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ExoRNAi, a new tool to probe plant gene function exposes contrasting roles for sugar exudation in host-finding by plant pathogens

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Abstract

Here we present a simple and rapid new method for RNAi-induced knockdown of genes in tomato seedlings, through treatment with an aqueous solution of double-stranded RNA (exoRNAi). The exoRNAi method is used to assess the involvement of tomato Sugar Transporter Protein genes, *stp1* and *stp2* on the root exudation of glucose, fructose and xylose; monosaccharide constituents of tomato root exudate. Plant parasitic nematodes (PPNs) are responsible for an estimated 12.3% loss in crop production globally, which equates to financial losses of approximately £100 billion annually. Our data show that infective juveniles of the promiscuous PPN, *Meloidogyne incognita* are attracted to glucose and fructose, but not xylose. Glucose and fructose also agonise serotonergic stylet thrusting in *M. incognita* infective juveniles; a key parasitic behaviour necessary for invasion and parasitism of host plants. In contrast, infective juveniles of the selective Solanaceae PPN, *Globodera pallida* are not attracted to tested monosaccharides, nor do the monosaccharides stimulate stylet thrusting. We demonstrate that knockdown of both *SlSTP1* and *SlSTP2* in tomato seedlings by the exoRNAi method is robust and specific, and that corresponding reductions of glucose and fructose, but not xylose, in collected exudate, correlate directly with reduced infectivity and stylet thrusting of *M. incognita*. Knockdown of *SlSTP1* or *SlSTP2* have no impact on the infectivity or stylet thrusting of *G. pallida.* The exoRNAi platform can underpin future efforts to understand the early stages of plant-pathogen interactions in tomato, and potentially other crop plants.

Introduction

RNA interference (RNAi) is widely used for the analysis of plant gene function, primarily through the transgenic production of dsRNA constructs *in planta*, and secondarily through Virus-Induced Gene Silencing (VIGS) (Watson *et al.,* 2005). Crude lysate from *Escherichia coli* expressing virus-specific dsRNA have been used to protect plants from viral pathology (Tenllado *et al.,* 2003), however the approach has not yet been validated as a reverse genetics tool appropriate for the study of gene function. Transgenic *in planta* RNAi is currently viewed as a specialist approach predicated upon the availability of sufficient expertise, and requiring a greater investment of time and resources relative to the other

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approaches, limiting widespread adoption. VIGS may provide advantages with respect to time investment and ease of application, yet suffers from particular nuances of viral infection, such as heterogenous expression levels and localisation of dsRNA constructs throughout the plant; both of which can also vary as a factor of time. Null phenotypes resulting from VIGS must also be interpreted against a background of viral pathology and stress, which may be difficult if the interaction between gene knockdown and the biotic stress of viral infection have non-additive effects. Here we present another approach to triggering RNAi in plants, which we term exogenous (exo)RNAi. In this approach, aqueous dsRNA is delivered exogenously to tomato seedlings, requiring only rudimentary molecular biology equipment and capability and avoiding the need to use transgenic plants, or plants infected with a viral pathogen.

Plant root exudate comprises a complex mixture of compounds including volatile and soluble chemicals which may derive from intact or damaged root cells, or sloughed-off root border cells (Dakora and Phillips, 2002). It has been estimated that 11% of photosynthetically-assimilated carbon is released as root exudate (Jones *et al.,* 2009). The monosaccharides glucose, fructose and xylose represent the major sugar component of tomato root exudates (Kamilova *et al.,* 2006). Plant parasitic nematodes (PPNs) are responsible for an estimated 12.3% loss in crop production globally each year (Sasser and Freckman, 1987), and are attracted to host plants by components of plant root exudate. Here we purposed to assess the chemosensory response of root knot nematode, *Meloidogyne incognita* (a promiscuous pathogen of flowering plants), and potato cyst nematode, *Globodera pallida* (a selective pathogen of Solanaceae plants) to each of the three major monosaccharide sugars of tomato plant root exudate, and to assess the efficacy of exoRNAi against *SlSTP1* and *SlSTP2*, known transporters of monosaccharide sugars in tomato (Gear *et al.,* 2000).

