3' Probe: 5'GGGAAACTCGACTGCCTATT3'	95bp	
TRE1	F: 5'GCAGTCGCCGCTACTACATA3' R: 5'CAGCGTCCCTATGTTGGTCT3' P: 5'ACGCTCTCAACCCCCACTAC3'	116bp	
TRE2	F: 5'CTAGTCGCTGGTTTGTCC3' R: 5'CCTACTTCGTCATGCCAC3' Probe: 5'TGGAATGCCGACTTGCTATCAC3'	226bp	
RPL5[10]	F: 5'CTGGTGTGATTGCTGATGATATTG3' R: 5'TCCACAGGTTTGGTGGATTT3' Probe: 5'AGCTGACCCAACCCATGTCAAGAA3'	109 bp	

[9] * T7 RNA polymerase promoter fragment: TAATACGACTCACTATAGGG

[10] Ribosomal protein large subunit 5 (RPL5) was used as the reference gene for normalization, according to Ibanez and Tamborindeguy (2016).

2-2-2 Amplification of transcripts

Polymerase chain reaction (PCR) amplification was carried out using standard procedures (Sambrook 2012) with REDTaq^{R} ReadyMix^{TM} PCR reaction mix with MgCl2 (Sigma-Aldrich^{R}, USA). Each PCR reaction contained 12.5µL of the mix, 2µL of PoP cDNA, 1µL of primers, and 9.5µL of nuclease-free water. Cycling conditions were: 95^{o}C for 2min, (95^{o}C for 20sec, annealing temperature for 20sec, 72^{o}C for 30sec) 30 cycles, and 72^{o}C for 2min. The annealing temperature was calculated by TmCalculator[11]. The quality and expected size of the amplicons were monitored by electrophoresis. 5µL of the sample was loaded on a 1.5% agarose gel stained with GelRed^{TM} (BIOGENERICA SRL, Mascalucia (CT), Italy). Agarose gel electrophoresis fractionation of bands was carried out in 1x Tris-Acetate-EDTA (TAE) buffer, pH 8.0, with 100volts for 45min.

2-2-3 PCR product purification

The PCR products were gel-purified using Thermo Scientific GeneJET PCR Purification Kit. Buffer was added 1:1 and mixed thoroughly. The solution was transferred to a GeneJET purification column prior to centrifugation for 45sec at 14000rpm. The supernatant was discarded, 700µL wash buffer was added, and the column was centrifuged for 45sec. The column was centrifuged for 1min to completely remove residual wash buffer and transferred to a clean 1.5 mL microcentrifuge tube. Nuclease-free water 50µL was added to the column and centrifuged for 1min. The GeneJET purification column was discarded and the purified DNA was stored at -20^{o}C.

[11] https://tmcalculator.neb.com/

2-2-4 Ligation

Cloning was carried out using the pGEM^{R}-T Easy Plasmid Vector System I Promega (Cat. # A1360). The LUC sequence was ligated into psiCHECK™-2 plasmid (Promega Corporation, USA). Each reaction reagent contained 5µL of 2x Rapid ligation buffer, 1µL of pGEM^{R}-T Easy vector (50ng), purified PCR product, 1µL of T4 DNA Ligase (3 Weiss units/µL), and up to 10µL of deionized water. The reactions were mixed gently by pipetting and incubated overnight at 4^{o}C. The amount of purified PCR product was calculated using NEBiocalculator[12].

2-2-5 LB media preparation

To make LB media containing ampicillin and X-gal, 12.5g Luria Broth (LB) and 7.5g bactoagar was added to 500mL of double distilled water and mixed well. The bottle was autoclaved and was allowed to cool to 70^{o}C in the water bath before adding the ampicillin and X-gal. Then 800µL of X-gal and 500µL of ampicillin were added to the media. The media was poured into plates, allowed to cool, and stored at 4^{o}C.

2-2-6 Transformation

The DH5a competent cells were placed on ice for 5min, and cells were mixed by gently flicking the tubes. The laminar flow hood was cleaned with 70% ethanol. The SOC media was allowed to equilibrate under the laminar flow hood. The ligation reaction was centrifuged to collect contents at the bottom of the tube. 10µL of the ligation reaction was added to the competent cell tube. The competent cell tube gently flicked before and after adding the ligation reaction. The tubes were

[12] https://nebiocalculator.neb.com/#!/ligation

placed on ice for 20min. Heat shock in a 42ºC water bath for 90sec was done to weaken cell walls. Tubes were placed on ice for 10min. 400µL room temperature SOC media was added to each tube. The tubes were incubated at 37ºC and 225rpm for one hour. For each gene, two plates containing ampicillin and X-gal were incubated at 37ºC to get a little warm. The tubes were centrifuged at 5000rpm for 5min, and the supernatant was removed. The pellet was suspended in 100µL of SOC. Under laminar flow hood, 10µL of the suspension was pipetted to an LB plate, and 90µL of the suspension was pipetted to another LB plate and a spreader was used to distribute the suspension evenly. Plates were incubated at 37°C overnight and placed at 4ºC for two hours to allow full-color development.

2-2-7 Colony PCR

Colony PCR is a technique for rapidly screening bacterial colonies that have grown up on selective media following a transformation step to see if the desired genetic construct is present. A few white colonies from each media were separately added to PCR mixtures containing M13 primers. The PCR run was set up at 94ºC for 5 min, (94ºC for 1min, 53ºC for 1min, 72ºC for 3min) 40 cycles, 72ºC for 10min. The quality and expected size of the amplicons were visualized by agarose gel electrophoresis, and colonies harboring an insert of the expected size were selected for confirmation by DNA sequencing.

2-2-8 DNA sequencing

The amplicon sequences were confirmed by sequencing. 20 µL PCR product from colony PCR was used for DNA sequencing through Eton[13]. Sequence analysis was carried out using Geneious

[13] www.etonbio.com

Prime software version 8 (https://www.geneious.com/prime/), and sequences were mapped to the reference genome.

2-2-9 Minipreparation

Plasmid DNA minipreps are used for small-scale isolation of plasmid DNA from bacteria. Single colonies were picked up from the backup plates using a 100µL tip and suspended in 4mL of liquid LB media containing ampicillin. The tip was dipped and left in the tubes. The tubes were placed in the shaker incubator at 200rpm at 37ºC overnight. Next, 1mL of the suspension was added to a 1.5mL tube and centrifuged for 1min at 15000rpm, and the supernatant was removed. This step was repeated three times. Each time the suspension was added to the pellet in the same tube. Then the pellet was used for plasmid isolation. The rest of the suspension was stored in 50% glycerol at -80ºC.

