RNA interference against gut osmoregulatory genes in phloem-feeding insects

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Abstract

In planta RNAi (i.e. plants engineered to synthesize active RNAi molecules) has great potential as a strategy to control insect crop pests. This study investigated the impact of RNAi against osmoregulatory genes expressed in the gut of two phloem-feeding species, the green peach aphid *Myzus persicae* and the potato/tomato psyllid *Bactericera cockerelli*. The target genes comprising candidate gut sucrase, aquaporin and sugar transporter genes were identified by mining insect genomic and transcriptomic datasets for genes orthologous to empirically-tested osmoregulatory genes of the pea aphid *Acyrtosiphon pisum*. Insects feeding on plants with RNAi against the target genes exhibited elevated hemolymph osmotic pressure (a predicted effect of perturbed osmotic function) and some reduction in performance, especially offspring production in *M. persicae* and mortality in *B. cockerelli*, associated with up to 50% reduction in mean expression of the target genes. The effects were particularly pronounced for insects treated with RNAi against multiple osmoregulatory genes, i.e. combinatorial RNAi, suggesting that the partial silencing of multiple genes with related roles can yield greater functional impairment than RNAi against a single gene. These results demonstrate the potential of RNAi against osmoregulatory genes, but further advances to improve the efficacy of RNAi in phloem-feeding insects are required to achieve effective pest control.

Keywords: aquaporin; *Bactericera cockerelli*; *Myzus persicae*; RNAi; sucrase; sugar transporter

1. Introduction

Insects that feed on plant phloem sap are generally deleterious to their plant hosts by both direct damage (including chlorosis, necrosis, wilting, stunting and gall formation) and transmission of plant viruses and phytoplasmas (Dolling, 1991; Jones, 2014; Sugio and Hogenhout, 2012). All phloem-feeding insects are members of the order Hemiptera (Douglas, 2003), and many, including various species of aphids, whiteflies, psyllids, scale insects, leafhoppers, planthoppers and heteropteran bugs, are important crop pests (Edwards and Gatehouse, 2007). The increasing incidence of resistance to chemical insecticides (Bass et al., 2014; Whalon et al., 2010) is creating a strong demand for novel strategies to control these insect pests. This demand is compounded by the limited success of traditional plant breeding programs to generate durably resistant cultivars (Ragsdale et al., 2011; Alvarez et al., 2006; Merrill et al., 2014) and the dearth of naturally-occurring Bt toxins that are active against hemipterans, although recombinant Bts with greater toxicity are being developed (Chougule et al., 2013; Porcar et al., 2009).

In planta RNAi (i.e. plants engineered to synthesize active RNAi molecules) has great potential for the control of agricultural insect pests, including phloem-feeding hemipterans (Burand and Hunter, 2013; Scott et al., 2013; Xue et al., 2012). As first demonstrated for chewing insect pests, the western corn rootworm *Diabrotica virgifera virgifera* (Baum et al., 2007) and the cotton bollworm *Helicoverpa zea* (Mao et al., 2007), insects can suffer high mortality when feeding on plants producing RNAi constructs targeting essential insect genes. Statistically significant effects of *in planta* RNAi on gene expression of hemipteran target genes have been demonstrated, for example for the potato/tomato psyllid *Bactericera cockerelli* (Wuriyanghan and Falk, 2013), the planthopper *Nilaparvata lugens* (Zha et al., 2011), the aphid

63 *Myzus persicae* (Elzinga et al., 2014; Pitino et al., 2011) and the whitefly *Bemisia tabaci* (Thakur
64 et al., 2014). These studies have variously used virus-induced gene silencing (VIGS), transient
65 *Agrobacterium*-mediated transformation and stable transgenic plants; and they have focused
66 principally on genes required for cell function (e.g. V-ATPase, actin), genes that are expressed
67 specifically in the gut (e.g. digestive enzymes and nutrient transporters), or genes coding salivary
68 proteins. Essential genes expressed in the insect gut are particularly promising candidate targets
69 because the delivery of ingested RNAi molecules does not depend on systemic spread of RNAi
70 in the insect body (Scott et al., 2013; Wuriyangan and Falk, 2013; Yu et al., 2013).

71 This study investigates the efficacy of *in planta* RNAi against genes that function in
72 osmoregulation and are expressed in the gut of phloem-feeding hemipterans. Osmoregulation is
73 a critical physiological function for all phloem-feeding insects because phloem sap has an
74 exceptionally high osmotic pressure, linked to its high sugar content (Douglas, 2003). Insect
75 osmoregulatory function protects against the transfer of water from body fluids down the
76 osmotic gradient to the gut, and the consequent dehydration and death (Karley et al., 2005).
77 Three classes of insect genes expressed in the gut have been implicated in osmoregulation: (1) a
78 gut sucrase-transglucosidase, identified and characterized in the pea aphid *Acyrtosiphon pisum*,
79 which mediates the hydrolysis of ingested sucrose to its constituent monosaccharides, glucose
80 and fructose, and oligosaccharide synthesis from glucose (Cristofolletti et al., 2003; Price et al.,
81 2007); (2) a gut aquaporin that facilitates the flux of water down its osmotic gradient,
82 characterized in the leafhopper *Cicadella viridis* (Le Caherec et al., 1996), the pea aphid
83 (Shakesby et al., 2009), the whitefly *Bemisia tabaci* (Mathew et al., 2011), the potato/tomato
84 psyllid *Bactericera cockerelli* (Ibanez et al., 2014) and the western tarnished plant bug (*Lygus*
85 *hesperus*) (Fabrick et al. 2014); and (3) sugar transporters that remove monosaccharides from the

gut lumen, characterized in the pea aphid (Price and Gatehouse, 2014; Price et al., 2010) and brown planthopper *Nilaparvata lugens* (Kikuta et al., 2010).