Results

Nematode chemosensory and stylet thrusting response to sugars

Meloidogyne incognita infective stage juveniles were attracted to glucose (CI: 0.33 ±0.07; P<0.01) and fructose (CI: 0.39 ±0.09; P<0.01), but not xylose (CI: 0.04 ±0.09; P>0.05) as compared to control treated worms (Fig 1A). Glucose (125.1% ±5.5; P<0.001) and fructose (124.8% ±5.4; P<0.001) also triggered an elevated level of serotonin-triggered stylet thrusting in treated juveniles; xylose failed to trigger any significant response (99.36% ±10.87; P>0.05) when compared to control treatments (Fig 1B). *Globodera pallida* infective stage juveniles were mildly repelled by glucose (CI: -0.23 ±0.09; P>0.05), and did not appreciably respond to fructose (CI: 0.15 ±0.08; P>0.05), or xylose (CI: -0.19 ±0.09; P>0.05) as compared to control treated worms (Fig 1C). Glucose (118.6% ±9.7; P>0.05), fructose (107.2% ±7.3; P>0.05), or xylose (119.6% ±8.6; P>0.05) had no significant impact on the frequency of serotonin-triggered stylet thrusting in *G. pallida* infective juveniles when compared to control treatments (Fig 1D).

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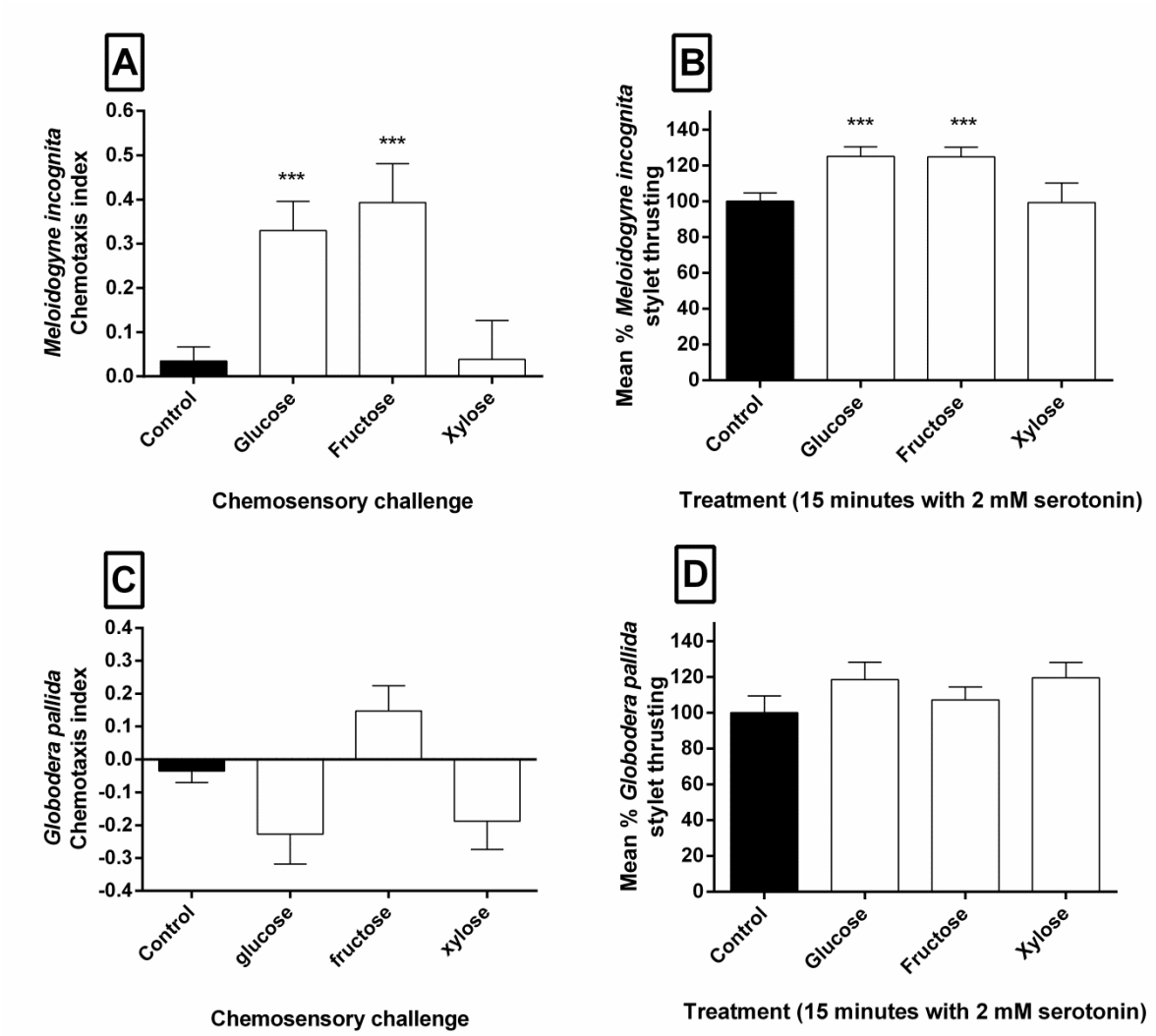