To resuspend cells, lyse, and neutralize, 250µL of resuspension solution was added to the pelleted cells and vortexed at room temperature to be homogenized. The lysis solution of 250µL was added and tubes were inverted 4-6 times before 350µL of neutralization solution was added. Tubes were inverted 4-6 times and centrifuged for 5min at 14000rpm. For DNA binding, ~700µL of the supernatant was loaded to GeneJET^{TM} spin column and centrifuged for 1min, and the flow-through was discarded. 500µL of wash solution was added and the column was washed by centrifugation for 1min, and the process was repeated. The flow-through was discarded and the empty column was centrifuged for 1min. To elute purified DNA, the column was transferred into a new tube, and

50µL of warm water was added, followed by incubation for 2min and centrifugation for 2min, after which the eluent was collected.

2-3 dsRNA Synthesis

The dsRNA was synthesized *in vitro* with the MEGAscript™ T7 Transcription Kit (catalog # AM1334, ThermoFisher, Waltham, MA). dsRNA coding regions of target genes were amplified using the REDTaq^{R} ReadyMix^{TM} PCR Reaction Mix and T7 primers, so the first strand of dsRNA was generated. The quality and expected size of the amplicons were monitored by electrophoresis. Then, the second strand of dsRNA was transcribed using 10X reaction buffer, ATP, GTP, CTP, UTP, T7 enzyme, and 12.5µL of PCR product. The reactions were kept at 37^{o}C for 13 hours. The template DNA was removed by a TURBO DNA-free kit (Ambion, TX, USA), and the dsRNA was precipitated by adding 30µL nuclease-free water and 30µL lithium chloride (7.5M lithium chloride, 50mM EDTA). The samples were placed at -20^{o}C for one hour and then centrifuged at 4^{o}C for 15min at 16,000g. The supernatant was dumped, and pellets were washed with 1mL of 70% ethanol. The ethanol was discarded, and the dsRNA was suspended in 20µL nuclease-free water. The quality and quantity of dsRNA was determined using NanoDrop™ 2000/2000c Spectrophotometer. Then, the dsRNA was incubated at 95^{o}C for 2.5min and gradually cooled to allow annealing of the DNA strands. The integrity and expected size of dsRNA were evaluated by agarose gel electrophoresis, as described above.

2-4 dsRNA Delivery

Each experiment consisted of at least three biological replicates. Third instar PoP nymphs were collected from infested leaflets taken from a CLso infected colony and transferred to a plastic petri

dish to allow a starvation period of six hours. The Petri dishes were sealed with a parafilm strip because nymphs will detect air flows and try their best to escape. To assemble sucrose feeding chambers, two plastic cups and two layers of parafilm stretched 4-times the original size. 200µL of 20% (w:v) of sucrose solution containing green food coloring (McCormick Food Color), was pipetted onto the parafilm at a final concentration of 100ng/µl. A round disk of filter paper with a 2.8cm diameter, previously UV-treated on both sides, was put on top. The second layer of parafilm was stretched as a top layer to form a sachet (solution + filter paper disk inside). Fifty 3rd instar starved for six hours were transferred to the feeding chamber using a fine horsehair brush. The chamber was sealed by adding the second cup on top and parafilm was stretched to hold the cup on place (Fig. 5).

Previously, to assess the potential correlation between dsRNA concentration and the extent of gene knockdown post-ingestion access by PoP to a range of seven dsRNAs concentrations: 0.1, 1, 10, 100, 150, 200, and 500ng/µL were tested for three target genes *vATPase-A*, *Snf7*, and *CHC*. A two-day ingestion access period on the dsRNAs (200µL) was allowed, followed by qPCR amplification to quantify knockdown, six days post-ingestion. Expression for each concentration was normalized to the group receiving the 200ng/µL Luciferase dsRNA non-target control. The results indicated that the minimal concentration of the dsRNA for optimal knockdown of the target genes was 100ng/µL and that increasing the dsRNA concentration to 500ng/µL did not increase knockdown (Mondal et al. submitted).

The LUC dsRNA was included at the same concentration as target gene(s) as a non-target control for each experiment, and water was used as a negative control. Nymphs PoP were allowed a 48-h ingestion-access on the sucrose solution. Psyllids were transferred to the tomato plants' three-leaf

stage and incubated until they molted teneral adults. Mortality was scored on days 0, 3, 5, 7, and 9 (alive or dead). The live PoP teneral adults were pooled, ten each were collected into 1.5mL microfuge tube containing 2- of 20mm zirconium beads and stored at -80°C until RNA isolation.

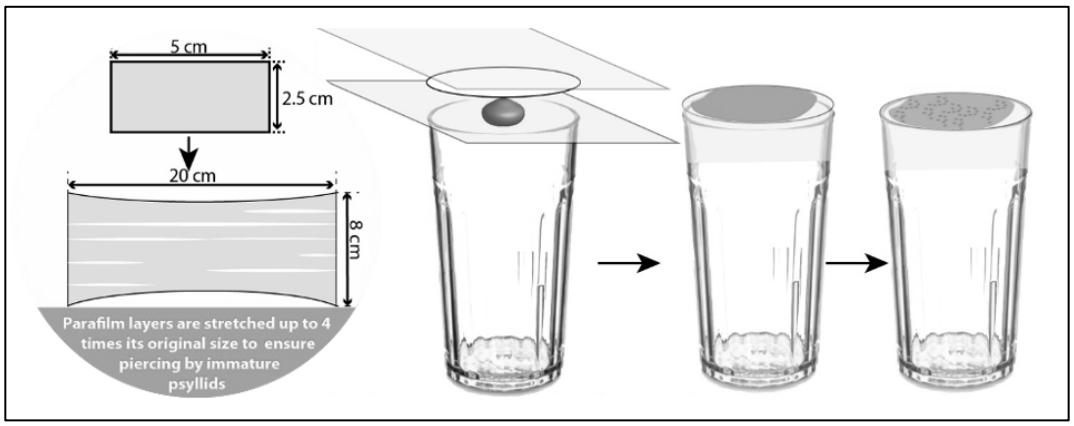

Fig. 5. Sucrose feeding chamber used for dsRNA ingestion for the RNAi experiments (Brown Lab).

The stability of dsRNA was evaluated by visualizing 5µL of the sucrose solution, 0dpi[14], on an agarose gel (1%) through electrophoresis. The integrity and expected size of the dsRNA indicated that this approach helps screen RNAi inducers against PoP (Fig. 6).

