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

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17 **Abstract**

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

29

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

31

32

33 RNA interference (RNAi) is widely used for the analysis of plant gene function,
34 primarily through the transgenic production of dsRNA constructs in planta, and
35 secondarily through Virus-Induced Gene Silencing (VIGS) (Watson et al., 2005).
36 Previous findings by Wolniak and colleagues have shown that exogenous dsRNA
37 can silence genes of the water fern *Marsilea vestita* (Klink and Wolniak, 2001), and
38 crude lysate from *Escherichia coli* expressing virus-specific dsRNA have also been
39 used to protect plants from viral pathology (Tenllado et al., 2003). Here we present a
40 similar approach to triggering RNAi in tomato seedlings, which we term exogenous
41 (exo)RNAi. In this approach, aqueous dsRNA is delivered exogenously to tomato
42 seedlings.

43 Plant root exudate comprises a complex mixture of compounds including
44 volatile and soluble chemicals which may derive from intact or damaged root cells, or
45 sloughed-off root border cells (Dakora and Phillips, 2002). It has been estimated
46 that 11% of photosynthetically-assimilated carbon is released as root exudate (Jones
47 et al., 2009). The monosaccharides glucose, fructose and xylose represent the
48 major sugar component of tomato root exudates (Kamilova et al., 2006). Plant
49 parasitic nematodes (PPNs) are responsible for an estimated 12.3% loss in crop
50 production globally each year (Sasser and Freckman, 1987), and are attracted to
51 host plants by components of plant root exudate. Here we assess the
52 chemosensory response of the root knot nematode, *Meloidogyne incognita* (a
53 promiscuous pathogen of flowering plants), and the potato cyst nematode,
54 *Globodera pallida* (a selective pathogen of Solanaceae plants) to each of the three
55 major monosaccharide sugars of tomato plant root exudate, and the efficacy of
56 exoRNAi against *STP1* and *STP2*, known transporters of monosaccharide sugars in
57 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,

83 respectively) and *STP2* (4.90 µg/ml ± 1.45; P <0.01 and 10.90 µg/ml ± 1.07; P <0.05,
84 respectively) dsRNA treated seedlings. No significant changes in xylose exudate
85 concentration were observed across treatment groups (Figs. 2B - D).

86 Root exudates collected from tomato seedlings which had been treated with
87 either *STP1* or *STP2* dsRNA were less capable of stimulating stylet thrusting in *M.*
88 *incognita* relative to exudates collected from control dsRNA treated seedlings (13.92
89 ± 5.10%, P <0.001; and 17.53 ± 8.12%, P <0.001, respectively) (Fig. 3A). No
90 significant difference in stylet thrusting frequency was observed for *G. pallida*
91 juveniles when exposed to root exudates from *STP1* or *STP2* dsRNA-treated
92 seedlings, relative to control treated seedlings (108.2 ± 38.87%, P >0.05; and 77.34
93 ± 30.84%, P >0.05, respectively) (Fig. 3B).

94 When exoRNAi-treated seedlings were challenged by *M. incognita* infection,
95 significant reductions in percentage infection levels relative to control (*neo*) dsRNA
96 treatment were observed for both *STP1* (14.15% ± 4.77; P <0.01) and *STP2*
97 (27.08% ± 7.32; P <0.05) dsRNA treatments (Fig. 3C). Knockdown of *STP1*
98 (100.2% ± 17.03; P >0.05) or *STP2* (90.26% ± 23.19; P >0.05) did not significantly
99 reduce the percentage infection levels of *G. pallida* relative to *neo* dsRNA treatment
100 (Fig. 3D).