The focus of this study was two major phloem-feeding pests: the aphid *M. persicae*, which is the most important aphid pest world-wide as a result of its extreme polyphagy and capacity to vector many plant viruses (van Emden and Harrington, 2007); and the psyllid *B. cockerelli*, which vectors the devastating plant pathogen Candidatus *Liberibacter psyllae* among solanaceous plants including economically-important crops (Butler and Trumble, 2012). The specific aims of this study were to identify candidate osmoregulatory genes in these insects by mining insect genomic and transcriptomic datasets; and to quantify the effects of RNAi against these genes on osmotic function, as determined by the osmotic pressure of insect hemolymph and insect performance. The RNAi constructs were designed both singly and in combination, to investigate the impacts of targeting multiple genes with complementary predicted functions on insect osmotic function.

2. Materials and Methods

2.1 Plants and Insects

Nicotiana benthamiana seeds were germinated in Metro Mix 360 (Scotts, Marysville, OH, USA) and transplanted after 2 weeks to Cornell mix (by weight, 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocote slow-release fertilizer [Scotts], and 1% Unimix [Peters, Everris, The Netherlands]) in Conviron growth chambers (Conviron, Winnipeg, Canada) in 20 × 40-cm nursery flats with a photosynthetic photon flux density of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 23 °C with 16 h light: 8 h dark and 50% relative humidity. *Agrobacterium*-mediated transient expression was performed using plants that were 4 to 5 weeks old. Seeds of *Solanum lycopersicum* cv.

Florida Lanai (tomato) were planted in Cornell mix and grown in Conviron chambers as described above.

A parthenogenetic red tobacco-adapted lineage of the green peach aphid *Myzus persicae* was obtained from S. Gray (USDA Plant Soil and Nutrition Laboratory, Ithaca, NY, USA) (Hayward et al., 2011) and maintained on tobacco plants with a 16 h day ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 23-24 °C) and an 8 h night (19-23 °C) at 50-60% relative humidity. A laboratory culture of the potato/tomato psyllid *Bactericera cockerelli* derived from a population in Texas USA and provided by C. Tamborindeguy (Texas A&M University) was maintained on tomato plants under 14 h:10 h light:dark cycle with 28 °C during the light and 25.5 °C during the dark.

2.2 Identification of target genes

Candidate aquaporin (AQP) and sucrose (SUC) genes in *M. persicae* and *B. cockerelli*, and the candidate gut sugar transporter *ST4* in *M. persicae* were identified by reciprocal blasts between the translated sequences of *A. pisum* RefSeq genes *ApAQP1* (ACYPI006387, Gene ID: 100165436), *ApSUC1* (ACYPI000002, Gene ID: 100144774) and *ApST4* (ACYPI001980, Gene ID: 100160702), against the draft *M. persicae* genome (<http://www.aphidbase.com/>) and a published *B. cockerelli* transcriptome (Naccappa et al., 2012). For the *B. cockerelli* analysis, raw transcript reads were assembled using Trinity (version: trinityrnaseq_r20131110) with default parameters (Haas et al., 2013) and the translated amino acid sequences of the longest ORFs were used. The sequences of *MpAQP1* and *MpSUC1* were determined by the Sanger method using *M. persicae* cDNA and primers listed in Table S1.

To identify candidate *B. cockerelli* sugar transporter (ST) genes, sequences with the sugar transporter domain (PF00083) were identified in the *B. cockerelli* transcriptome using HMMER

v. 3 ($e \leq 0.001$) (Eddy, 2011) and then filtered by InterProScan to retain only those with sugar transporter signature (Zdobnov et al., 2001). We adopted a three-step strategy to obtain candidate *B. cockerelli* ST genes with osmoregulatory function. First, the phylogenetic relationship between the *B. cockerelli* ST genes and *A. pisum* ST genes identified by Price and Gatehouse (2014) was analyzed using a Neighbor Joining (NJ) phylogenetic tree constructed in MEGA 4.0.2 with 1000 bootstrap replicates (Tamura et al., 2007). Then, the *A. pisum* ST genes with $\geq$ five-fold greater expression in the gut relative to the whole body were identified by analysis of RNAseq transcriptome datasets of *A. pisum* whole body and guts (NCBI SRA: Accession No. SRP053295). Finally, the *B. cockerelli* ST genes related to the gut-enriched *A. pisum* ST genes were obtained by reference to the NJ phylogenetic tree.

We adopted a further criterion for assigning genes to candidate osmoregulatory function in the gut of *M. persicae* and *B. cockerelli*: that their expression was enriched $\geq$ two-fold in the gut relative to the whole body. For this test, the expression of candidate *AQP*, *SUC* and *ST* genes of *M. persicae* and *B. cockerelli* was tested by qRT-PCR with primers listed in Table S1. Total RNA was extracted by the RNeasy Mini kit (Qiagen, Venlo, Limburg, USA) following manufacturer's instructions from whole bodies and dissected guts of *M. persicae* (5 bodies and 60 guts of 5-day-old adult apterae) and *B. cockerelli* (10 bodies and 56 guts of 3-to-6-day-old adult insects). First-strand cDNAs were synthesized using random hexamers and SuperScript® II reverse transcriptase kit (Invitrogen, Grand Island, NY, USA). Each reaction comprised 10 μ l 2 $\times$ iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 2.4 μ l primer mix (2.5 μ M), 2.5 μ l cDNA and 5.1 μ l H₂O on C1000 Thermal cycler with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) under the following program: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 sec at 95°C, 1 min at 60°C, followed by a dissociation step (60 °C for 10 s, and then

0.5 °C was added for 10 s until 95 °C). Dissociation curves confirmed single gene-specific peaks without primer dimerization. All assays include 3 technical replicates with template-free and reverse transcriptase samples as negative controls. The $\Delta\Delta C_t$ method was adopted to quantify the target gene, normalized to the housekeeping gene RPL7 for *M. persicae* (Ramsey et al., 2014) and actin for *B. cockerelli* (Wuriyangan et al., 2011).