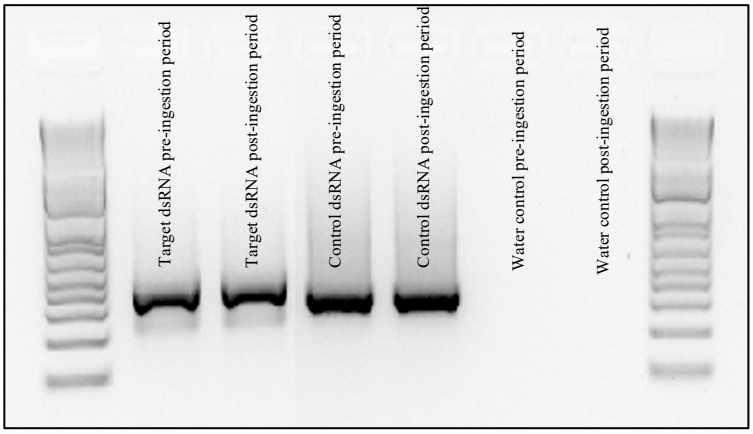

Fig. 6. Stability of the dsRNA in the sucrose solution 0dpi

[14] Days post-ingestion

2-5 RNA Isolation and cDNA Synthesis

Total nucleic acids from specimens were isolated using the Tri Reagent kit (Sigma T9424). The stored specimens at -80°C were homogenized in TRI (1mL per ten insects) in the Storm 24 Bullet Homogenizer for 4min. The samples were left at room temperature for 5min. Then the tubes were centrifuged at 12,000g for 10min at 4ºC. The supernatant was transferred to a new sterile tube (800µL) and 200µL chloroform was added. After vigorous shaking for 15 seconds, the samples were held at room temperature for 5min and centrifuged at 12,000g for 15min at 4ºC. The aqueous phase was transferred to a fresh tube, and an equal volume of isopropanol was added. Samples were incubated at room temperature for 25min with occasional mixing by inversion, and centrifuged at 12,000g for 15min at 4ºC. The supernatant was removed, the RNA pellet was washed by adding 1mL of 75% ethanol, followed by centrifugation at 15,000g for 10min at 4ºC. The supernatant was removed, the RNA pellet was air-dried, and the RNA was dissolved by the addition of 40µL of water.

The genomic DNA was removed using the RNA Clean & Concentrator^{TM}-5 kit (Zymo Research, Cat. # R1013). 5µL DNaseI and 5µL DNA digestion buffer were added to 40µL of RNA and incubated at room temperature for 15min. 100µL RNA binding buffer and 150µL 100% ethanol was added to the sample and mixed well. The mixture was added to the column. Then, the column was centrifuged for 30sec, and the flow-through was discarded. 400µL RNA prep buffer was added to the column. Then, the column was centrifuged for 30sec, and the flow-through was discarded. 700µL wash buffer was added to the column. Then, the column was centrifuged for 30sec, and the flow-through was discarded. The empty column was centrifuged for 90sec, and the flow-through was discarded. The column was transferred to a sterile tube, and 15µL of 80ºC water was added.

After 3min the column was centrifuged for 2min, and the RNA suspension was transferred to a sterile tube. The quality and quantity of isolated RNA were analyzed using a NanoDrop™ 2000/2000c Spectrophotometer.

The cDNA synthesis was carried out using 0.7µg RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814, Carlsbad, CA 92008 USA). Each reaction included 2µL of 10x RT buffer, 0.8µL of 25x dNTP Mix (100 mM), 2µL of 10x RT random primers, 1µL of MultiScribe™ reverse-transcriptase, 4.2µL of nuclease-free H2O, and 10µL of RNA. A PCR was run with 10min at 25°C, following 120min at 37°C, and 5min 85°C. the following steps to make a complementary DNA from the RNA template. The synthesized cDNA is then diluted with 200µL of nuclease free water.

2-6 Gene Knockdown Evaluation

The efficiency of each TaqMan primers-probe was evaluated for each gene target before gene knockdown quantification was carried out by qRT-PCR of the mRNA. The standard curve for each TaqMan primers and probe sets was established by making a 10-fold serial dilution of the cloned fragment of each template to evaluate the reaction efficiency. The acceptable threshold of reaction efficiency was established at >90%. The purified plasmid harboring an insert of each gene was PCR-amplified using the respective TaqMan primers and probe set. The reaction mixture contained 1µL of TaqMan primers-probe Master Mix (10µM), 25µL of the REDTaq^{R} ReadyMix^{TM} PCR reaction mix, 1µL purified plasmid (20ng/µL), and brought to 50µL total with nuclease-free water. The expected size of the amplicons was visualized by electrophoresis. The PCR product was purified and used to make the seven-fold dilution series.

A qPCR run was carried out with each TaqMan primers-probe, using the CFX96 Touch Deep Well Real-Time PCR System (Bio-Rad), and he diluted purified PCR products were used as the template. The reaction conditions included 2min at 50℃ as an initial step, 10min at 95℃ as an initial denaturation, followed by 40 cycles of 95℃ for 15s and 58℃ for 60s for the annealing of TaqMan primers-probe. The reaction mixture contained 10µL TaqPath™ qPCR Master Mix, CG (2x) (Applied Biosystems™), 1µL TaqMan primers-probe Master Mix (20x), and 4µL template, and 5µL of nuclease-free water in a final reaction volume of 20µL. Reactions were set up in triplicate technical replicates and no-template control (NTC) in 96-well Microseal PCR plates (Bio-Rad1, Hercules, CA, USA). The average of the Cq values for each dilution was obtained. Using Excel, the values plotted to determine the slope of the best line through at least three points. Then, the primer efficiency was calculated using the following equation: $(10^{(-1/\text{-slope})}) - 1$.

The effect of dsRNA on target transcript levels was quantified by qRT-PCR amplification for each biological replicate. Amplification reactions and cycling parameters were as described above, except diluted cDNA was used as the template. Relative gene expression of the PoP target genes was normalized to *RPL5* expression levels, and fold-change was quantified using CFX Maestro qPCR Analysis Software[15].

2-7 Statistical Analyses

Mortality rates from the water control were used as the baseline value to correct mortality for all of the treatments and the non-target control, according to formula of Schneider-Orelli's and Abbott's (Fig. 7). This correction using the water control is carried out because mortality can result

[15] www.bio-rad.com

from handling psyllids during collection and transfer. The corrected mortality was calculated 0-, 3-, 5-, 7-, and 9dpi. The student's t-test was carried out for qPCR analyses through the software to indicate significant groups (p-value <0.0.5), by which each experimental group was compared to the non-target control group.

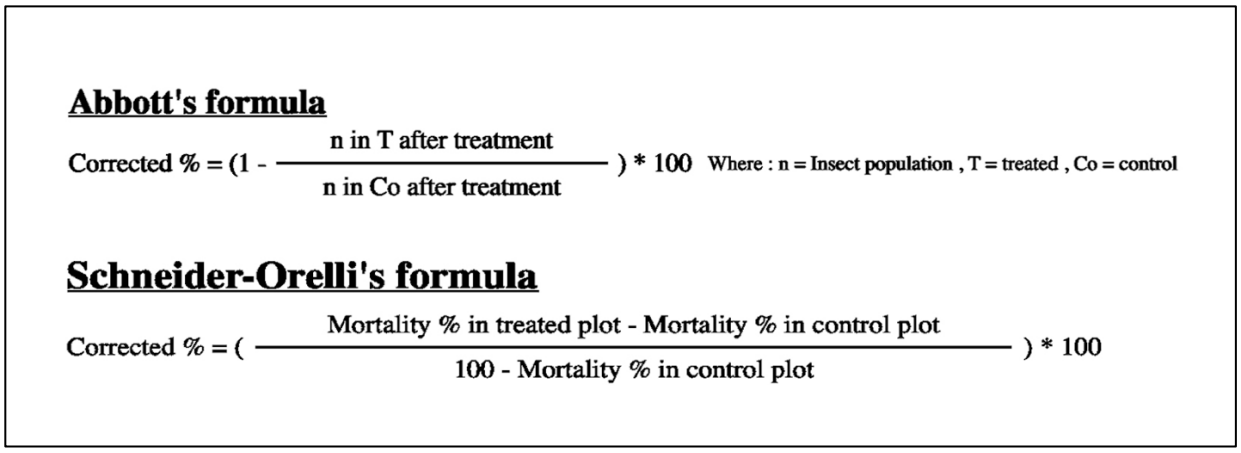

Fig. 7. The Schneider-Orelli's and Abbott's equations.