Figure 1. Glucose and fructose stimulate different chemotaxis and stylet thrusting responses in *M. incognita* and *G. pallida*. (A) Chemosensory response (chemosensory index) of *M. incognita* infective juveniles to glucose, fructose, xylose and control (water) assay challenge. Each data point represents the mean (±SEM) of 10 assays of 100 infective juveniles each. (B) Mean percentage (±SEM) stylet thrusting of glucose, fructose and xylose treated *M. incognita* infective stage juveniles (n=100) relative to control (2 mM serotonin in water). (C) Chemosensory response of *G. pallida* infective juveniles to glucose, fructose, xylose and control (water) assay challenge. (D) Mean percentage (±SEM) stylet thrusting of glucose, fructose and xylose treated *G. pallida* infective stage juveniles (n=100) relative to control (2 mM serotonin in water). ***, P<0.001.

exoRNAi impact on tomato gene expression,

Treatment of tomato seedlings with *SlSTP1* dsRNA triggered a significant reduction in *SlSTP1* transcript abundance (0.17 ±0.05; P<0.001), yet had no impact on *SlSTP2* abundance (1.037 ±0.13; P>0.05) relative to neomycin transferase (NEO) dsRNA treatment. Likewise, *SlSTP2* dsRNA induced significant reductions in *SlSTP2* transcript abundance (0.21 ±0.06; P<0.001), but not *SlSTP1* (0.94 ±0.05; P>0.05) relative to NEO dsRNA treatments (Fig 2).

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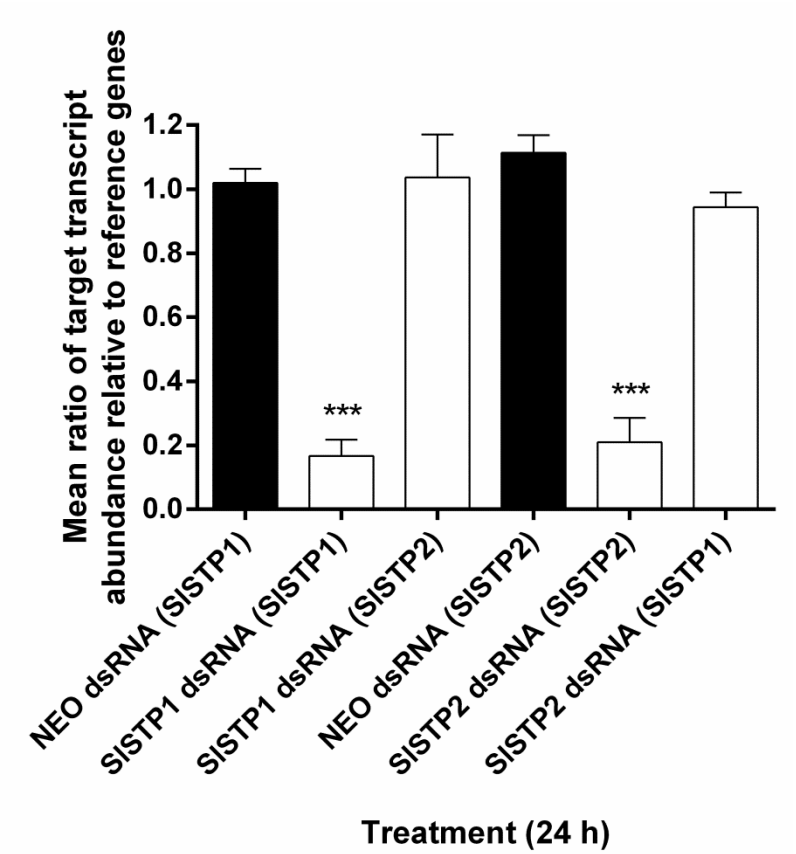

Figure 2. ExoRNAi induces target-specific silencing of tomato plant genes. Mean ratio of target transcript (in parentheses) abundance relative to three endogenous reference genes. Each data point represents the mean (±SEM) of three replicates of five seedlings each. ***, P<0.001.

exoRNAi impact on sugar exudation and nematode infectivity

Corresponding reductions in glucose and fructose exudate concentration were observed for both *SlSTP1* (5.10 µg/ml ±1.31; P<0.01 and 3.14 µg/ml ±0.92; P<0.01, respectively) and *SlSTP2* (4.90 µg/ml ±1.45; P<0.01 and 10.90 µg/ml ±1.07; P<0.05, respectively) dsRNA treated seedlings. No significant changes in xylose exudate concentration were observed across treatment groups (Fig 3A-C). When exoRNAi-treated seedlings were challenged by *M. incognita* infection assay, significant reductions in percentage infection levels relative to NEO dsRNA treatment were observed for both *SlSTP1* (14.15% ±4.77; P<0.01) and *SlSTP2* (27.08% ±7.32; P<0.05) dsRNA treatments (Fig 3D). Knockdown of *SlSTP1* (14.15% ±4.77; P>0.05) or *SlSTP2* (14.15% ±4.77; P>0.05) did not significantly reduce the percentage infection levels of *G. pallida* relative to NEO dsRNA treatment.

