ExoRNAi exposes contrasting roles for sugar exudation in host-finding by plant pathogens

Neil D. Warnock[a], Leonie Wilson[a], Juan V. Canet-Perez[a], Thomas Fleming[a], Colin C. Fleming[b], Aaron G. Maule[a], Johnathan J. Dalzell[a*]

[a]*School of Biological Sciences, Institute for Global Food Security, Queen's University Belfast, UK*

[b]*Plant Health and Environmental Protection Branch, Sustainable Agri-Food Sciences Division (SAFSD), Agri-Food and Biosciences Institute, Newforge Lane, Belfast, UK*

*Corresponding author.

E-mail address: j.dalzell@qub.ac.uk

Abstract

Plant parasitic nematodes (PPN) locate host plants by following concentration gradients of root exudate chemicals in the soil. We present a simple method for RNA interference (RNAi)-induced knockdown of genes in tomato seedling roots, facilitating the study of root exudate composition, and PPN responses. Knockdown of sugar transporter genes, *STP1* and *STP2* in tomato seedlings triggered corresponding reductions of glucose and fructose, but not xylose, in collected root exudate. This corresponded directly with reduced infectivity and stylet thrusting of the promiscuous PPN *Meloidogyne incognita*, however we observed no impact on the infectivity or stylet thrusting of the selective Solanaceae PPN *Globodera pallida.* This approach can underpin future efforts to understand the early stages of plant-pathogen interactions in tomato, and potentially other crop plants.

Keywords: exoRNAi, *Meloidogyne incognita*, *Globodera pallida*, Sugar, Root exudate

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). Previous findings by Wolniak and colleagues have shown that exogenous dsRNA can silence genes of the water fern *Marsilea vestita* (Klink and Wolniak, 2001), and crude lysate from *Escherichia coli* expressing virus-specific dsRNA have also been used to protect plants from viral pathology (Tenllado et al., 2003). Here we present a similar approach to triggering RNAi in tomato seedlings, which we term exogenous (exo)RNAi. In this approach, aqueous dsRNA is delivered exogenously to tomato seedlings.

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 assess the chemosensory response of the root knot nematode, *Meloidogyne incognita* (a promiscuous pathogen of flowering plants), and the 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 the efficacy of exoRNAi against *STP1* and *STP2*, known transporters of monosaccharide sugars in tomato (Gear et al., 2000).

Meloidogyne incognita infective stage juveniles were attracted to glucose (chemotaxis index (CI): 0.33 ± 0.07; *P* <0.001) and fructose (CI: 0.39 ± 0.09; *P* <0.001), but not xylose (CI: 0.04 ± 0.09; *P* >0.05) as compared with 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 with 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 respond to fructose (CI: 0.15 ± 0.08; *P* >0.05), or xylose (CI: -0.19 ± 0.09; *P* >0.05) as compared with 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 with control treatments (Fig. 1D). It was found that the monosaccharide sugars tested did not trigger stylet thrusting in either *M. incognita* or *G. pallida* when used in isolation (data not shown), suggesting that additional plant root exudate components are required. Our data indicate that glucose and fructose agonise the rate of stylet thrusting subsequent to onset.

Treatment of tomato seedlings with *STP1* dsRNA triggered a significant reduction in *STP1* transcript abundance (0.17 ± 0.05; *P* <0.001), yet had no impact on *STP2* abundance (1.037 ±0.13; *P* >0.05) relative to neomycin phosphotransferase (*neo*) dsRNA treatment. Likewise, *STP2* dsRNA induced significant reductions in *STP2* transcript abundance (0.21 ± 0.06; *P* <0.001), but not *STP1* (0.94 ± 0.05; *P* >0.05) relative to *neo* dsRNA treatments (Fig. 2A). Corresponding reductions in glucose and fructose exudate concentration were observed for both *STP1* (5.10 µg/ml ± 1.31; *P* <0.01 and 3.14 µg/ml ± 0.92; *P* <0.01,

respectively) and *STP2* (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 (Figs. 2B - D).

Root exudates collected from tomato seedlings which had been treated with either *STP1* or *STP2* 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. 3A). No significant difference in stylet thrusting frequency was observed for *G. pallida* juveniles when exposed to root exudates from *STP1* or *STP2* dsRNA-treated seedlings, relative to control treated seedlings (108.2 ± 38.87%, *P* >0.05; and 77.34 ± 30.84%, *P* >0.05, respectively) (Fig. 3B).