3- RESULTS AND DISCUSSION

This study aimed to screen the dsRNAs targets separately, in pairs, and stacked to determine the most influential groups to reduce potato psyllids survival. The goal was to identify the fewest dsRNAs necessary for high penetrance corresponding to mortality phenotype and characterize the RNAi activity of the different combinations as additive or synergistic. The RNAi penetrance, referring to the sensitivity level of PoP, results in relative gene knockdown efficiency (Paredes-Montero, Arif, and Brown 2022). The mortality was recorded by 9 days post-treatment. The target genes(s) dsRNA induced higher mortality than the Luciferase dsRNA controls in all the experiments, suggesting that the respective mRNA(s) is suitable RNAi target for potato psyllid. The effect of target gene(s) silencing on the nymph mortality of third instars of PoP carried out using 100 ng/μL of each target gene dsRNA revealed that the nymph mortality (%) increased after dsRNA ingestion, compared to the negative water and non-target Luciferase controls, respectively.

3-1 Single dsRNA Ingestion by PoP

In the experiments for which PoP ingested dsAGLU1 alone, mortality was 19.38 on 0dpi and almost doubled to 46.67 by 9dpi. In contrast, the mortality caused by ingestion of dsAQP2 started from approximately 10% and doubled by 9dpi, which was similar to the mortality documented from dsTRET1 ingestion by PoP. The highest mortality among single targets was observed for dsAGLU1. In the experiments for which the dsAQP2 or dsTRET1 was delivered, mortality was negligible, indicating ineffective knockdown (Fig. 8).

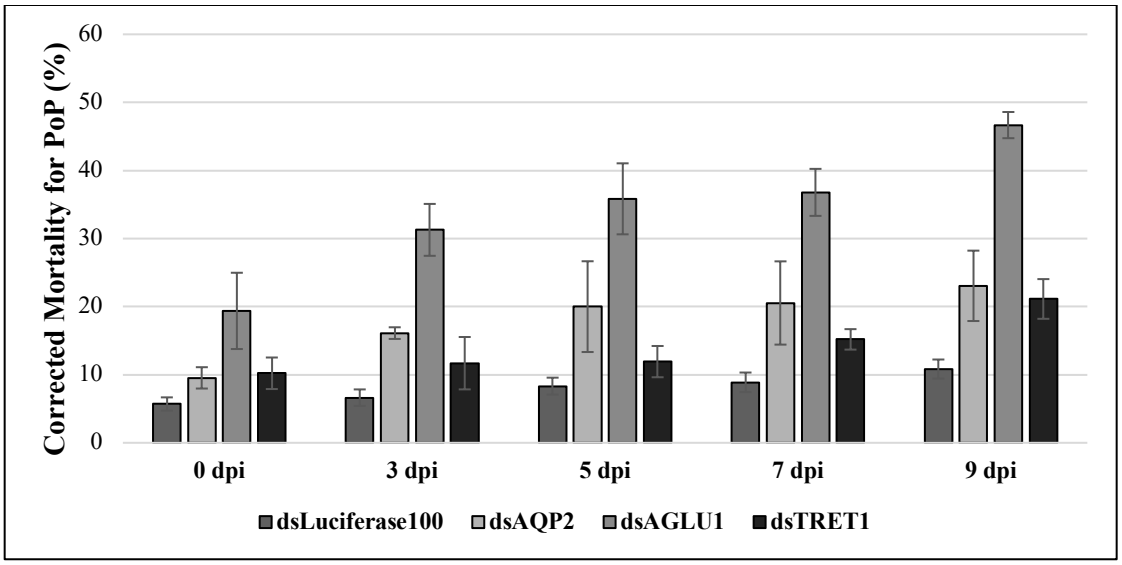

Fig. 8. Percentage of corrected mortality for PoP, on 0-, 3-, 5-, 7-, and 9dpi resulted from ingestion of single dsRNA in 20% sucrose solution carried out for three replicates. Corrected mortality was calculated using the formula of Abbott's and Schneider-Orelli's, and 'dpi' indicates days post-ingestion. The dsLuciferase100 is equal to 100ng/µL of Luciferase dsRNA. Bars and error bars indicate the mean and SE, respectively.

To determine if dsRNA ingested from sucrose solution induced target mRNA knockdown, RT-qPCR amplification was carried out to assess target mRNA levels in PoP teneral adults. The relative gene expression of each gene was determined at 9dpi. Silenced *AGLU1* resulted in 59.15% knockdown, compared to the dsLuciferase100 non-target negative control. The observed decrease in *AGLU1* transcripts was consistent with the levels of mortality observed. Knockdown of dsAQP2 resulted in a 22.1% decrease in the *AQP2* mRNA transcript, compared to the dsLuciferase100 non-target negative control. Relative knockdown result is consistent with mortality values. The partial *AQP2* silencing is possibly attributable to compensatory expression of a homolog in the gut or elsewhere in the psyllid body. This is consistent with previous study in which four AQPs were

evaluated for PoP knockdown that showed greater expression of *AQP2* transcript compared to knockdown of the other three targets (Ibanez, Hancock, and Tamborindeguy 2014).

In contrast, a 58.11% *TRET1* knockdown, compared to the dsLuciferase100[16] non-target negative control showed lower percent mortality for psyllids post-RNAi knockdown of *TRET1*, suggesting that *TRET1* silencing minimally affected psyllid survival. This observation may possibly be explained by the presence of parallel gene expression pathways for gut-expressed isoforms that compensate for the *TRET1* function. The TRET1 protein expressed may also have contributed to the apparent rescue phenomenon for PoP (Fig. 9). The qRT-PCR results demonstrated that post-dsRNA ingestion the mRNA targets exhibited knockdown of gene expression in PoP.