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

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

116 It is well established that plant root exudates mediate both positive and
117 negative interactions with commensal and pathogenic microbes (Badri et al., 2009),
118 insects (Walker et al., 2003), and other plants (Bais et al., 2006). PPNs also
119 respond to plant root exudates (Teillet et al., 2013). The present study aimed to
120 probe the involvement of monosaccharide sugars of tomato root exudate in the
121 attraction and activation of parasitic behaviours in the promiscuous root knot
122 nematode *M. incognita*, and the host-selective potato cyst nematode *G. pallida*.
123 STP1 and STP2 are known transporters of monosaccharide sugars (Gear et al.,
124 2000), and our data demonstrate that both play a role in regulating the level of
125 glucose and fructose (but not xylose) exudation from tomato seedling roots.
126 exoRNAi knockdown of each transporter significantly reduced the amount of glucose
127 and fructose secreted from plant roots, which corresponded with a decrease in *M.*
128 *incognita* infectivity, but not *G. pallida* infectivity. These results suggest that glucose
129 and fructose are important chemical cues which infective stage *M. incognita* use to
130 find host plants. These data indicate that glucose and fructose trigger host-finding
131 and stylet thrusting in promiscuous PPNs, as opposed to host-specific PPNs, an
132 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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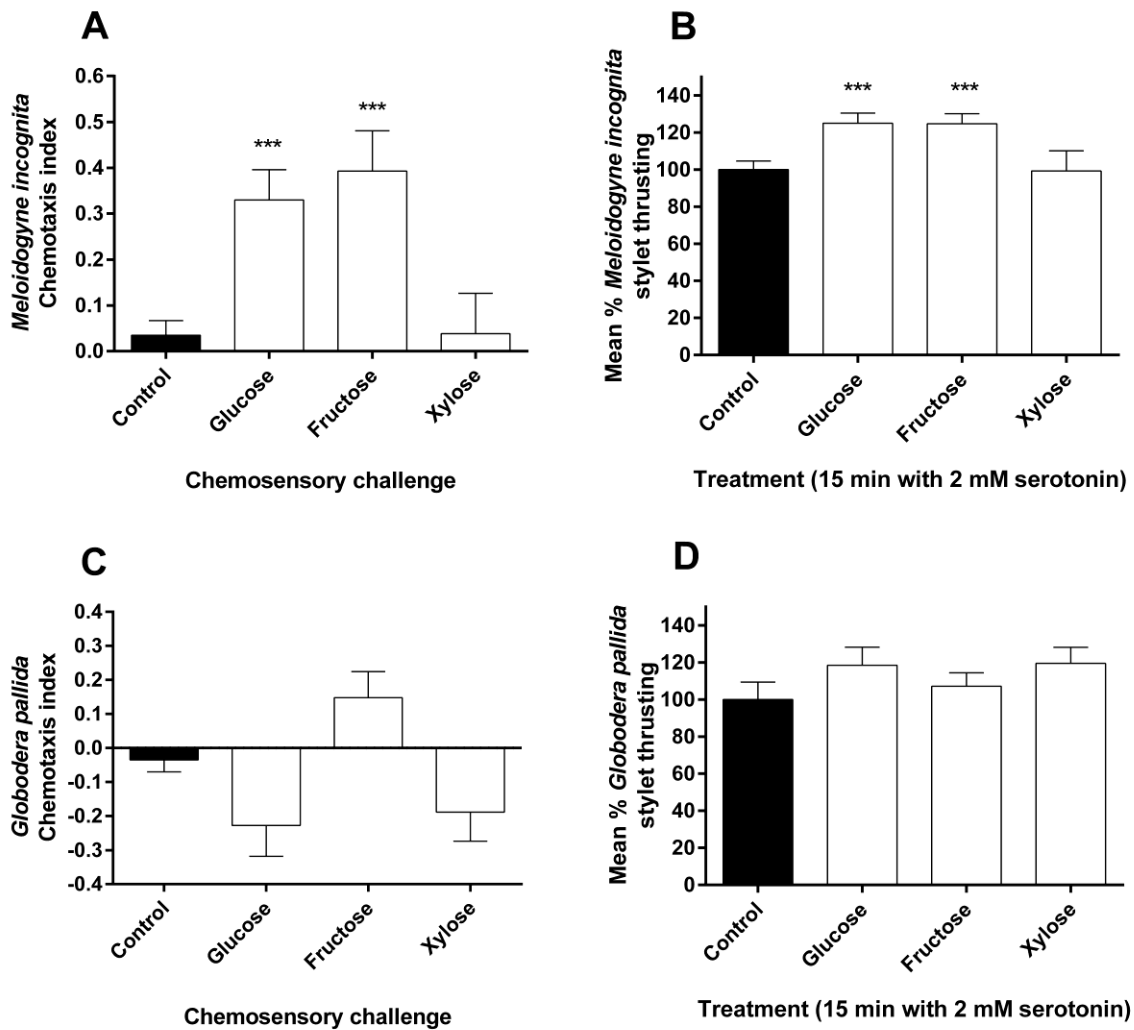