2.3 Transient expression of RNAi constructs in plants

To make *M. persicae* and *B. cockerelli* expression silencing constructs, mRNA and cDNA were prepared as described previously (Elzinga et al, 2014). Briefly, each sample of aphids and psyllids was frozen in liquid nitrogen in a 2 ml microcentrifuge tubes containing two 3-mm stainless steel balls (Abbot Ball Company, West Hartford, CT, USA) and ground to a fine powder using a Harbil model 5G-HD paint shaker (Fluid Management, Wheeling, IL, USA). RNA was extracted from homogenized insect tissue using TRI Reagent (Sigma, St. Louis, MO, USA) and purified with the SV Total RNA Isolation Kit with on-column DNase treatment (Promega, Madison, WI, USA). From 1 µg of RNA, cDNA was reverse-transcribed using SMART MMLV reverse transcriptase (Clontech, Mountain View, CA, USA), with oligo-dT12-18 as a primer.

PCR primers (Table S1) were designed to amplify 250-500 bp of the coding regions fused to attB1 and attB2 borders compatible for the Gateway cloning system (Invitrogen). Each selected fragment for gene silencing was analyzed by SOL Genomics Network VIGS tool (<http://www.solgenomics.net>) to avoid off-gene silencing in the target plants (Fernandez-Pozo et al., 2014). Due to the large number of predicted ST genes in *B. cockerelli*, 250 bp of each gene were fused into one fragment using gBlocks Gene Fragments (Integrated DNA Technologies,

178 Coralville, Iowa, USA). Using EX Taq DNA polymerase (Takara, Shiga, Japan), genes were
179 cloned into entry vector pDONR207 using BP Clonase (Invitrogen) and transformed into
180 *Escherichia coli* strain DH5 α bacteria. An LR reaction with LR Clonase (Invitrogen) was used to
181 move constructs into the *Tobacco Rattle Virus* (TRV) TRV2 fragment (Senthil-Kumar and
182 Mysore, 2014).

183 Plasmids carrying the two genomic segments of TRV (TRV1, and TRV2 containing the cloned
184 RNAi fragments) were transformed into *Agrobacterium tumefaciens* strains GV2260 or GV3101.
185 *A. tumefaciens* bacteria were cultured in 20 ml Luria Bertani (LB) medium supplemented with
186 kanamycin (50 mg ml⁻¹) and rifampicin (15 mg ml⁻¹). After two days, the bacterial cells were
187 transferred to induction medium (10 mM MgCl₂, 10 mM 2-(N-morpholino)ethanesulfonic acid
188 [MES] buffer pH 5.6, and 200 μ M acetosyringone) for 6 h at room temperature, and then
189 transferred to infiltration medium (10 mM MES buffer pH 5.5). *A. tumefaciens* cultures with
190 plasmids encoding TRV1 and TRV2 (with RNA corresponding to the gene of interest) were
191 mixed (1:1) and infiltrated to the leaves with a needleless 1 ml tuberculin syringe. Strain
192 GV2260 was used to infiltrate leaves of *N. benthamiana* plants three weeks after germination
193 (Vaghchhipawala et al., 2011), with constructs targeting *M. persicae* gene expression. Strain
194 GV3101 was used to infiltrate *S. lycopersicum* (tomato) cotyledons one week after germination,
195 with constructs targeting *B. cockerelli* gene expression. Gene silencing occurs in newly-
196 developed non-inoculated leaves and can persist in tobacco and tomato plants for more than two
197 years (Senthil-Kumar et al, 2014). As a visual indicator of the progression of virus infection, a
198 previously described TRV2 clone with a fragment of the *N. benthamiana* *PDS* (phytoene
199 desaturase) gene (Velasquez et al., 2009; Vaghchhipawala et al., 2011) was used to cause a
200 photobleaching phenotype in both *N. benthamiana* and *L. esculentum*. Both species showed the

photobleaching phenotype starting 9 days after infiltration and continuing the three to five weeks during which insect bioassays were performed. Additional controls consisted of a 365-bp fragment of the jellyfish green fluorescent protein (GFP), which causes no off-target silencing in either *N. benthamiana* or *S. lycopersicum* (Vaghchhipawala et al., 2011), and, for *S. lycopersicum*, an empty vector control plasmid. The empty vector control was omitted for *N. benthamiana* because of differences in growth of plants infected with the empty virus vector and those carrying TRV2-*GFP* or other inserts. Primers used for gene cloning are listed in Table S1.

To confirm expression down-regulation in insects raised on plants expressing RNAi constructs, RNA was prepared as described above. After DNase treatment, 1 µg RNA was reverse-transcribed using SMART MMLV reverse transcriptase (Clontech) and 400 nM oligo-dT12-18 primer (Qiagen). Reactions were performed with 5 µl Power SYBR Green (Applied Biosystems, Grand Island, NY, USA) and 800 nM primer mix in the 7900HT instrument (Applied Biosystems) with an initial incubation at 95 °C for 10 min. The following cycle was repeated 40 times: 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The CT values were quantified and analyzed according to the standard curve method. Primers used for gene expression analysis are listed in Table S1.

2.4 Osmotic pressure of hemolymph

Each adult insect was immersed in water-saturated paraffin oil and legs were cut off using micro-scissors. The exuding hemolymph from the cut openings was collected using a silanized capillary tube, between two drops of oil, to avoid evaporation. The osmotic pressure of 0.05-0.5 nl samples was determined by freezing point depression in a Nanoliter Osmometer (Otago Osmometers Ltd, New Zealand), calibrated against 0-0.6 M sodium chloride.