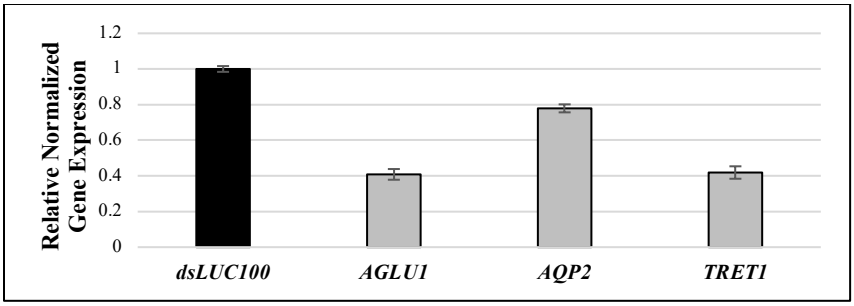

Fig. 9. Silencing of the target genes as a single dsRNA treatment. The relative gene expression level of PoP target genes normalized to the expression value of the *RPL5* gene, and the changes were evaluated using CFX Maestro qPCR analysis software (www.bio-rad.com). The student's t-test significant groups (p-value <0.0.5) are indicated by the asterisk (*) when the experimental group was compared to the control group. The *dsLUC100* is equal to 100ng/µL of Luciferase dsRNA.

[16] The dsLuciferase100 is equal to 100 ng/µL of Luciferase dsRNA

3-2 Pair dsRNA Ingestion by PoP

To determine if knockdown can result in an apparent additive or synergistic outcome, different combinations of dsRNAs were tested. Simultaneous silencing of *AGLU1* and *TRET1* resulted in 35.21% of nymphs' PoP mortality on 3dpi and slightly increased by 9dpi to 46.70%. Following 5dpi of dsRNAs, PoP nymphal mortality reached 40%, a cut-off mortality caused by dsRNA to be considered as a promising target. Rapid onset of mortality post-ingestion of dsRNA is a key for effective psyllid control by reducing reproduction and potentially reducing the rate of CLas transmission. These were among the most significant mortality values for both single and dsRNA pairs and were comparable to values observed with dsAGLU1 alone. Although the mortality increased when dsAGLU1 was co-delivered with dsTRET1, results suggested that dsTRET1 was not a potential target for mortality based on the observation that a similar percentage mortality occurred when dsAGLU1 was delivered alone and together with dsTRET1.

In contrast, the mortality caused by RNAi-mediated knockdown of *AGLU1* and *AQP2* started with 23.32% on 3dpi and didn't reach the mortality threshold by 9dpi. Likewise, the PoP mortality caused by co-delivery of dsAQP2 and dsTRET1 started with 23.39% on 3dpi and didn't reach the mortality threshold by 9dpi. According to the results, it appears that *AQP2* and *TRET1* have additive interaction because the effect of two genes is comparable to the sum of the effect of the two genes taken separately (Fig. 10).

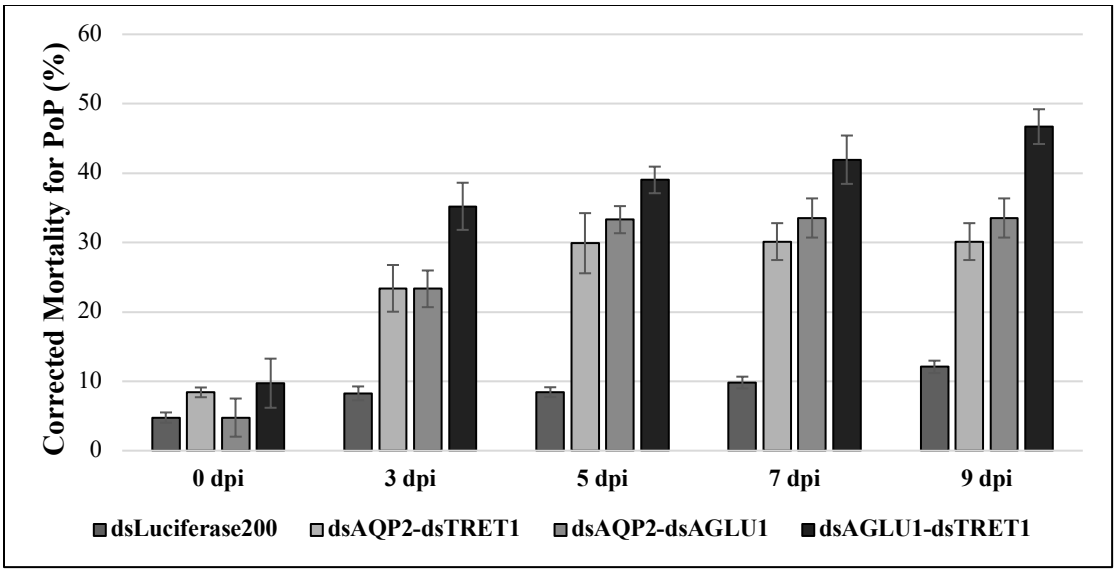

Fig. 10. Percentage of corrected mortality for PoP, on 0-, 3-, 5-, 7-, and 9dpi resulted from ingestion of pair dsRNA in 20% sucrose solution. The experiment was done in three replicates. Corrected mortality was calculated using the formula of Abbott's and Schneider-Orelli's, and 'dpi' indicates days post-ingestion. The dsLuciferase200 is equal to 200ng/µL of Luciferase dsRNA. Bars and error bars are shown for the mean and SE, respectively.

A 41.22% and 31.52% gene knockdown were observed for *AGLU1* and *TRET1* in co-ingestion of dsAGLU1 and dsTRET1, respectively, compared to dsLuciferase200 non-target negative control. The treatment of dsAQP2-dsTRET1 resulted in 31.45% and 34.06% gene knockdown of *AQP2* and *TRET1*, respectively, compared to dsLuciferase200 non-target negative control. Moreover, co-ingestion of dsAGLU1 and dsAQP2 resulted in 26.25% and 28.45% gene knockdown for *AGLU1* and *AQP2*, respectively, compared to dsLuciferase200 non-target negative control (Fig. 11).