205

206 **Fig. 1.** Glucose and fructose stimulate different chemotaxis and stylet thrusting
207 responses in *Meloidogyne incognita* and *Globodera pallida*. (A) Chemosensory
208 response (chemosensory index) of *M. incognita* infective juveniles to glucose,
209 fructose, xylose and control (water) assay challenge. Each data point represents the
210 mean (±S.E.M.) of 10 assays of 100 infective juveniles each. (B) Mean percentage
211 (±S.E.M.) stylet thrusting of glucose, fructose and xylose treated *M. incognita*
212 infective stage juveniles (*n* = 100) relative to control (2 mM serotonin in water). **(**C)
213 Chemosensory response of *G. pallida* infective juveniles to glucose, fructose, xylose
214 and control (water) assay challenge. (D) Mean percentage (±S.E.M.) stylet thrusting
215 of glucose, fructose and xylose treated *G. pallida* infective stage juveniles (*n* = 100)

216 relative to control (2 mM serotonin in water). An agar slurry (0.25% agar, pH 7) was
217 used to flood Petri dishes for chemosensory assays. Specifically, 3 ml of agar slurry
218 was poured to provide the medium through which the infective stage juveniles could
219 move. Sugar plugs were prepared by dissolving 50 mM of the relevant sugar
220 (glucose / fructose / xylose) in 0.25% agar and allowed to set. Plugs were picked
221 with a Pasteur pipette which had been cut half way down the pipette barrel, and
222 placed onto one side of a Petri dish, with a negative plug (water instead of 50 mM
223 sugar) on the other. *Meloidogyne incognita* J2s were hatched from eggs in spring
224 water (pH 7), and *G. pallida* cysts were hatched in 0.6 mM sodium orthovanadate
225 (pH 7). One hundred *M. incognita* or *G. pallida* infective stage juveniles were
226 suspended in 5 µl of water, and spotted onto the centre point of each dish. A Petri
227 dish lid was marked with two parallel vertical lines 0.5 cm either side of the centre
228 point forming a 1 cm 'dead zone' that ran vertically along the lid. Assay plates were
229 set onto the lid for scoring of nematode positions following a 2 h assay period. Only
230 nematodes outside the dead zone were counted. The distribution of plant parasitic
231 nematode (PPN) infective stage juveniles was used to generate the chemotaxis
232 index (Hart, 2006) for each assay plate which formed one replicate. For the stylet
233 thrusting assay, 100 *M. incognita* or *G. pallida* infective stage juveniles were
234 suspended in 20 µl of water (autoclaved and adjusted to pH 7) containing 2 mM
235 serotonin and 50 mM of glucose, fructose or xylose (Sigma-Aldrich, UK). Worms
236 were incubated in this solution for 15 min, pipetted onto a glass slide with a
237 coverslip, and stylet thrusts were counted in randomly selected infective stage
238 juveniles for 1 min each, slides where counted for a maximum of 15 min as variability
239 was found to increase after 15 min causing a deterioration in the quality of the
240 results. Control treatments were expressed as a percentage, including technical