2.5 Insect bioassays

Three weeks after infiltration of TRV expression silencing constructs, new leaves that were virus-infected were used for insect bioassays. Clip cages (1.5 cm diameter) were attached to the abaxial side of fully-developed *N. benthamiana* leaves, and single *M. persicae* adults were allowed to deposit offspring over 24 h. Three nymphs were retained in each cage and allowed to develop to adulthood over 7 days. At this point all adult aphids in each cage were collected and pooled into one RNA preparation for gene expression analysis by qRT-PCR. Average weight of the adult aphids in each cage was determined on a Sartorius model ME 5 precision balance (Data Weighing Systems, Inc., Elk Grove, IL, USA). Hemolymph osmotic pressure was measured from individual insects. The number of offspring deposited per adult aphid in each cage over the subsequent 8 days was scored.

For *S. lycopersicum* experiments, newly-developed leaves, distal to the virus inoculation site, were used for insect bioassays three weeks after the time of inoculation. Three pairs of teneral adult *B. cockerelli* were transferred to 30 replicate clip-cages (28 ml plastic portion cup with top diameter 43 mm, bottom diameter 30 mm, height 33 mm), each attached to the upper side of a lower expanded leaf of the test tomato plants. The number of live insects and deposited eggs were counted on day-4 and day-8. Gene expression and hemolymph osmotic pressure determinations were conducted on one pair of insects per cage on day-4, and the remaining live insects at day-8.

2.6 Statistical analysis

All data sets were checked for normal distributions by the Anderson Darling test and homogeneity of variances by the Levine and Bartlett tests. The insect performance and osmotic

pressure datasets and qRT-PCR datasets for *M. persicae* reared on RNAi-plants met these criteria, and were analyzed by ANOVA with Tukey's *post hoc* test, or by t-tests, as appropriate. The qRT-PCR datasets for *B. cockerelli* reared on RNAi-plants displayed heterogeneous variances and were analyzed by Kruskal-Wallis test. Statistical comparisons were conducted with JMP software (SAS Institute, Miami, FL, USA) and Minitab 17.

3. Results

3.1 Candidate osmoregulatory genes in *Myzus persicae* and *Bactericera cockerelli*

To identify candidate aquaporin and sucrase genes with osmoregulatory function in the guts of *M. persicae* and *B. cockerelli*, we made use of the sequence of the aquaporin and sucrase genes of *Acyrtosiphon pisum* (*ApAQPI* and *ApSUCI*), which have empirically validated osmoregulatory functions (Price et al., 2007; Shakesby et al. 2009). tBLASTx query of the *A. pisum* sequences against the draft *M. persicae* genome and *B. cockerelli* transcriptome generated single reciprocal best hits. Because the *M. persicae* sequences, which we named *MpAQPI* and *MpSUCI*, were derived from genomic data, the presence and sequence of the cognate transcripts were confirmed by Sanger sequencing of cDNA obtained from our *M. persicae* culture (Fig. S1A, B). The *B. cockerelli* cDNA sequences were designated *BcAQPI* (following the nomenclature of Ibanez et al. (2014)) and *BcSUCI*. *BcAQPI* expression has been shown previously to be upregulated in the *B. cockerelli* gut, relative to the whole body (Ibanez et al., 2014); and qRT-PCR analysis using the cDNA of the whole body and dissected guts of *M. persicae* and *B. cockerelli* as templates confirmed that transcripts of *MpAQPI*, *MpSUCI* and *BcSUCI* are $\geq$ two-fold enriched in the gut (Table 1).

Our strategy to identify a sugar transporter (ST) with osmoregulatory function in *M. persicae* was based on published evidence that *ST4* in *A. pisum* (*ApST4*) is selectively expressed in the gut and the cognate protein transports glucose with high affinity (Price and Gatehouse, 2014). Reciprocal tBLASTx of *ApST4* against the draft *M. persicae* genome yielded a single *M. persicae* ortholog, *MpST4*. The sequence of *MpST4*, determined by Sanger sequencing, is provided in Fig. S1C. The expression of *MpST4* was enriched $\geq$ two-fold in the gut, relative to the whole body (Table 1).

The search for *B. cockerelli* genes with a sugar transporter domain by HMMER and InterProScan in the *B. cockerelli* transcriptome yielded 39 candidate *ST* genes (Fig. 1). Preliminary analysis identified a single ortholog of *ApST4*, p30480c1s1, but the expression of this gene was not enriched in the *B. cockerelli* gut (Table S2), suggesting that it may not make a significant contribution to the gut osmoregulatory function. To identify other candidate gut sugar transporters of *B. cockerelli*, we interrogated our *A. pisum* gut transcriptome dataset (NCBI SRA: Accession No. SRP053295) for genes enriched in the gut. Adopting the stringent criterion of $\geq$ five-fold gut enrichment relative to the whole body, *ApST4* and five other genes (*ApST1*, *ApST2*, *ApST6*, *ApST11* and *ApST21*) were selected (Fig. S2). A single *B. cockerelli* ortholog of *ST11* was identified, together with a single gene allied to *ApST1* and multiple genes allied to *ApST21* (Fig. 1), generating a total of 11 *B. cockerelli* *ST* genes (additional to *BcST4*) for consideration. The expression of 5 of these genes, *BcST1*, *BcST11*, *BcST21a*, *BcST21b* and *BcST21c* were enriched $\geq$ two-fold in the *B. cockerelli* gut (Fig. 1, Table S2).

The candidate osmoregulatory genes of *M. persicae* and *B. cockerelli* obtained from this analysis (Table 1) were taken forward to investigate the effects of *in planta* RNAi on insect gene expression, osmotic function and performance.