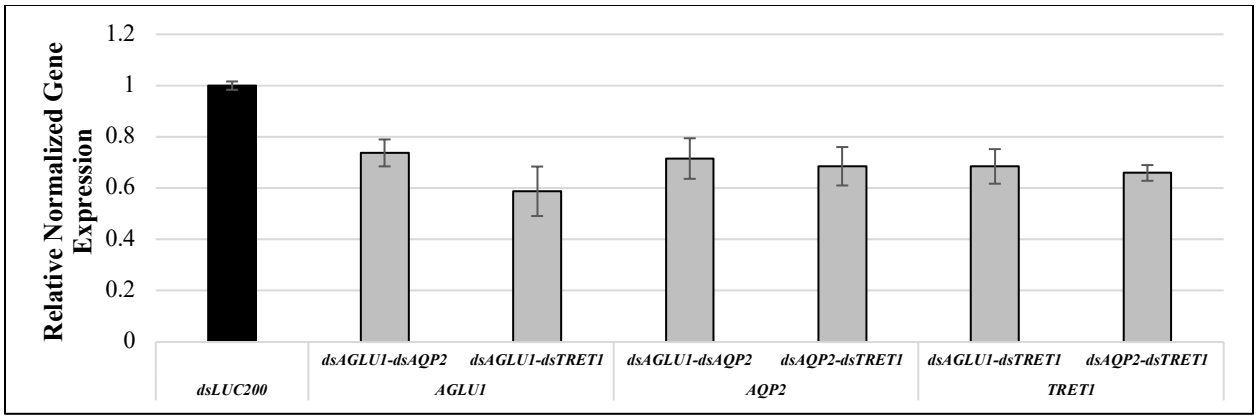

Fig. 11. Silencing of the target genes as a single dsRNA treatment. The relative gene expression level of PoP target genes normalized to the expression value of the *RPL5* gene, and the changes were evaluated using CFX Maestro qPCR analysis software (www.bio-rad.com). The student's t-test significant groups (p-value <0.0.5) are indicated by the asterisk (*) when the experimental group was compared to the control group. The *dsLUC200* is equal to 200ng/µL of Luciferase dsRNA.

3-3 Stacked dsRNA Ingestion by PoP

It has been contended that the RNAi effectiveness and durability for insect pest control can be improved by stacking dsRNA constructs against multiple targets in the pest species (Luo et al. 2017). This approach is valuable where the target genes have different molecular, but related, physiological functions. The incomplete suppression of multiple contributing gene functions can synergistically mediate physiological collapse. Such a scenario appears to be the most likely explanation for the enhanced efficacy of stacking dsRNA constructs against multiple osmoregulation genes in the aphid and PoP (Tzin et al. 2015). Stacked of dsAGLU1, dsAQP2, and dsTRET1 resulted in 21.36% PoP mortality on 3dpi and 30% on 5dpi and surged to 53.18% by 9dpi (Fig. 12).

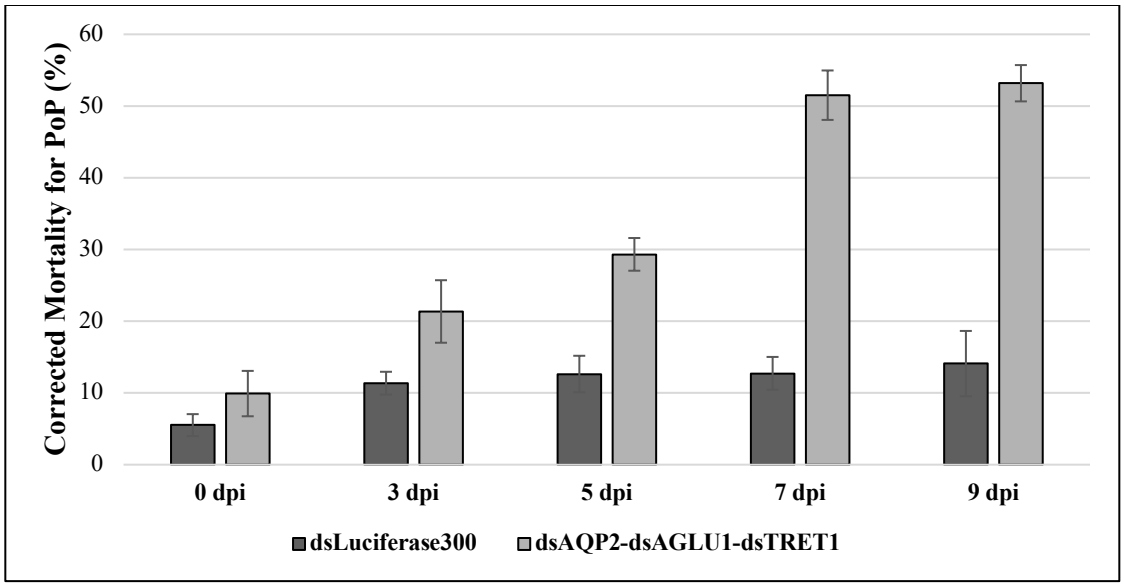

Fig. 12. Percentage of corrected mortality for PoP, on 0-, 3-, 5-, 7-, and 9dpi resulted from ingestion of pair dsRNA in 20% sucrose solution. The experiment was done in three replicates. Corrected mortality was calculated using the formula of Abbott's and Schneider-Orelli's, and 'dpi' indicates days post-ingestion. The dsLuciferase300 was delivered at 300ng/µL of Luciferase dsRNA. Bars and error bars indicate the mean and SE, respectively.

Trehalase is results from the breakdown of glucose from trehalose. Ingestion of trehalase dsRNA, which was tested in two combinations, resulted in both the earliest and the highest level of mortality for dsAGLU1-dsTRET1 and dsAQP2-dsAGLU1-dsTRET1 treatments, respectively. Previous studies have shown that *TRE1* and *TRE2* are compensatory, based on the increased expression of each gene, when other gene was silenced (Zhao et al. 2016).

The addition of dsTRE1 and dsTRE2 resulted in 37.64% and 33.75% PoP mortality on 3dpi in four and five stacked dsRNA treatments, respectively. The PoP mortality by 3dpi significantly increased compared to mortality for the triply-stacked dsRNA treatment, which was 21.26%. Here,

PoP mortality by 9dpi was increased by about 12% compared to the triply-stacked dsRNA treatment, at 53.18% (Fig. 13).