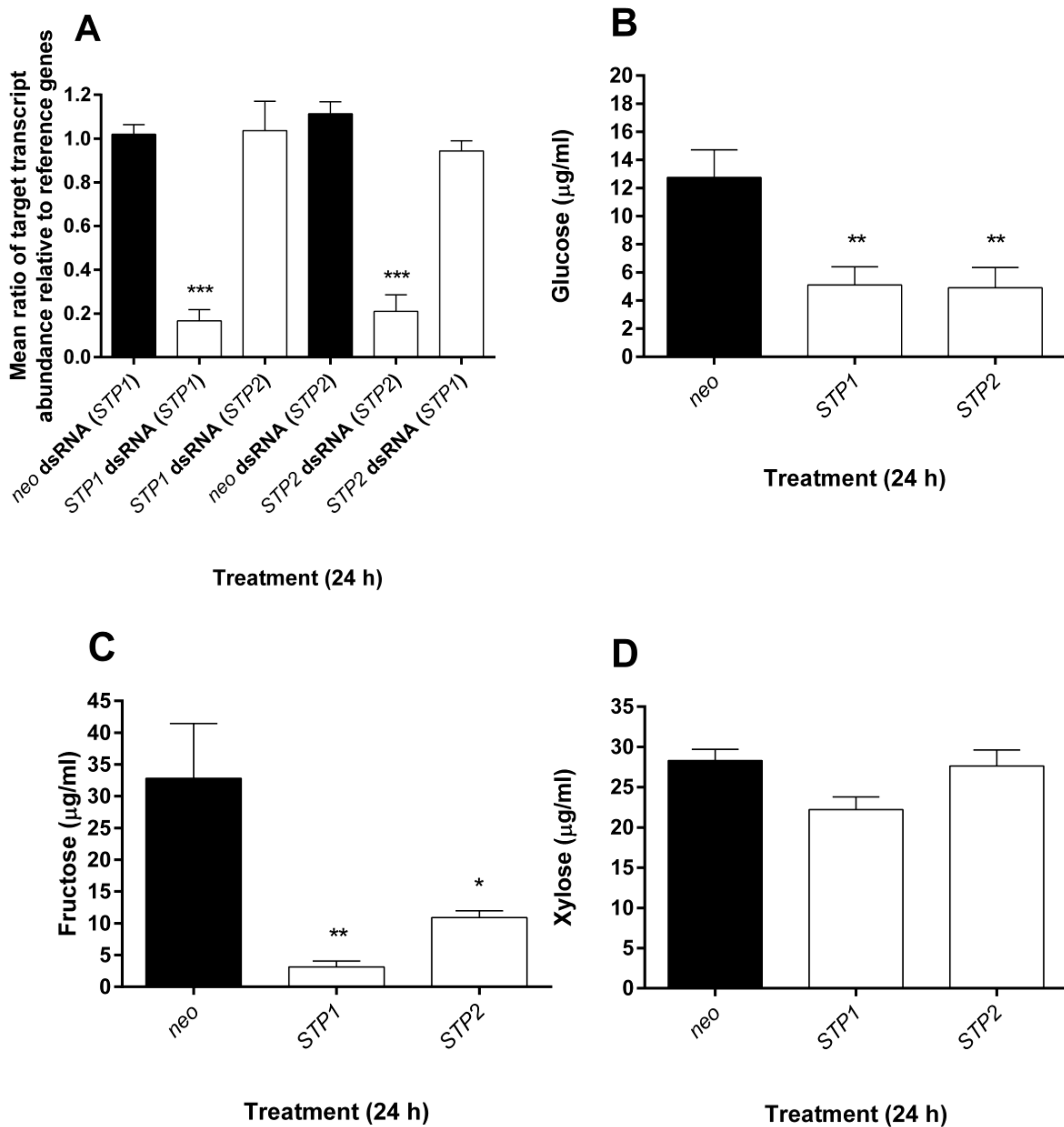
241 variation, and experimental treatments were normalised to control percentages

242 across individual experiments and days. Chemosensory and stylet thrusting results

243 were analysed by one-way ANOVA and Tukey's Honestly Significant Difference test

244 using Graphpad Prism 6. Probabilities of less than 5% ($P < 0.05$) were deemed

245 statistically significant *, P <0.05; **, P <0.01; ***, P <0.001.