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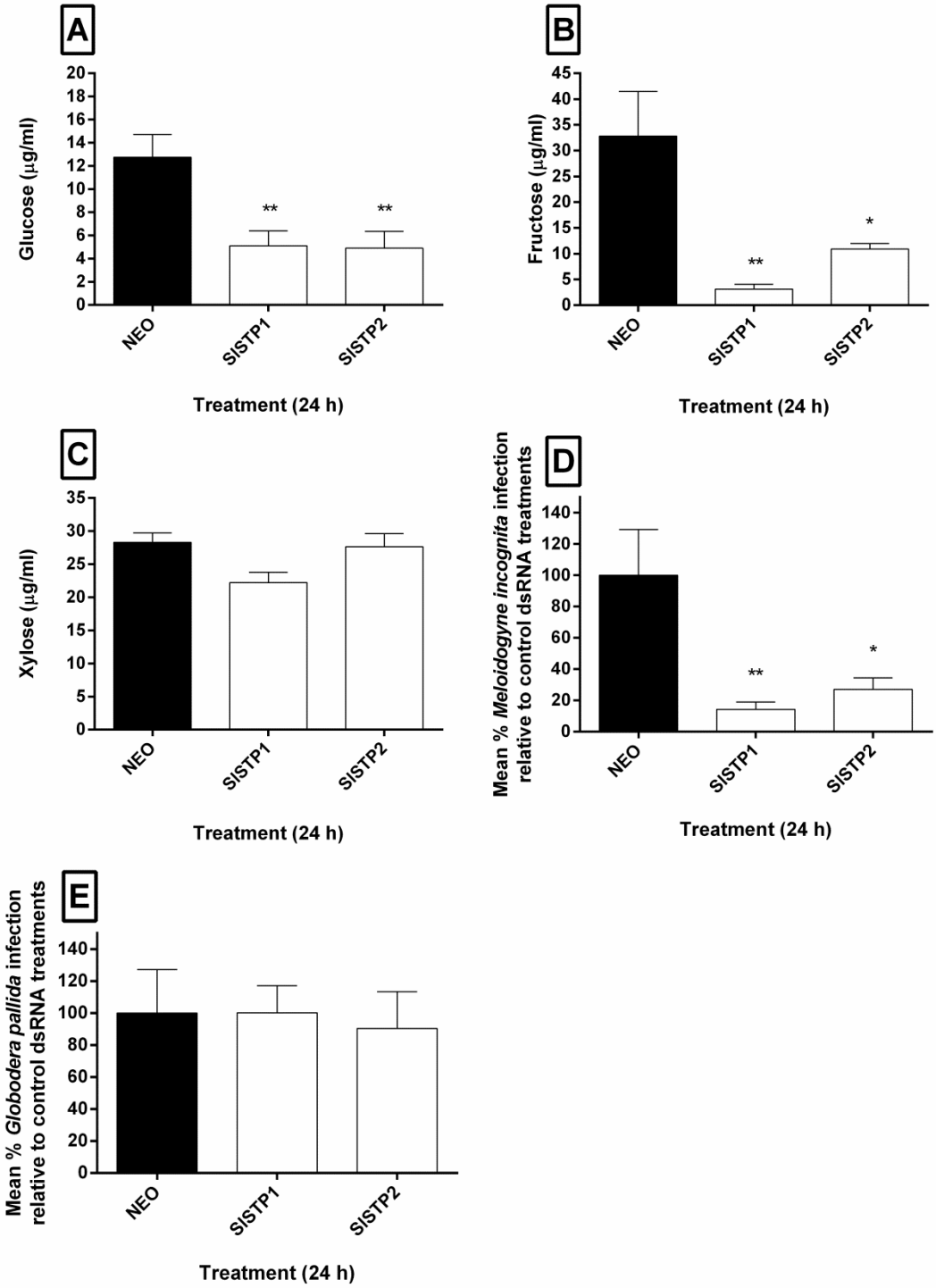