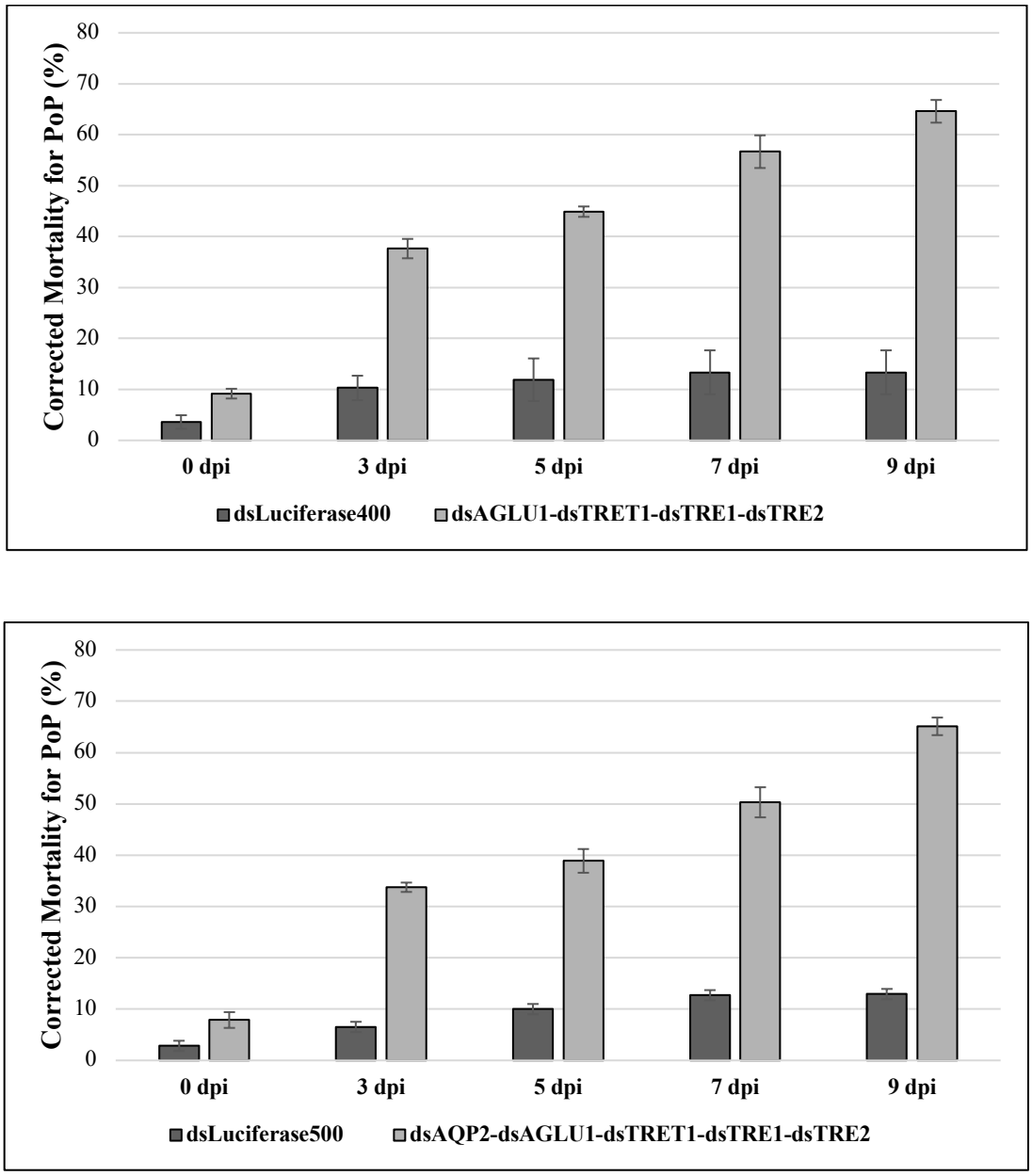

Fig. 13. Percentage of corrected mortality for PoP, on 0-, 3-, 5-, 7-, and 9dpi resulted from ingestion of pair dsRNA in 20% sucrose solution. The top graph represents the corrected mortality of PoP resulted from stacked of 4 dsRNA. The bottom graph represents the corrected mortality of PoP resulted from stacked of 5 dsRNA. The experiment was done in three replicates. Corrected mortality was calculated using the formula of Abbott's and

Schneider-Orelli's, and 'dpi' indicates days post-ingestion. The dsLuciferase400 and dsLuciferase500 represent 400- and 500ng/µL Luciferase dsRNA. Bars and error bars indicate the mean and SE, respectively.

Co-ingestion of three dsRNAs, including dsAQP2, dsAGLU1, and dsTRET1, resulted in 45.28%, 40.40%, and 39.94% knockdown in *AGLU1*, *AQP2*, and *TRET1* expression, respectively, compared to dsLuciferase300 non-target negative control.

When trehalase dsRNA was included, RNAi-mediated knockdown of *AQP2* showed a 29.01% for the five stacked dsRNA. The gene *AGLU1* showed 41.09% knockdown in *AGLU1* expression among the stacked four dsRNAs, and 47.17% in the stack of five dsRNAs. The relative expression of *TRE2* was reduced by 20.16% for the stacked five dsRNAs, and 53.23% in the stacked of four dsRNAs, compared to the ds Luciferase non-target control. The dsTRET1 caused 34.16% and 51.16% gene knockdown of *TRET1* for the stacked four and five dsRNAs, respectively. Knockdown of *TRE1* resulted in 39.27% gene silencing for the four stacked dsRNAs, compared to 63.78% for the five stacked dsRNAs (Fig. 14 and Table3).

Table3. Target genes knockdown in stacked of four and five treatments

Target Gene	Knockdown[17] (%)	
	5dsRNA	4dsRNA
AGLU1	47.17	41.09
AQP2	-	29.01
Tret1	5116	34.16
TRE1	63.78	39.27
TRE2	53.23	20.16

[17] The relative gene expression level of PoP target genes normalized to the expression value of the *RPL5* gene, and the changes were evaluated using CFX Maestro qPCR analysis software (www.bio-rad.com). The student's t-test significant groups (p-value <0.0.5) are indicated by the asterisk (*) when the experimental group was compared to the control group.