246

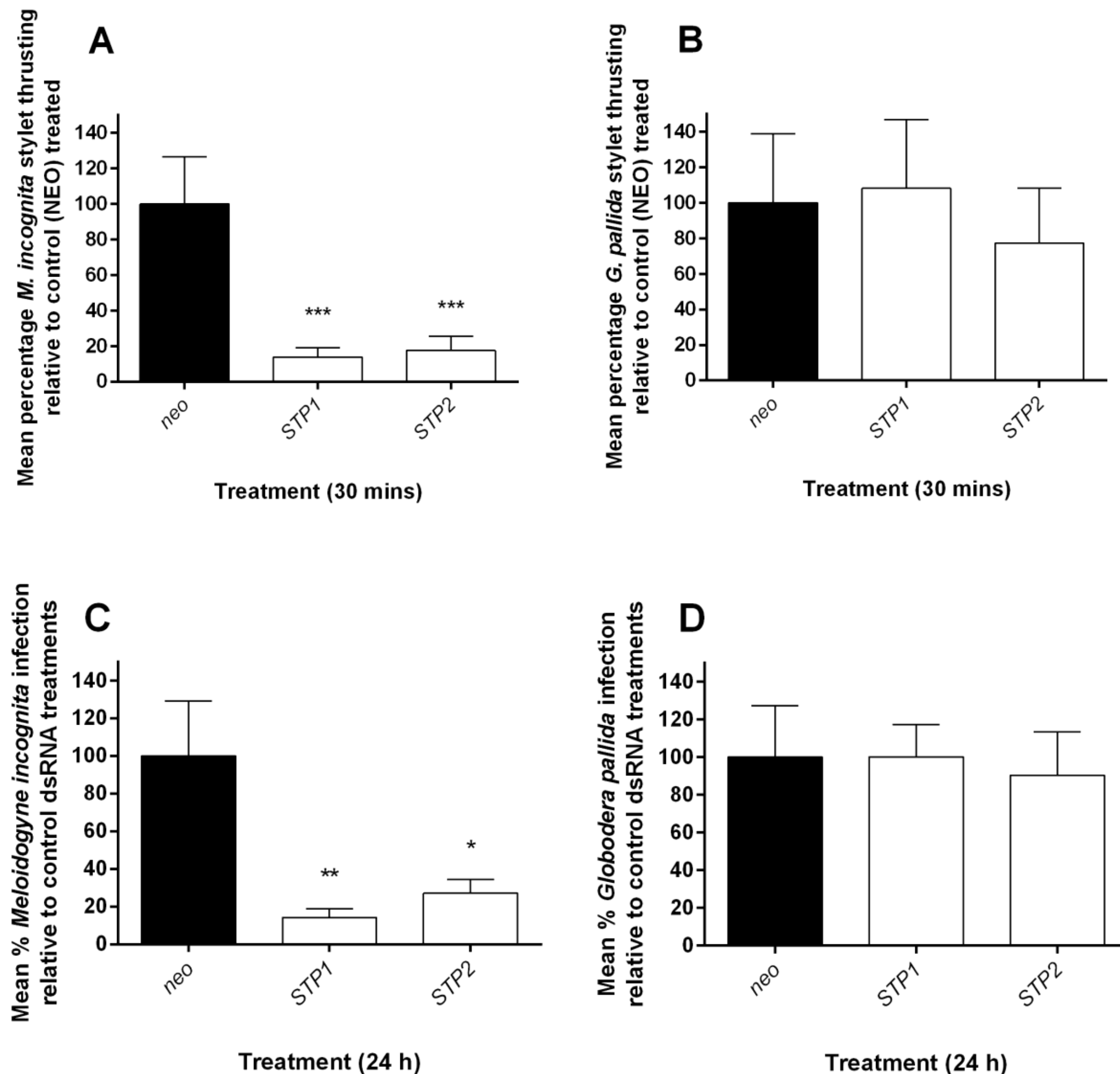
247 **Fig. 2.** Exogenous RNA interference (exoRNAi) induces target-specific knockdown

248 of tomato sugar transporter genes (*STP*); alters root exudate composition. (A) Mean

249 ratio of target transcript (in parentheses) abundance relative to three endogenous
250 reference genes. Each data point represents the mean (±S.E.M.) of three replicates
251 of five seedlings each. Forward and reverse primers including 5' T7-recognition sites
252 were used to generate specific amplicons for dsRNA synthesis to expressed
253 sequence tag (EST)-supported fragments of *stp1* (Solyc02g079220.2), and *stp2*
254 (Solyc09g075820.2) (Reuscher et al., 2014). Primers for dsRNA synthesis were as
255 follows: (Neomycin Phosphotransferase, *neo*F 5'
256 -GGTGGAGAGGCTATTCGGCT-3', *neo*FT7 5'-
257 TAATACGACTCACTATAGGGGTGGAGAGGCTATTCGGCT -3', *neo*R 5'-
258 CCTTCCCGCTTCAGTGACAA-3', *neo*RT7 5'-
259 TAATACGACTCACTATAGGCCTTCCCGCTTCAGTGACAA -3'); (Sugar Transporter
260 1, *stp1*F 5'- CTGCTGTGATCACTGGTGGA-3', *stp1*FT7 5'-
261 TAATACGACTCACTATAGGCTGCTGTGATCACTGGTGGA -3', *stp1*R 5'-
262 ATTCCCCTGGAGTTCCATTT-3', *stp1*RT7 5'-
263 TAATACGACTCACTATAGGATTCCCCTGGAGTTCCATTT -3'); (Sugar Transporter
264 2, *stp2*F 5'- ACGTTCTCTCCACCGTTGTC -3', *stp2*FT7 5'-
265 TAATACGACTCACTATAGGACGTTCTCTCCACCGTTGTC -3', *stp2*R 5'-
266 CTACGAAGATTCCCCAACCA-3', *stp2*RT7 5'-
267 TAATACGACTCACTATAGGCTACGAAGATTCCCCAACCA-3');
268 PCR products were assessed by gel electrophoresis, and purified using the
269 Chargeswitch PCR clean-up kit (Life Technologies, UK). dsRNA was synthesised
270 using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega,
271 UK), and quantified by Nanodrop 1000 spectrophotometer. Tomato cv. Moneymaker
272 seeds (Suttons, UK) were sterilised by 30 min treatment in dilute bleach, followed by
273 five, 15 min washes in 1 ml deionised water. Seeds were germinated on 0.5X MS