3.2 RNAi of osmoregulation genes in *Myzus persicae*

Aphids were caged on *N. benthamiana* plants infected with TRV carrying constructs designed to reduce expression of aquaporin (*MpAQPI*), sucrose (*MpSUC1*), sugar transporters (*MpST4*), either singly or in combination. TRV2-*GFP* was used as a negative control construct. Expression of each target gene was significantly reduced in insects feeding on plants expressing RNAi constructs directed against the target gene provided in isolation (*MpAQPI*), in combination with other osmoregulatory genes (*MpST4*) or under both RNAi-treatments (*MpSUC1*) (Fig. 2).

M. persicae feeding from plants with RNAi against *MpSUC1*, *MpST4* and all target genes in combination displayed significantly elevated hemolymph osmotic pressure (Fig. 3A), which is the key physiological indicator of osmotic perturbation (Karley et al., 2005). Furthermore, the effect of combined RNAi against all three genes on hemolymph osmotic pressure was significantly greater than for each function tested individually. This effect was associated with depressed adult weight and reproductive output of the aphids on plants expressing the RNAi constructs against the osmoregulatory genes (Fig. 3B&C). A significant reduction of aphid weight was only observed when expression of more than one gene was targeted (Fig. 3B), but reproductive output was significantly depressed in aphids reared on plants with RNAi against each of the genes tested singly (Fig. 3C).

3.3 RNAi of candidate osmoregulation genes in *Bactericera cockerelli*

Adult *B. cockerelli* were fed for 8 days on tomato plants expressing RNAi constructs targeting candidate insect osmoregulatory genes. These were delivered into the plants by *Agrobacterium*-mediated infection with the TRV expression silencing vectors. The expression of the *B. cockerelli* osmoregulatory genes, quantified by qRT-PCR, did not differ significantly between

insects reared on plants with RNAi against any of the osmoregulatory genes and plants with TRV2-*GFP* (Fig. S3). Despite this, the osmotic pressure of *B. cockerelli* hemolymph varied significantly with plant treatment at both times of assay (4 and 8 days after transfer to the test plants). *Post hoc* analysis revealed a significant increase in osmotic pressure for insects on plants with RNAi against the full set of osmoregulatory genes at day-4 and *STs* at day-8 (Fig. 4A). Over the 8 days, egg production per live female did not differ significantly across the treatments (Fig. S4), but survivorship varied significantly with plant treatment. The significance of the χ^2 -test was lost by elimination of just one treatment, the TRV2-*AQP* plants, on which 50% of the insects died, pointing to the importance of AQP function for survival of *B. cockerelli* (Fig. 4B).

4. Discussion

In planta RNAi against osmoregulatory genes had statistically significant effects on the performance, especially reproductive output of the aphid *M. persicae* and mortality of the psyllid *B. cockerelli*. These effects can be attributed to interference with osmoregulatory gene function, rather than nonspecific effects, because the effects of the experimental treatments were, first, compared to plants transformed with non-target RNAi (TRV2-*GFP*) and, second, associated with increased osmotic pressure of the insect hemolymph, indicative of osmotic dysfunction. This result indicates that the bioinformatic strategy adopted in this study to identify osmoregulatory genes was broadly effective. However, definitive biochemical evidence for the molecular function of the genes tested must await analysis of properties of the recombinant proteins in isolation.

The effects of RNAi on hemolymph osmotic pressure were generally small. For *B. cockerelli*, this might be attributable, at least partly, to the disproportionate death of insects displaying the greatest increase in hemolymph osmotic response. Even so, the effects of RNAi against osmoregulatory genes on insect performance in this study were considerably less severe than the results obtained for pea aphids feeding on a diet supplemented with a chemical inhibitor of the gut sucrase, in which all the insects died within three days (Karley et al., 2005). The likely reason is that the RNAi treatment was only partially effective. As with other studies on hemipterans using RNAi delivered by injection or orally via diet or transformed plants, average gene expression knockdown in this study rarely exceeded 50-60% (Christiaens and Smagghe, 2014). Contributory factors to incomplete knock-down of transcript abundance may include degradation of RNAi molecules in the insect gut, poor delivery to insect cells, incomplete activation of the RNAi machinery, and upregulation of transcription rates in response to RNAi-mediated degradation of transcripts (Christiaens et al., 2014; Finn et al., 2011). The variation in proportionate knock-down among genes in *M. persicae* and the statistical significance of the differences among *M. persicae*, but not *B. cockerelli*, genes should, however, be interpreted with caution because the relationship between transcript and protein abundance can vary widely, depending on the rates of mRNA translation and degradation; and RNAi molecules can interfere directly with translation, independently of their degradation of transcripts (Huvenne and Smagghe, 2010; Scott et al., 2013; Yu et al., 2013). An additional source of uncertainty arises from the imperfect systemic spread of the RNAi effects through the insect (Wuriyangan and Falk, 2013), such that gene expression data obtained for the whole insect following oral delivery of RNAi (as in this study) may underestimate the expression knock-down in the gut, and overestimate the effect of RNAi in other tissues.

This study investigated the impact of RNAi against multiple genes with predicted function in osmoregulation, both in the TRV2-STs treatment against five *B. cockerelli* sugar transporter genes, and in the several treatments combining sucrose, aquaporin and sugar transporters. This approach of combinatorial RNAi is being applied, especially in medical applications (Afonin et al., 2014; Grimm and Kay, 2007; Sindhu et al., 2012), and it has two potential advantages. First, the partial silencing of multiple genes with related roles can result in greater impairment of function than RNAi against a single gene. For example, significantly greater hemolymph osmotic pressure was obtained by combinatorial RNAi than mono-RNAi for *M. persicae* in this study. Second, combinatorial RNAi may reduce the evolution of resistant genotypes by requiring simultaneous mutations in all targeted genes. In this regard, however, it may prove more effective to combine RNAi (against a single or multiple genes) with strategies that have different modes of action. This is because the insect pest populations may respond to selection exerted by RNAi-based control strategies by displaying reduced functionality of the RNAi machinery (which functions in defense against RNA viruses) and increased dependence on complementary anti-viral strategies (Nachappa et al., 2012), which can be particularly important in relation to orally-acquired double-stranded RNA molecules (Ferreira et al., 2014).