When exoRNAi-treated seedlings were challenged by *M. incognita* infection, significant reductions in percentage infection levels relative to control (*neo*) dsRNA treatment were observed for both *STP1* (14.15% ± 4.77; *P* <0.01) and *STP2* (27.08% ± 7.32; *P* <0.05) dsRNA treatments (Fig. 3C). Knockdown of *STP1* (100.2% ± 17.03; *P* >0.05) or *STP2* (90.26% ± 23.19; *P* >0.05) did not significantly reduce the percentage infection levels of *G. pallida* relative to *neo* dsRNA treatment (Fig. 3D).

These data demonstrate that the exogenous application of aqueous dsRNA onto tomato seedlings is sufficient to trigger specific gene knockdown. However, we found that different experimental populations of tomato seedlings could display wide variation in the expression of both sugar transporter genes, and reference genes which resulted in high S.E.M. values. This made it difficult to resolve gene knockdown levels for a number of experiments which used isolated batches of tomato seeds. This may be due to variation in the susceptibility of tomato seedlings

to exoRNAi, as has been observed for Tobacco Rattle Virus (TRV) VIGS approaches in tomato (Liu et al., 2002), or it could indicate that larger replicates of seedlings are required to consistently resolve gene expression data post exoRNAi. The use of isogenic tomato seed lines may resolve this issue. It should also be noted that attempts to silence phytoene desaturase in order to observe a bleaching phenotype in the cotyledons were unsuccessful (data not shown). This may indicate that only genes expressed in the tomato root are susceptible to this approach, but warrants further investigation.

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). PPNs 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 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. These data indicate that glucose and fructose trigger host-finding and stylet thrusting 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). The demonstration that STP1 and STP2 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.

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References

Badri DV, Vivanco JM. 2009 Regulation and function of root exudates. Plant Cell Environ 32, 666 - 681.

Badri DV, Quintana N, El Kassis EG, Kim HK, Choi YH, Sugiyama A, Verpoorte R, Martinoia E, Manter DK, Vivanco JM. 2009 An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol 151, 2006 - 2017.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. 2006 The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol 57, 233 - 266.

Bybd DW JR, Kirkpatrick TJR, Barker KR. 1983. An Improved Technique for Clearing and Staining Plant Tissues for Detection of Nematodes. J. Nematol. 15,142 - 143.

Dakora FD, Phillips DA. 2002 Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245, 35 – 47.

Dekkers BJ, Willems L, Bassel GW, van Bolderen-Veldkamp RP, Ligterink W, Hilhorst HW, Bentsink L. 2012. Identification of reference genes for RT-qPCR expression analysis in *Arabidopsis* and tomato seeds. Plant Cell Physiol 53,28 - 37.

Gear ML, McPhillips ML, Patrick JW, McCurdy DW. 2000 Hexose transporters of tomato: molecular cloning, expression analysis and functional characterization. Plant Mol Biol 44, 687 - 697.

Hart AC 2006 Behavior. WormBook, doi/10.1895/wormbook.1.87.1, http://www.wormbook.org.

Jones DL, Nguyen C, Finlay RD. 2009 Carbon flow in the rhizosphere: carbon trading at the soil-root interface. Plant Soil 321, 5 – 33.

Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg B. 2006 Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol Plant Microbe In 19, 250 - 256.

Klink VP, Wolniak SM. 2001 Centrin is necessary for the formation of the motile apparatus in spermatids of *Marsilea*. Mol. Biol. Cell 12, 761 - 776.

Liu Y, Schiff M, Dinesh-Kumar SP. 2002 Virus-induced gene silencing in tomato. Plant J 31, 777 – 786.

Pfaffl MW. 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res 2001 29, e45.

Reuscher S, Akiyama M, Yasuda T, Makino H, Aoki K, Shibata D, Shiratake K. 2014 The sugar transporter inventory of tomato: genome wide identification and expression analysis. Plant Cell Physiol 55, 1123 - 1141.

Sasser JN, Freckman DW. 1987 Vistas on Nematology. In: Veech, J. A. (Ed.), A world perspective on nematology: the role of the society pp 7-14.

Teillet A, Dybal K, Kerry BR, Miller AJ, Curtis RHC, Hedden P. 2013 Transcriptional changes of the root-knot nematode *Meloidogyne incognita* in response to *Arabidopsis thaliana* root signals. PLoS One 8, e61259. doi:10.1371/journal.pone.0061259.