Figure 3. ExoRNA interference (exoRNAi) of tomato seedling sugar transporters alters root exudate and plant nematode infection. Exudate concentration of (A) glucose, (B) fructose and (C) xylose across NEO (double stranded [ds]RNA control), *SlSTP1* and *SlSTP2* dsRNA treated tomato seedlings. Each data point represents the mean (±SEM) of three replicates of ten seedlings each. (D) Mean percentage *M. incognita* infection levels of *SlSTP1* and *SlSTP2* dsRNA treated tomato seedlings normalised to NEO double stranded

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(ds)RNA (control) treated seedlings. (E) Mean percentage *G. pallida* infection levels of *SlSTP1* and *SlSTP2* dsRNA treated tomato seedlings normalised to NEO double stranded (ds)RNA (control) treated seedlings. Each data point represents the mean (±SEM) of ten seedlings challenged with 500 infective stage juveniles each. *, P<0.05; **, P<0.01; ***, P<0.001.

exoRNAi impact on nematode stylet thrusting

Root exudates collected from tomato seedlings which had been treated with either *SlSTP1* or *SlSTP2* dsRNA were less capable of stimulating stylet thrusting in *M. incognita* relative to exudates collected from control dsRNA treated seedlings (13.92 ±5.10%, P<0.001; and 17.53 ±8.12%, P<0.001, respectively. Fig 4A). No significant difference in stylet thrusting frequency was observed for *G. pallida* juveniles when exposed to root exudates from *SlSTP1* or *SlSTP2* dsRNA-treated seedlings, relative to control treated seedlings (108.2 ±38.87%, P>0.05; and 77.34 ±30.84%, P>0.05, respectively). In general, *G. pallida* juveniles were less readily activated by tomato seedling exudates, accounting for much higher variation, even after extended exudate exposure times of 24 hours (Fig 4B).

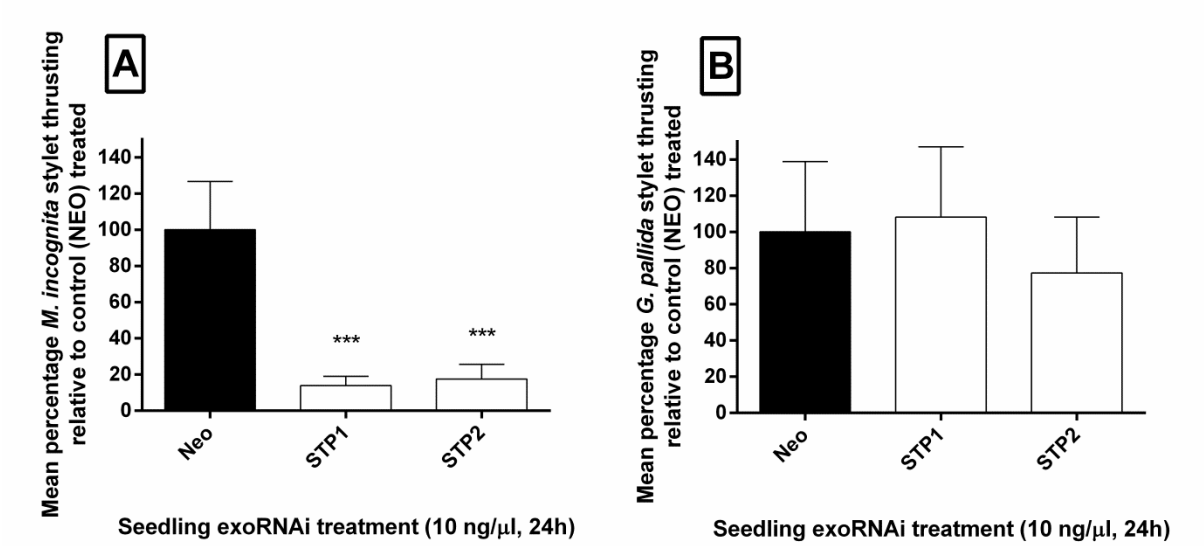

Figure 4. ExoRNA interference (exoRNAi) of tomato seedling sugar transporters alters plant nematode stylet thrusting behaviour. Mean percentage (±SEM) stylet thrusting of (A) *M. incognita* and (B) *G. pallida* infective stage juveniles in response to collected seedling exudates (n=100), relative to control (NEO dsRNA). ***, P<0.001.