In conclusion, this study has demonstrated the potential of *in planta* RNAi against insect osmoregulation genes as a contribution to the control of phloem-feeding pest species, with statistically significant effects on important indices of insect fitness, including reproductive output and mortality. Nevertheless, further advances in the efficacy of RNAi against hemipteran pests are required before this strategy can be considered for pest control.

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387 **References**

388 Alvarez, A. E., Tjallingii, W. F., Garzo, E., Vleeshouwers, V., Dicke, M., Vosman, B., 2006.

389 Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to
390 the aphid *Myzus persicae*. Ent. Exp. Appl. 121, 145-157.

391 Afonin, K.A., Viard, M., Koyfman, A.Y., Martins, A.N., Kasprzak, W.K., Panigaj, M., Desai, R.,

392 Santhanam, A., Grabow, W.W., Jaeger, L., et al., 2014. Multifunctional RNA nanoparticles.

393 Nano Lett. 14, 5662-5671.

394 Bass, C., Puinean, A.M., Zimmer, C.T., Denholm, I., Field, L.M., Foster, S.P., Gutbrod, O., Nauen,

395 R., Slater, R., Williamson, M.S., 2014. The evolution of insecticide resistance in the peach potato
396 aphid, *Myzus persicae*. Insect Biochem. Mol. Biol. 51, 41-51.

397 Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck,

398 G., Munyikwa, T., Pleau, M., et al., 2007. Control of coleopteran insect pests through RNA
399 interference. Nat. Biotechnol. 25, 1322-1326.

400 Burand, J.P., Hunter, W.B., 2013. RNAi: future in insect management. J. Invertebr. Pathol. 112
401 Suppl, S68-74.

402 Butler, C.D., Trumble, J.T., 2012. The potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera:

403 Trioxidae): life history, relationship to plant diseases, and management strategies. Terr. Arthr.

404 Rev. 5, 87-111.

405 Chougule, N.P., Li, H., Liu, S., Linz, L.B., Narva, K.E., Meade, T., Bonning, B.C., 2013.
 406 Retargeting of the *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests. Proc.
 407 Natl Acad. Sci. U S A 110, 8465-8470.
 408 Christiaens, O., Smagghe, G. 2014. The challenge of RNAi-mediated control of hemipterans. Curr.
 409 Op. Insect Sci. 6, 15-21.
 410 Christiaens, O., Swevers, L., and Smagghe, G. 2014. dsRNA degradation in the pea aphid
 411 (*Acyrtosiphon pisum*) associated with lack of response in RNAi feeding and injection assay.
 412 Peptides 53, 307-314.
 413 Cristofolletti, P.T., Ribeiro, A.F., Deraison, C., Rahbe, Y., Terra, W.R., 2003. Midgut adaptation
 414 and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrtosiphon*
 415 *pisum*. J. Insect Physiol. 49, 11-24.
 416 Dolling, W.R. (1991). The Hemiptera (Oxford, UK: Oxford University Press).
 417 Douglas, A.E., 2003. The nutritional ecology of aphids. Adv. Insect Physiol. 31, 73-140.
 418 Eddy, S.R., 2011. Accelerated profile HMM searches. PLoS Comput. Biol. 7, e1002195
 419 Edwards, M.G., Gatehouse, A.M.R., 2007. Biotechnology in crop protection: towards sustainable
 420 insect control. In Novel Biotechnologies for Biocontrol Agent Enhancement and Management,
 421 M. Vurro, and J. Gressel, eds. (New York: Springer).
 422 Elzinga, D.A., De Vos, M., Jander, G., 2014. Suppression of plant defenses by a *Myzus persicae*
 423 (green peach aphid) salivary effector protein. Mol. Plant Microbe Interact. 27, 747-756.
 424 Fabrick, J.A., Pei, J., Hull, J.J., Yool, A.J., 2014. Molecular and functional characterization of
 425 multiple aquaporin water channel proteins from the western tarnished plant bug, *Lygus*
 426 *hesperus*. Insect Biochem. Mol. Biol. 45, 125-140.

427 Fernandez-Pozo, N., Menda, N., Edwards, J.D., Saha, S., Tecle, I.Y., Strickler, S.R., Bombarely,
 428 A., Fisher-York, T., Pujar, A., Foerster, H., et al., 2014. The Sol Genomics Network (SGN)-from
 429 genotype to phenotype to breeding. *Nucl. Acids Res.* 43, D1036-D1041.
 430 Ferreira, A.G., Naylor, H., Esteves, S.S., Pais, I.S., Martins, N.E., Teixeira, L., 2014. The Toll-
 431 dorsal pathway is required for resistance to viral oral infection in *Drosophila*. *PLoS Pathog.* 10,
 432 e1004507.
 433 Finn, R.D., Clements, J., Eddy, S.R., 2011. HMMER web server: interactive sequence similarity
 434 searching. *Nucl. Acids Res.* 39, W29-37.
 435 Grimm, D., Kay, M.A., 2007. Combinatorial RNAi: a winning strategy for the race against
 436 evolving targets? *J. Amer. Soc. Gene Therapy* 15, 878-888.
 437 Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B.,
 438 Eccles, D., Li, B., Lieber, M., et al., 2013. De novo transcript sequence reconstruction from
 439 RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8, 1494-
 440 1512.
 441 Hayward, A., Padmanabhan, M., Dinesh-Kumar, S.P., 2011. Virus-induced gene silencing in
 442 *Nicotiana benthamiana* and other plant species. *Meth. Mol. Biol.* 678, 55-63.
 443 Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi
 444 for pest control: a review. *J. Insect Physiol.* 56, 227-235.
 445 Ibanez, F., Hancock, J., Tamborindéguy, C., 2014. Identification and expression analysis of
 446 aquaporins in the potato psyllid, *Bactericera cockerelli*. *PLoS One* 9, e111745.
 447 Jones, R.A., 2014. Trends in plant virus epidemiology: opportunities from new or improved
 448 technologies. *Virus Res.* 186, 3-19.