Tenllado F, Martínez-García B, Vargas M, Díaz-Ruíz JR. 2003 Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. BMC Biotechnol,3,3.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002 Accurate normalization of real-time quantitative RT-PCR data by

197 geometric averaging of multiple internal control genes. Genome Biol.,3,
198 RESEARCH0034.
199 Walker TS, Bais HP, Grotewold E, Vivanco JM. 2003 Root exudation and
200 rhizosphere biology. Plant Physiol 132, 44 - 51.
201 Watson JM, Fusaro AF, Wang M, Waterhouse PM. 2005 RNA Silencing
202 Platforms in Plants. FEBS Lett 579, 5982 - 5987.
203
204

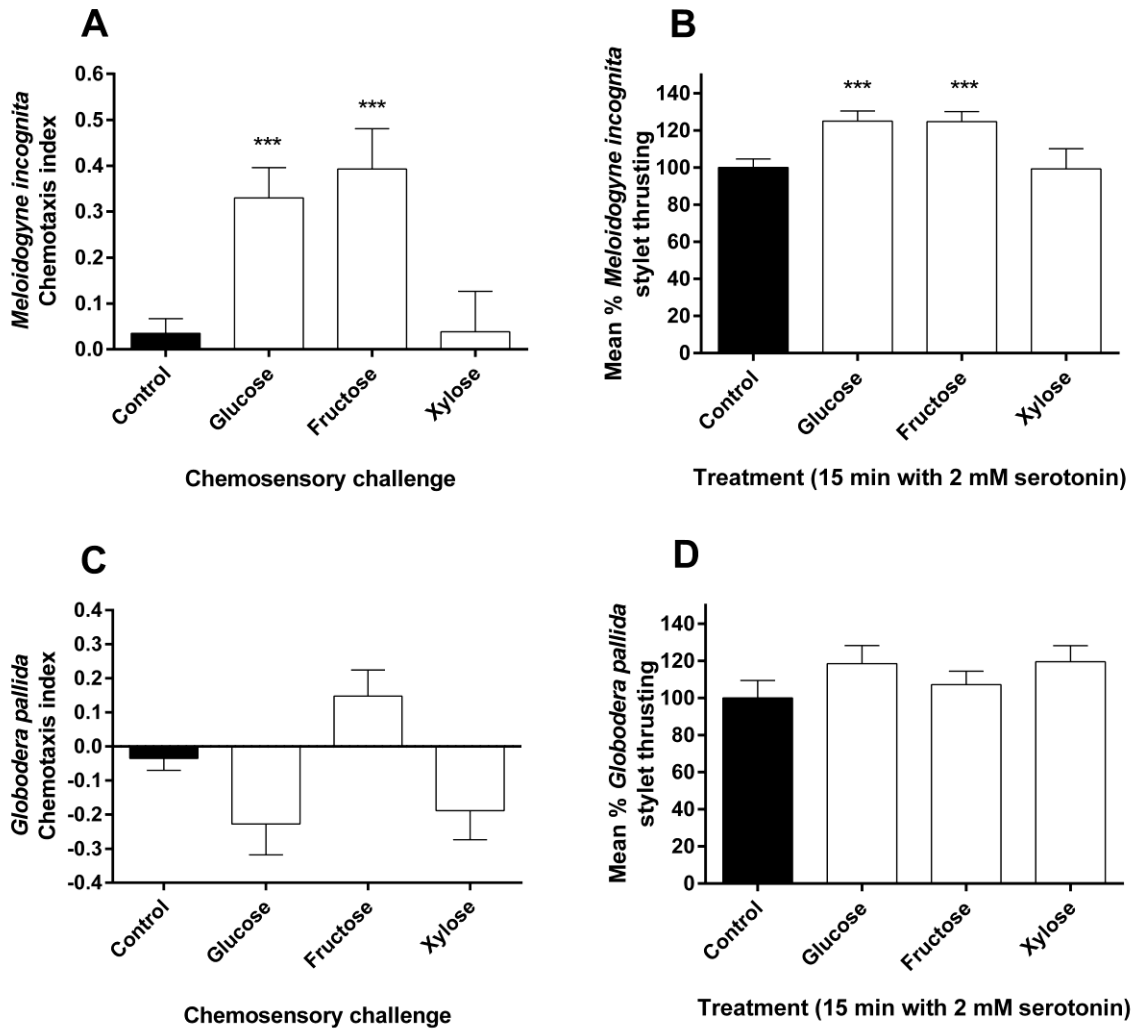


Fig. 1. Glucose and fructose stimulate different chemotaxis and stylet thrusting responses in *Meloidogyne incognita* and *Globodera pallida*. (A) Chemosensory response (chemosensory index) of *M. incognita* infective juveniles to glucose, fructose, xylose and control (water) assay challenge. Each data point represents the mean (±S.E.M.) of 10 assays of 100 infective juveniles each. (B) Mean percentage (±S.E.M.) 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 (±S.E.M.) stylet thrusting of glucose, fructose and xylose treated *G. pallida* infective stage juveniles (*n* = 100)

relative to control (2 mM serotonin in water). An agar slurry (0.25% agar, pH 7) was used to flood Petri dishes for chemosensory assays. Specifically, 3 ml of agar slurry was poured to provide 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. *Meloidogyne incognita* J2s were hatched from eggs in spring water (pH 7), and *G. pallida* cysts were hatched in 0.6 mM sodium orthovanadate (pH 7). One hundred *M. incognita* or *G. pallida* infective stage juveniles were suspended in 5 µl of 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 2 h assay period. Only nematodes outside the dead zone were counted. The distribution of plant parasitic nematode (PPN) infective stage juveniles was used to generate the chemotaxis index (Hart, 2006) for each assay plate which formed one replicate. For the stylet thrusting assay, 100 *M. incognita* or *G. pallida* infective stage juveniles were suspended in 20 µl of water (autoclaved and adjusted to pH 7) containing 2 mM serotonin and 50 mM of glucose, fructose or xylose (Sigma-Aldrich, UK). Worms were incubated in this solution for 15 min, pipetted onto a glass slide with a coverslip, and stylet thrusts were counted in randomly selected infective stage juveniles for 1 min each, slides where counted for a maximum of 15 min as variability was found to increase after 15 min causing a deterioration in the quality of the results. Control treatments were expressed as a percentage, including technical

variation, and experimental treatments were normalised to control percentages across individual experiments and days. Chemosensory and stylet thrusting results 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 *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