Discussion

Here we demonstrate that the exogenous application of aqueous double stranded (ds)RNA onto tomato seedlings is sufficient to trigger specific gene knockdown via the RNA interference (RNAi) pathway. The exoRNAi approach provides a simple and rapid methodology for the analysis of tomato seedling gene function which will facilitate the reverse genetics study of gene function in non-specialist laboratories. Furthermore, we

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believe that inducible RNAi approaches, such as exoRNAi, should be considered preferable to constitutive transgenic RNAi methods which may increase the likelihood of false positive phenotypes resulting from knock-on effects from specific gene knockdown on other non-target genes and pathways as a factor of time. The exoRNAi method reduces the financial and time costs associated with the study of gene function in tomato, and potentially other crop plant species. The simplicity of the procedure, and timescale required for exoRNAi indicates that it could also be useful for high-throughput screening processes. In particular, this approach could be of great value to crop plants which are difficult to manipulate genetically. In this proof of principle study, we have used the exoRNAi approach to examine the interaction between tomato sugar transporters, sugar exudation, and plant parasitism.

Plant root exudation is an energetically expensive process for plants (Badri and Vivanco, 2009), with an estimated 11% of photosynthetically-assimilated carbon released in the form of various root exudate constituents (Jones *et al.,* 2009). It is well established that plant root exudates mediate both positive and negative interactions with commensal and pathogenic microbes (Badri *et al.,* 2009), insects (Walker *et al.,* 2003), and other plants (Bais *et al.,* 2006). Plant parasitic nematodes also respond to plant root exudates (Teillet *et al.,* 2013). The present study aimed to probe the involvement of monosaccharide sugars of tomato root exudate for involvement in the attraction and activation of parasitic behaviours in the promiscuous root knot nematode *M. incognita*, and the host-selective potato cyst nematode *G. pallida*.

STP1 and STP2 are known transporters of monosaccharide sugars (Gear *et al.,* 2000), and our data demonstrate that both play a role in regulating the level of glucose and fructose (but not xylose) exudation from tomato seedling roots. exoRNAi knockdown of each transporter significantly reduced the amount of glucose and fructose secreted from plant roots, which corresponded with a decrease in *M. incognita* infectivity, but not *G. pallida* infectivity. These results suggest that glucose and fructose are important chemical cues which infective stage *M. incognita* use to find host plants. The role of glucose and fructose in *M. incognita* host-finding was further demonstrated through *in vitro* assays which validated *M. incognita* attraction towards both glucose and fructose. Glucose and fructose also triggered an increase in the rate of serotonin-triggered stylet thrusting, a key parasitic behaviour associated with parasite activation and plant infectivity (Teillet *et al.,* 2013). Xylose had no impact on chemosensory behaviour, or stylet thrusting of *M. incognita,* and silencing of both *SlSTP1* and *SlSTP2* had little impact on the amount of xylose secreted from the seedlings. In contrast, the selective Solanaceae PPN, *G. pallida* did not utilise monosaccharide components of tomato root exudate to locate a host, nor do the monosaccharides play any role in the activation or enhancement of stylet thrusting. Glucose and fructose appear to trigger host-finding and activation behaviours in promiscuous PPNs, as opposed to host-specific PPNs, an observation which is consistent with the ubiquitous nature of monosaccharide sugars in plant root exudates (Kamilova *et al.,* 2006), which could render these exudate components as uninformative to selective pathogens. Our data suggest that manipulation of sugar exudation from crop plant roots

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could provide a novel means of promiscuous PPN control, but not selective PPNs which likely utilise more diagnostic, and less ubiquitous root exudate cues. The demonstration that *SlSTP1* and *SlSTP2* are specifically involved in the exudation of both monosaccharides from tomato roots is an important finding which can underpin future efforts to study the link between plant root transporters, and chemical constituents of root exudates. These data provide an important proof of concept validation of the exoRNAi approach, which promises a quick and easy method for gene functional studies in tomato, and potentially other crop plant species.

Materials and Methods

Nematode maintenance and collection

Meloidogyne incognita was maintained on tomato cv. Moneymaker (23°C, 18h light, 6h dark). Roots were harvested from infected plants and washed thoroughly in water. Egg masses were removed by treatment in 2.5% sodium hypochlorite for two minutes. Eggs were collected by washing the hypochlorite-treated roots through a series of nested sieves (150 µM, 53 µM and 38 µM mesh sizes respectively) with water. Eggs were hatched in DEPC-treated spring water (pH 7), and hatched infective stage juveniles were used immediately for downstream applications. *Globodera pallida* (pathotype Pa2/3) were collected from potato cv. Cara which had been grown under glasshouse conditions. Infective juveniles were hatched from cysts in potato root exudate, in complete darkness at 16°C, and were used immediately for downstream applications.