449 Karley, A.J., Ashford, D.A., Minto, L.M., Pritchard, J., Douglas, A.E., 2005. The significance of
 450 gut sucrase activity for osmoregulation in the pea aphid, *Acyrtosiphon pisum*. J. Insect Physiol.
 451 51, 1313-1319.

452 Kikuta, S., Kikawada, T., Hagiwara-Komoda, U., Nakashima, N. Noda, H., 2010. Sugar transporter
 453 genes in the brown planthopper, *Nilaparvarta lugens*. Insect Biochem. Mol. Biol. 40, 805-813.

454 Le Caherec, F., Deschamps, S., Delamarche, C., Pellerin, I., Bonnec, G., Guillam, M.T., Thomas,
 455 D., Gouranton, J., Hubert, J.F., 1996. Molecular cloning and characterization of an insect
 456 aquaporin functional comparison with aquaporin 1. Eur. J. Biochem. 241, 707-715.

457 Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., Chen, X.Y.,
 458 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs
 459 larval tolerance of gossypol. Nat. Biotechnol. 25, 1307-1313.

460 Mathew, L.G., Campbell, E.M., Yool, A.J., Fabrick, J.A., 2011. Identification and characterization
 461 of functional aquaporin water channel protein from alimentary tract of whitefly, *Bemisia tabaci*.
 462 Insect Biochem. Mol. Biol. 41, 178-190.

463 Merrill, S. C., Randolph, T. L., Peairs, F. B., Michels, G. J., Walker, C. B., 2014. Examining the
 464 competitive advantage of *Diuraphis noxia* (Hemiptera: Aphididae) biotype 2 over biotype 1. J.
 465 Econ. Ent. 107, 1471-1475.

466 Nachappa, P., Levy, J., Tamborindeguy, C., 2012. Transcriptome analyses of *Bactericera*
 467 *cockerelli* adults in response to "Candidatus *Liberibacter solanacearum*" infection. Mol. Gen.
 468 Genom. 287, 803-817.

469 Pitino, M., Coleman, A.D., Maffei, M.E., Ridout, C.J., Hogenhout, S.A., 2011. Silencing of aphid
 470 genes by dsRNA feeding from plants. PLoS One 6, e25709.

471 Porcar, M., Grenier, A.M., Federici, B., Rahbe, Y., 2009. Effects of *Bacillus thuringiensis* delta-
 472 endotoxins on the pea aphid (*Acyrtosiphon pisum*). Appl. Environ. Microbiol. 75, 4897-4900.
 473 Price, D.R., Gatehouse, J.A., 2014. Genome-wide annotation and functional identification of aphid
 474 GLUT-like sugar transporters. BMC Genomics 15, 647.
 475 Price, D.R., Tibbles, K., Shigenobu, S., Smertenko, A., Russell, C.W., Douglas, A.E., Fitches, E.,
 476 Gatehouse, A.M., Gatehouse, J.A., 2010. Sugar transporters of the major facilitator superfamily
 477 in aphids; from gene prediction to functional characterization. Insect Mol. Biol. 19 Suppl 2, 97-
 478 112.
 479 Price, D.R.G., Karley, A.J., Ashford, D.A., Isaacs, H.V., Pownall, M.E., Wilkinson, H.S.,
 480 Gatehouse, J.A., Douglas, A.E., 2007. Molecular characterisation of a candidate gut sucrase in
 481 the pea aphid, *Acyrtosiphon pisum*. Insect Biochem. Mol. Biol. 37, 307-317.
 482 Scott, J.G., Michel, K., Bartholomay, L.C., Siegfried, B.D., Hunter, W.B., Smagghe, G., Zhu, K.Y.,
 483 Douglas, A.E., 2013. Towards the elements of successful insect RNAi. J. Insect Physiol. 59,
 484 1212-1221.
 485 Ragsdale, D. W., Landis, D. A., Brodeur, J., Heimpel, G. E., Desneux, N., 2011. Ecology and
 486 management of the soybean aphid in North America. Annu. Rev. Entomol. 56, 375-399.
 487 Ramsey, J.S., Elzinga, D.A., Sarkar, P., Xin, Y-R., Ghanim, M., Jander, G. 2014. Adaptation to
 488 nicotine feeding in *Myzus persicae*. J. Chem. Ecol. 40, 869-877.
 489 Senthil-Kumar, M., Mysore, K.S., 2014. Tobacco rattle virus-based virus-induced gene silencing in
 490 *Nicotiana benthamiana*. Nat. Protoc. 9, 1549-1562.
 491 Shakesby, A.J., Wallace, I.S., Isaacs, H.V., Pritchard, J., Roberts, D.M., Douglas, A.E.. 2009. A
 492 water-specific aquaporin involved in aphid osmoregulation. Insect Biochem. Mol. Biol. 39, 1-10.

493 Sindhu, A., Arora, P., Chaudhury, A., 2012. Illuminating the gateway of gene silencing:
 494 perspective of RNA interference technology in clinical therapeutics. *Mol. Biotechnol.* 51, 289-
 495 302.