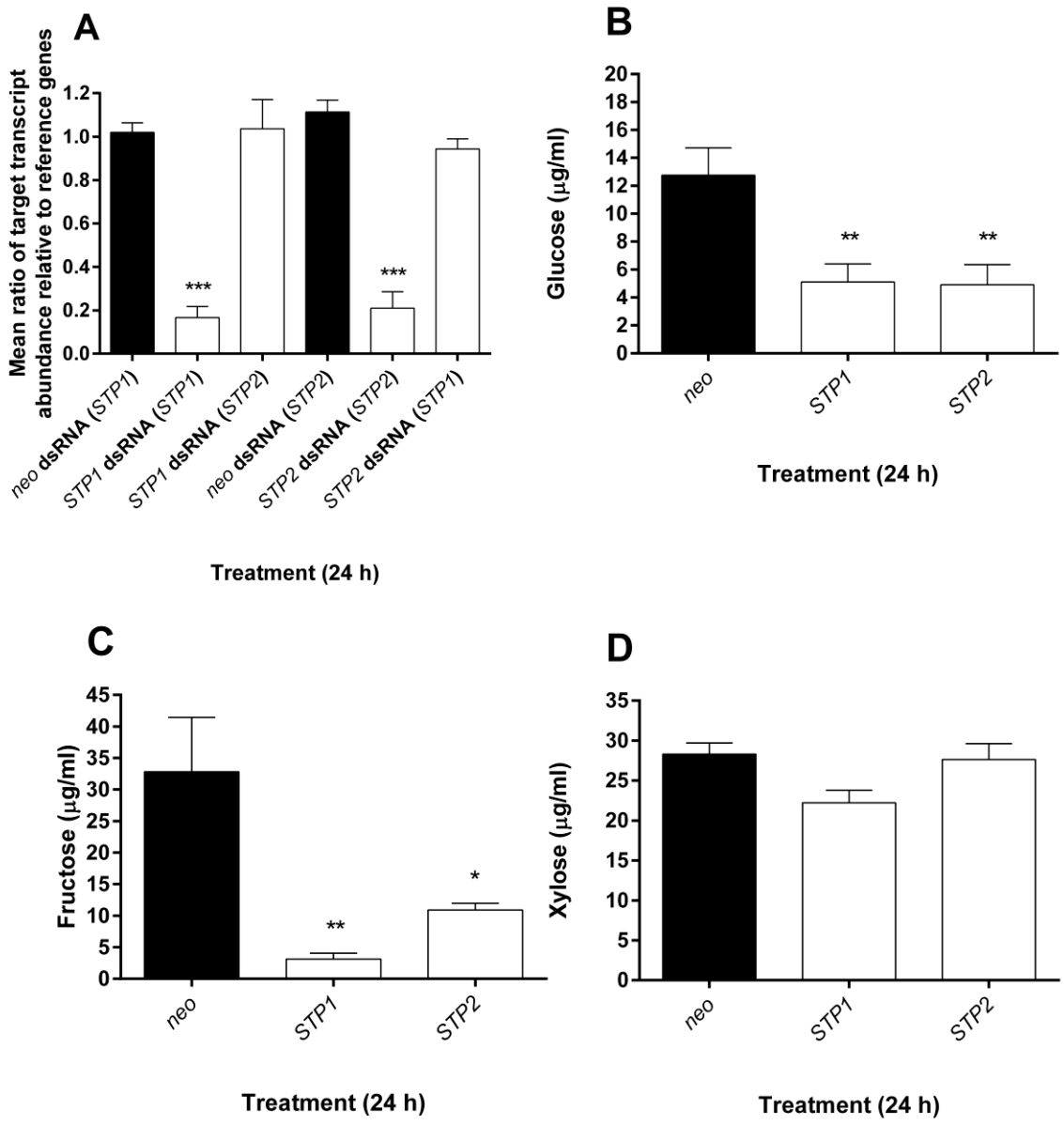


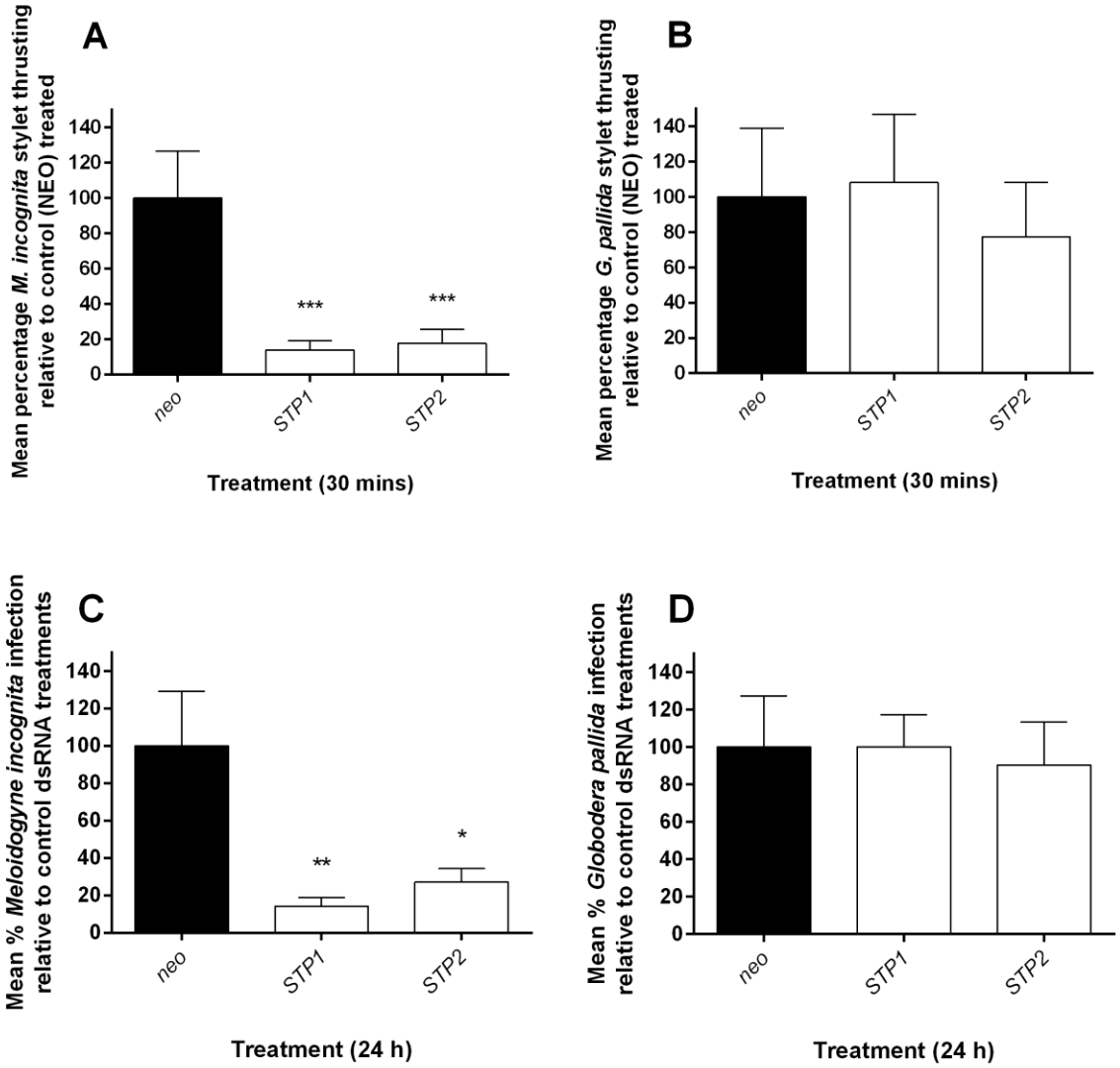
Fig. 2. Exogenous RNA interference (exoRNAi) induces target-specific knockdown of tomato sugar transporter genes (*STP*); alters root exudate composition. (A) Mean