Nematode stylet thrusting assay

100 *M. incognita* or *G. pallida* infective stage juveniles were suspended in 20 µl of spring water (autoclaved and adjusted to pH 7) containing 2 mM serotonin and 50 mM of glucose, fructose or xylose (Sigma-Aldrich). Worms were incubated in this solution for 15 minutes, pipetted onto a glass slide with a coverslip, and stylet thrusts were counted in randomly selected infective stage juveniles for 1 minute each. In order to keep counts consistent, worms were left on the slide no longer than 20 minutes. The experiment was replicated five times to give stylet thrust counts for 100 infective stage juveniles per treatment. Control treatments were expressed as a percentage, including technical variation, and experimental treatments were normalised to control percentages across individual experiments and days. We found quite a bit of inter- and intra-experimental variability in the response of *M. incognita* infective stage juveniles to serotonin which may be linked to the age of juvenile nematodes, the density of nematodes per hatching plate, or the time of day. This variability was not so prominent in *G. pallida* tests. Data were analysed by One-way ANOVA and Tukey's Honestly Significant Difference test using Graphpad Prism 6. Probabilities of less than 5% ($P < 0.05$) were deemed statistically significant. Nematode stylet thrusting was also assessed in response to seedling exudates (without the addition of exogenous serotonin). Exudates were collected as below, and *M. incognita* juveniles were exposed to the exudate

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for 30 minutes prior to the counting of stylet thrusting frequency. *G. pallida* juveniles required increased exudate exposure before stylet thrusting was triggered, and so data were collected 24h post-exposure for this species. Even after 24h exposure we found that many *G. pallida* juveniles did not initiate stylet thrusting at all, accounting for the high SEM values observed in figure 4B.

Nematode Chemosensory Assay

An agar slurry was prepared by autoclaving a 0.25% agar solution using spring water, and adjusted to pH 7. It was then agitated overnight by magnetic stirrer until it had a smooth consistency. Assay plates were prepared by filling 6 cm Petri dish with 10 ml of 0.25% spring water agar which was allowed to solidify. Subsequently, 3 ml of agar slurry was poured on top providing the medium through which the infective stage juveniles could move. Sugar plugs were prepared by dissolving 50 mM of the relevant sugar (glucose / fructose / xylose) in 0.25% agar and allowed to set. Plugs were picked with a Pasteur pipette which had been cut half way down the pipette barrel, and placed onto one side of a Petri dish, with a negative plug (water instead of 50 mM sugar) on the other. 100 *M. incognita* or *G. pallida* infective stage juveniles were suspended in 5 µl of spring water, and spotted onto the centre point of each dish. A Petri dish lid was marked with two parallel vertical lines 0.5 cm either side of the centre point forming a 1 cm 'dead zone' that ran vertically along the lid. Assay plates were set onto the lid for scoring of nematode positions following a two hour assay period. Only nematodes outside the dead zone were counted. The distribution of *M. incognita* infective stage juveniles was used to generate the chemotaxis index (Hart, 2006) for each assay plate which formed one replicate. Ten replicate assays were performed, and were analysed by One-way ANOVA and Tukey's Honestly Significant Difference test using Graphpad Prism 6. Probabilities of less than 5% ($P < 0.05$) were deemed statistically significant.

Double-stranded RNA synthesis and tomato seedling treatment

Forward and reverse primers (Table 1) including 5' T7-recognition sites were used to generate specific amplicons for dsRNA synthesis to EST-supported fragments of *LeSTP1* (Solyc02g079220.2), and *LeSTP2* (Solyc09g075820.2) (Reuscher *et al.,* 2014). PCR products were assessed by gel electrophoresis, and cleaned using the Chargeswitch PCR clean-up kit (Life Technologies). dsRNA was synthesised using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega), and quantified by Nanodrop 1000 spectrophotometer. Tomato cv. Moneymaker seeds (Suttons) were sterilised by 30 minute treatment in dilute bleach, followed by five 15 minute washes in 1 ml deionised water. Seeds were germinated on 0.5X MS salts, 0.6% agar plates at 23°C, and taken for exoRNAi treatment on the first day of radicle emergence. Ten seedlings were used per well of a 24-well plate (SPL Lifesciences), and incubated with 300 µl of 10 ng/µl dsRNA solution for 24h at 23°C, in darkness.