496 Sugio, A., Hogenhout, S.A., 2012. The genome biology of phytoplasma: modulators of plants and
 497 insects. *Curr. Op. Microbiol.* 15, 247-254.

498 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
 499 Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599.

500 Thakur, N., Upadhyay, S.K., Verma, P.C., Chandrashekar, K., Tuli, R., Singh, P.K., 2014.
 501 Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-
 502 ATPase A Gene. *PLoS ONE* 9, e87235.

503 Vaghchhipawala, Z., Rojas, C.M., Senthil-Kumar, M., Mysore, K.S., 2011. Agroinoculation and
 504 agroinfiltration: Simple tools for complex gene function analyses. In *Plant Reverse Genetics*, A.
 505 Pereira, ed. (Humana Press), pp. 65-76.

506 Van Emden, H.F., Harrington, R., 2007. *Aphids as Crop Pests*, CABI, Wallingford, UK, 717 pp.

507 Velasquez, A.C., Chakravarthy, S., Martin, G.B., 2009. Virus-induced gene silencing (VIGS) in
 508 *Nicotiana benthamiana* and tomato. *J. Vis. Exp.* 28, 1292.

509 Whalon, M.E., Mota-Sanchez, D., Hollingworth, R., 2010. Analysis of global pesticide resistance
 510 in arthropods. In *Global Pesticide Resistance in Arthropods*, M.E. Whalon, D. Mota-Sanchez,
 511 and R. Hollingworth, eds. (Wallingford, UK: CABI International).

512 Wuriyangan, H., Rosa, C., Falk, B.W., 2011. Oral delivery of double-stranded RNAs and siRNAs
 513 induces RNAi effects in the potato/tomato psyllid, *Bactericera cockerelli*. *PLoS ONE* 6,
 514 e27736.

515 Wuriyanghan, H., Falk, B.W., 2013. RNA interference towards the potato psyllid, *Bactericera*
516 *cockerelli*, is induced in plants infected with recombinant tobacco mosaic virus (TMV). PLoS
517 ONE 8, e66050.

518 Xue, X.Y., Mao, Y.B., Tao, X.Y., Huang, Y.P., Chen, X.Y., 2012. New approaches to agricultural
519 insect pest control based on RNA interference. Adv. Insect Physiol. 42, 74-117.

520 Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G., 2013.
521 Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Sci. 20, 4-14.

522 Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., He, G., 2011. Knockdown of midgut genes by
523 dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata*
524 *lugens*. PLoS One 6, e20504.

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Figure Legends

Fig. 1. Neighbor-joining tree of sugar transporters identified in the genome of *A. pisum* and transcriptome of *B. cockerelli* with 1000 bootstrap replications. Bootstrap values >90% are marked as black nodes, and 71-89% as grey nodes. * candidate *B. cockerelli* sugar transporter genes with enriched expression in the gut (see Table S2). Scale bar represents 0.05 substitutions per amino acid site.

Fig. 2. Expression of candidate osmoregulatory genes in *M. persicae* reared from birth to day-7 on *N. benthamiana* plants with different RNAi constructs. Median (horizontal bar) and ANOVA results are shown, with different letters referring to treatments with significantly different expression by Tukey's HSD test. A. *MpAQPI*, B. *MpSUC1*, C. *MpST4*. The expression level of each gene has been normalized to the GFP control which is set to 1.

Fig. 3. Performance of *M. persicae* reared on *N. benthamiana* plants with different RNAi constructs. (A) Osmotic pressure of the aphid hemolymph. (B) Weight of 7-day-old aphids. (C) Reproductive output per aphid over first 8 days of reproduction. ANOVA results are shown, with different letters referring to treatments with significantly different expression by Tukey's HSD test.

Fig. 4. Performance of adult *B. cockerelli* feeding for 4 days and 8 days on tomato plants expressing TRV2 constructs candidate psyllid osmoregulatory genes and two negative controls (empty TRV2 vector and TRV2-GFP). (A) Osmotic pressure of hemolymph at day-4 and day-8.

549 For each time, treatments with significantly different mean values by Tukey's HSD post hoc
550 tested are indicated by different letters. (B) Female mortality over 8 days.

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552 Table 1 Candidate osmoregulation genes used in experiments

553

Table 1 Candidate osmoregulation genes used in experiments

Function	Gene name	Sequence ID ^a	log ₂ (fold difference in expression gut/whole body (mean $\pm$ s.e., n=3)
(a) <i>Myzus persicae</i>			
Aquaporin	<i>MpAQP1</i>	KR047100	1.69 $\pm$ 0.096
Sucrase	<i>MpSUC1</i>	KR047101	1.83 $\pm$ 0.038
Sugar transporter	<i>MpSt4</i>	KR047102	1.78 $\pm$ 0.058
(b) <i>Bactericera cockerelli</i>			
Aquaporin	<i>BcAQP2</i>	bc30878c0s1	n.d. ^b
Sucrase	<i>BcSUC1</i>	bc30260c0s1	6.09 $\pm$ 0.056
Sugar transporter	<i>BcST1</i>	bc 30953c0s1	2.09 $\pm$ 0.009
Sugar transporter	<i>BcST11</i>	bc 24114c0s1	1.15 $\pm$ 0.085
Sugar transporter	<i>BcST21a</i>	bc 13079c0s1	1.83 $\pm$ 0.151
Sugar transporter	<i>BcST21b</i>	bc 29796c0s1	1.00 $\pm$ 0.038
Sugar transporter	<i>BcST21c</i>	bc 30077c0s1	3.70 $\pm$ 0.167

^a NCBI Accession Number for *M. persicae* genes; contig number from assembled transcriptome for *B. cockerelli*

^b Not determined: Ibanez et al. (2014) has demonstrated that expression of this gene significantly enriched in the gut of the same *B. cockerelli* culture as used in this study.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Graphical Abstract