ratio of target transcript (in parentheses) abundance relative to three endogenous reference genes. Each data point represents the mean (±S.E.M.) of three replicates of five seedlings each. Forward and reverse primers including 5' T7-recognition sites were used to generate specific amplicons for dsRNA synthesis to expressed sequence tag (EST)-supported fragments of *stp1* (Solyc02g079220.2), and *stp2* (Solyc09g075820.2) (Reuscher et al., 2014). Primers for dsRNA synthesis were as follows: (Neomycin Phosphotransferase, *neo*F 5' -GGTGGAGAGGCTATTCGGCT-3', *neo*FT7 5'-TAATACGACTCACTATAGGGGTGGAGAGGCTATTCGGCT -3', *neo*R 5'-CCTTCCCGCTTCAGTGACAA-3', *neo*RT7 5'-TAATACGACTCACTATAGGCCTTCCCGCTTCAGTGACAA -3'); (Sugar Transporter 1, *stp1*F 5'- CTGCTGTGATCACTGGTGGA-3', *stp1*FT7 5'-TAATACGACTCACTATAGGCTGCTGTGATCACTGGTGGA -3', *stp1*R 5'-ATTCCCCTGGAGTTCCATTT-3', *stp1*RT7 5'-TAATACGACTCACTATAGGATTCCCCTGGAGTTCCATTT -3'); (Sugar Transporter 2, *stp2*F 5'- ACGTTCTCTCCACCGTTGTC -3', *stp2*FT7 5'-TAATACGACTCACTATAGGACGTTCTCTCCACCGTTGTC -3', *stp2*R 5'-CTACGAAGATTCCCCAACCA-3', *stp2*RT7 5'-TAATACGACTCACTATAGGCTACGAAGATTCCCCAACCA-3');

PCR products were assessed by gel electrophoresis, and purified using the Chargeswitch PCR clean-up kit (Life Technologies, UK). dsRNA was synthesised using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, UK), and quantified by Nanodrop 1000 spectrophotometer. Tomato cv. Moneymaker seeds (Suttons, UK) were sterilised by 30 min treatment in dilute bleach, followed by five, 15 min 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 post radicle emergence. Ten seedlings were used per well of a 24-well plate (SPL Lifesciences, UK), and incubated with 300 µl of 10 ng/µl dsRNA solution for 24h at 23°C, in darkness. The root tissue of five seedlings were snap frozen in liquid nitrogen, representing one biological replicate. Total RNA was isolated using Trizol reagent, and treated with the Turbo DNase free kit (Life Technologies). cDNA was synthesised using the High-capacity RNA-to-cDNA kit (Applied Biosciences, UK) according to manufacturer's instructions using the maximum input concentration of RNA. Three biological replicates were performed for each treatment. Quantitative (q)RT-PCR primers were as follows: (Sugar Transporter 1, q*stp1*F 5'-ATGTTGCTGGATTCGCTTGGTC-3', q*stp1*R 5'-TGTGCAGCTGATCGAATTTCCAG-3'); (Sugar Transporter 2, q*stp2*F 5'-ATTATGGCTGCTACCGGAGGTC-3', q*stp2*R 5'-TGTAACACCACCAGAAACTCCAAC-3'); (Elongation Factor, q*efα*F 5'-TACTGGTGGTTTTGAAGCTG-3', q*efα*R 5'-AACTTCCTTCACGATTTCATCATA-3'); (SAND protein family, q*sand*F 5'- TTGCTTGGAGGAACAGACG-3', q*sand*R 5'-GCAAACAGAACCCCTGAATC-3'); (Sugar Transporter 41, q*stp41*F 5'-ATGGAGTTTTTGAGTCTTCTGC -3', q*stp41*R 5'-GCTGCGTTTCTGGCTTAGG -3') (Dekkers et al., 2012). 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. Each individual reaction comprised 5 µl Faststart SYBR Green mastermix (Roche Applied Science, UK), 1 µl each of the forward and reverse primers (10 µM), 1 µl water, 2 µl cDNA. PCR reactions were conducted in triplicate for each individual cDNA using a Rotorgene Q thermal cycler under the following conditions: (95°C x 10 min, 40 x