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RNA extraction, cDNA synthesis and qRT-PCR

Five seedlings were snap frozen in liquid nitrogen per biological replicate, and total RNA isolated using Trizol reagent. Total RNA was treated with the Turbo DNase free kit (Life Technologies), and cDNA was synthesised using the High-capacity RNA-to-cDNA kit (Applied Biosciences) according to manufacturer's instructions, and using the maximum input concentration of RNA. Three biological replicates were performed for each treatment. Primer sets to be used for qPCR were optimised for working concentration, annealing temperature and analysed by dissociation curve for contamination or non-specific amplification by primer–dimer as standard (Table 1). Each individual reaction comprised 5 µl Faststart SYBR Green mastermix (Roche Applied Science), 1 µl each of the forward and reverse primers (10 mM), 1 µl water, 2 µl cDNA. PCR reactions were conducted in triplicate for each individual cDNA using a Rotorgene Q thermal cycler according to the following conditions: [95°C x 10 min, 40 x (95°C x 20s, 60°C x 20s, 72°C x 20s) 72°C x 10 min]. The PCR efficiency of each specific amplicon was calculated using the Rotorgene Q software, and quantification of each target amplicon calculated by an augmented comparative Ct method (Pfaffl, 2001), relative to the geometric mean of three endogenous reference genes (Vandesompele *et al.,* 2002) (see Table 1). Ratio-changes in transcript abundance were calculated relative to control dsRNA treated seedlings in each case, and data were analysed by ANOVA and Tukey's Honestly Significant Difference test using Graphpad Prism 6. Probabilities of less than 5% ($P < 0.05$) were deemed statistically significant.

Exudate collection and sugar quantification

Ten seedlings per biological replicate were rinsed thoroughly with three washes of 2 ml deioinised water each, post-dsRNA treatment. 500 µl of deionised water was then added to each replicate of ten seedlings. Plates were sealed with parafilm, covered above and below with a sheet of tin foil and incubated for 24 hours at 23°C. The exudate solution was collected by pipette and transferred to a hydrophobically-lined microcentrifuge tube (Anachem) prior to quantification. The sugars were quantified colorimetrically at 340 nm using Glucose (HK), and Fructose assay kits from Sigma-Aldrich, and the Xylose assay kit from Megazyme as per manufacturer's instructions.

Nematode infection assays

Agar slurry was prepared by autoclaving a 0.55% agar solution with Spring water which had been autoclaved and adjusted to pH 7. The agar was agitated for six hours at room temperature, until it had a smooth consistency. 500 *M. incognita* or *G. pallida* infective stage juveniles were added to each well of a 6 well plate (SPL Lifesciences) with one exoRNAi treated seedling embedded within 3 ml of agar slurry. Plates were sealed with parafilm, covered above and below with a sheet of tin foil and incubated for 24 hours at 23°C. Seedlings were subsequently removed from the slurry, gently washed several times by immersion in deionised water, and stained using acid fuschin (Bybd *et al.,* 1983). The number of invading PPN juveniles was counted for each seedling using a light microscope.

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Control treatments were expressed as a percentage, including technical variation, and experimental treatments were normalised to control percentages.

Acknowledgements

This work was supported financially by Queen's University Belfast. Dalzell is supported by an Early Career Fellowship from The Leverhulme Trust. Warnock was supported by a Gates Foundation Grand Challenges Grant, Wilson was supported by a EUPHRESCO fellowship, Canet-Perez was supported by an Invest Northern Ireland Proof-of-Concept award and Fleming by a Department of Agriculture and Rural Development studentship award.

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Table 1. Primer sequences.

Primer Designation	Sequence
SlSTP1F	CTGCTGTGATCACTGGTGGA
SlSTP1R	ATTCCCCTGGAGTTCCATTT
SlSTP1Ft7	TAATACGACTCACTATAGGCTGCTGTGATCACTGGTGGA
SlSTP1Rt7	TAATACGACTCACTATAGGATTCCCCTGGAGTTCCATTT
qSlSTP1F	ATGTTGCTGGATTCGCTTGGTC
qSlSTP1R	TGTGCAGCTGATCGAATTTCCAG
SlSTP2F	ACGTTCTCTCCACCGTTGTC
SlSTP2R	CTACGAAGATTCCCCAACCA
SlSTP2Ft7	TAATACGACTCACTATAGGACGTTCTCTCCACCGTTGTC
SlSTP2Rt7	TAATACGACTCACTATAGGCTACGAAGATTCCCCAACCA
qSlSTP2F	ATTATGGCTGCTACCGGAGGTC
qSlSTP2R	TGTAACACCACCAGAAACTCCAAC
qSlTIP41F	ATGGAGTTTTTGAGTCTTCTGC
qSlTIP41R	GCTGCGTTTCTGGCTTAGG
qSlEFαF	TACTGGTGGTTTTGAAGCTG
qSlEFαR	AACTTCCTTCACGATTTCATCATA
qSlSANDF	TTGCTTGGAGGAACAGACG
qSlSANDR	GCAAACAGAACCCCTGAATC