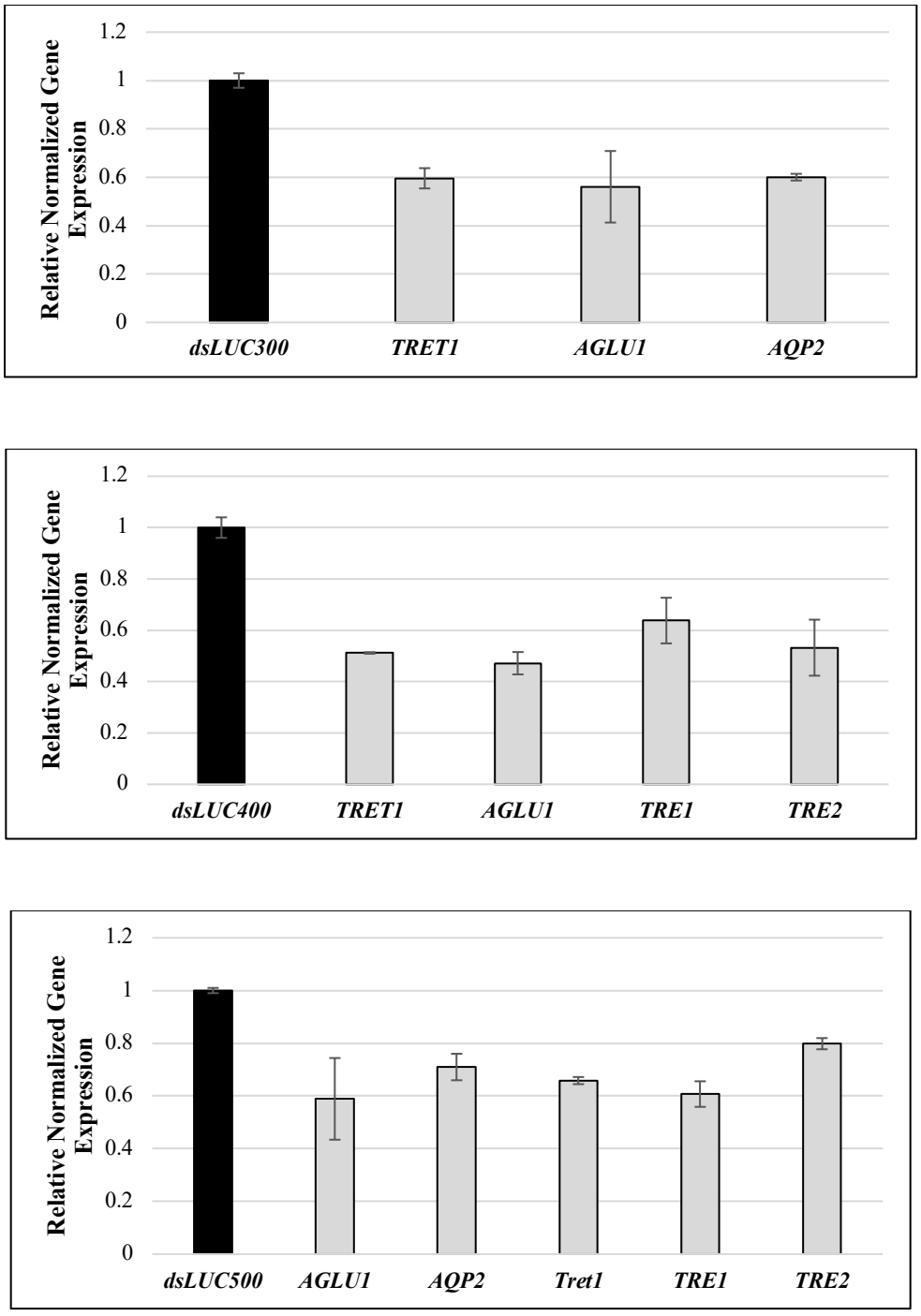

Fig. 14. Silencing of the target genes as a single dsRNA treatment. The top graph represents the relative normalized gene expression of psyllids in stacked of three dsRNA treatment. The middle graph represents the relative normalized gene expression of psyllids in stacked of four dsRNA treatment. The bottom graph represents the relative normalized gene expression of psyllids in stacked of five dsRNA treatment. The relative gene expression level

of PoP target genes normalized to the expression value of the *RPL5* gene, and the changes were evaluated using CFX Maestro qPCR analysis software (www.bio-rad.com). The student's t-test significant groups (p-value <0.0.5) are indicated by the asterisk (*) when the experimental group was compared to the control group. The *dsLUC200* represents 200ng/µL of Luciferase dsRNA.

4- CONCLUSIONS

The use of RNA interference to achieve gene knockdown in eukaryotes, including arthropods and nematodes, initially was used in functional or 'reverse-genetics' studies. More recently, it has been shown that RNAi has considerable potential to control insect pests and insect vectors of plant pathogens they transmit such as plant viruses and the fastidious bacterium, Liberibacter. In this scenario, insect ingest dsRNA, for example, through artificial feeding devices, which allows the dsRNA to be delivered to directly to the gut. The dsRNA must be delivered from the gut lumen into the gut cells for the target gene silencing, occurring through ingestion of a sucrose diet. An advantage of studying RNAi through feeding experiments is that the whole process of non-cell-autonomous RNAi can be evaluated.

Several important factors influencing the success of RNAi on targets are the dsRNA concentration, the sequence nucleotide of the target, the length of the dsRNA fragment, the constancy of the silencing effect, and the life stage the target insect. A proper delivery system for dsRNAs might be the most significant limitation of RNAi studies for insects. dsRNA delivery methods have been demonstrated for sap-sucking insects, such as sharpshooters, aphids, whiteflies, and psyllids (Vyas et al. 2017; Paredes-Montero, Arif, and Brown 2022; Chen et al. 2016; Tzin et al. 2015; L. Zhao et al. 2016). This study demonstrated specific RNAi effects in PoP by the oral acquisition of dsRNA from a sucrose diet. The 'doses' or concentrations of dsRNA required for mortality or the development of a corresponding phenotype varies among insect genera and even species. Here, 100 ng/µL of target gene(s) dsRNA was presented to PoP in a sterile 20% sucrose solution. Third instar nymphs were allowed a 2-d feeding-access to the sucrose solution containing dsRNA. The dsRNA was shown to be highly stable in the sucrose solution, beyond when psyllids were transferred to tomato plants, indicating the viability of this approach to screen gene targets for

mortality or other phenotypes in psyllids. Oral delivery of dsRNAs, combined with qRT-PCR analysis of gene expression, offers a feasible approach for screening dsRNAs as biopesticides for psyllid abatement.

Pest control through RNAi can be performed in different ways, including host-induced gene silencing (HIGS), spray-induced gene silencing (SIGS), or virus-induced gene silencing (VIGS) (Christiaens et al. 2020). HIGS entails the transgenic crop's design that expresses the dsRNA specific for the pest. The first commercial RNAi product targeting an insect pest is a transgenic corn crop, which expresses a hairpin dsRNA targeting the *snf7* gene in the Western corn rootworm, *Diabrotica virgifera*. Virus-induced gene silencing (VIGS) is based on engineered viruses to produce the desired dsRNA in the pest. The virus's infection and replication would result in the dsRNA production in the insect cells (Kolliopoulou et al. 2017). The Flock house virus (FHV) has been modified to express Drosophila melanogaster-specific dsRNA (Taning et al. 2018). SIGS, spray-based dsRNAs pesticide, is a non-transgenic method that dsRNAs can be absorbed from leaves, roots, and injected trunks (Joga et al. 2016). Since dsRNA has low persistence in the environment, SIGS needs unique formulations to increase its stability. Besides, the target pests' exposure through SIGS might be lower than transgenic plants because plants will continuously express the dsRNA. Therefore, spray-based applications might become a reality for insects more sensitive to dsRNA ingestion through dietary uptake (Christiaens et al. 2020).

This study screened the PoP mortality resulting from the ingestion of the target dsRNA. Among the targets screened for knockdown, dsAGLU1, dsAGLU1-dsTRET1, dsAGLU1-dsAQP2-dsTRET1, dsAGLU1-dsTRET1-dsTRE1-dsTRE2, and dsAGLU1-dsAQP2-dsTRET1-dsTRE1-dsTRE2 treatments yielded >40% PoP mortality. Based on PoP ‘Fast track screening’ results, the

AGLU1, *TRET1*, and *TRE1/2* targets were selected for evaluation in the ACP–CLas system. RNAi-mediated knockdown of *AQP2* and *TRET1*, at 20-30% PoP mortality, fell below the 40% minimum threshold, and will not be tested in the ACP screen. Based on preliminary experiments in PoP, it is hypothesized that stacking dsTRE1/2 and dsAGLU1 will result in ACP genes knockdown and at least 40% mortality by 9dpi. For future studies, direct evidence of gene silencing by localization of target gene transcripts in the psyllid gut by *in situ* hybridization in dsRNA treated and untreated control psyllids is of interest to corroborate indirect evidence provided by qPCR knockdown and a mortality phenotype.

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