299 (95°C x 20s, 60°C x 20s, 72°C x 20s) 72°C x 10 min). The PCR efficiency of each
300 specific amplicon was calculated using the Rotorgene Q software, and quantification
301 of each target amplicon calculated by an augmented comparative Ct method (Pfaffl,
302 2001), relative to the geometric mean of three endogenous reference genes
303 (Vandesompele et al., 2002). Ratio-changes in transcript abundance were
304 calculated relative to control dsRNA treated seedlings in each case; Exudate
305 concentration of (B) glucose, (C) fructose and (D) xylose across *neo* (double
306 stranded (ds)RNA control), *STP1* and *STP2* dsRNA treated tomato seedlings. The
307 exudate solution was collected post RNAi by soaking in 1ml of double distilled water
308 for 24 h and transferred by pipette to a hydrophobically-lined microcentrifuge tube
309 (Anachem, UK) prior to quantification. The sugars were quantified colorimetrically at
310 340 nm using Glucose (HK), and Fructose assay kits from Sigma-Aldrich (UK), and
311 the Xylose assay kit from Megazyme (Ireland) as per manufacturer's instructions.
312 Each data point represents the mean (±S.E.M.) of three replicates of 10 seedlings
313 each. Data were analysed by ANOVA and Tukey's Honestly Significant Difference
314 test using Graphpad Prism 6. Probabilities of less than 5% ($P < 0.05$) were deemed
315 statistically significant *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



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317 **Fig. 3.** Exogenous RNA interference (exoRNAi) of tomato seedling sugar
318 transporters (STPs) differentially alters plant nematode infection and activation. (A)
319 Mean percentage (±S.E.M.) stylet thrusting of *Meloidogyne incognita* and (B)
320 *Globodera pallida* infective stage juveniles in response to collected seedling
321 exudates (*n* = 100), relative to control (neomycin phosphotransferase (*neo*) dsRNA).
322 Root exudate was collected and quantified as described in Fig. 2. Nematodes were
323 exposed to 50µl of dsRNA treated seedling exudate for 30 min, thrusts were counted
324 in randomly selected infective stage juveniles for 1 min each. (C) Mean percentage
325 *M. incognita* infection levels of *STP1* and *STP2* dsRNA treated tomato seedlings

normalised to control (*neo*) dsRNA treated seedlings. (D) Mean percentage *G. pallida* infection levels of *STP1* and *STP2* dsRNA treated tomato seedlings normalised to control (*neo*) dsRNA treated seedlings. Agar slurry was prepared by autoclaving a 0.55% agar solution which was subsequently adjusted to pH 7. The agar was agitated for 6 h at room temperature, until it had a smooth consistency. Five hundred *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 h at 23°C. Seedlings were subsequently removed from the slurry, gently washed several times by immersion in deionised water, and stained using acid fuchsin (Bybd et al., 1983). The number of invading plant parasitic nematode (PPN) juveniles was counted for each seedling using a light microscope. Control treatments were expressed as a percentage, including technical variation, and experimental treatments were normalised to control percentages. Each data point represents the mean (±S.E.M.) of 10 seedlings challenged with 500 infective stage juveniles each. *, P <0.05; **, P <0.01; ***, P <0.001.