274 salts, 0.6% agar plates at 23°C, and taken for exoRNAi treatment on the first day
275 post radicle emergence. Ten seedlings were used per well of a 24-well plate (SPL
276 Lifesciences, UK), and incubated with 300 µl of 10 ng/µl dsRNA solution for 24h at
277 23°C, in darkness. The root tissue of five seedlings were snap frozen in liquid
278 nitrogen, representing one biological replicate. Total RNA was isolated using Trizol
279 reagent, and treated with the Turbo DNase free kit (Life Technologies). cDNA was
280 synthesised using the High-capacity RNA-to-cDNA kit (Applied Biosciences, UK)
281 according to manufacturer's instructions using the maximum input concentration of
282 RNA. Three biological replicates were performed for each treatment. Quantitative
283 (q)RT-PCR primers were as follows: (Sugar Transporter 1, q*stp1*F 5'-
284 ATGTTGCTGGATTCGCTTGGTC-3', q*stp1*R 5'-
285 TGTGCAGCTGATCGAATTTCCAG-3'); (Sugar Transporter 2, q*stp2*F 5'-
286 ATTATGGCTGCTACCGGAGGTC-3', q*stp2*R 5'-
287 TGTAACACCACCAGAAACTCCAAC-3'); (Elongation Factor, q*efα*F 5'-
288 TACTGGTGGTTTTGAAGCTG-3', q*efα*R 5'-AACTTCCTTCACGATTTCATCATA-3');
289 (SAND protein family, q*sand*F 5'- TTGCTTGGAGGAACAGACG-3', q*sand*R 5'-
290 GCAAACAGAACCCCTGAATC-3'); (Sugar Transporter 41, q*stp41*F 5'-
291 ATGGAGTTTTTGAGTCTTCTGC -3', q*stp41*R 5'-GCTGCGTTTCTGGCTTAGG -3')
292 (Dekkers et al., 2012). Primer sets to be used for qPCR were optimised for working
293 concentration, annealing temperature and analysed by dissociation curve for
294 contamination or non-specific amplification by primer–dimer as standard. Each
295 individual reaction comprised 5 µl Faststart SYBR Green mastermix (Roche Applied
296 Science, UK), 1 µl each of the forward and reverse primers (10 µM), 1 µl water, 2 µl
297 cDNA. PCR reactions were conducted in triplicate for each individual cDNA using a
298 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 *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



316

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

326 normalised to control (*neo*) dsRNA treated seedlings. (D) Mean percentage *G.*
327 *pallida* infection levels of *STP1* and *STP2* dsRNA treated tomato seedlings
328 normalised to control (*neo*) dsRNA treated seedlings. Agar slurry was prepared by
329 autoclaving a 0.55% agar solution which was subsequently adjusted to pH 7. The
330 agar was agitated for 6 h at room temperature, until it had a smooth consistency.
331 Five hundred *M. incognita* or *G. pallida* infective stage juveniles were added to each
332 well of a 6-well plate (SPL Lifesciences) with one exoRNAi treated seedling
333 embedded within 3 ml of agar slurry. Plates were sealed with parafilm, covered
334 above and below with a sheet of tin foil and incubated for 24 h at 23°C. Seedlings
335 were subsequently removed from the slurry, gently washed several times by
336 immersion in deionised water, and stained using acid fuchsin (Bybd et al., 1983).
337 The number of invading plant parasitic nematode (PPN) juveniles was counted for
338 each seedling using a light microscope. Control treatments were expressed as a
339 percentage, including technical variation, and experimental treatments were
340 normalised to control percentages. Each data point represents the mean (±S.E.M.)
341 of 10 seedlings challenged with 500 infective stage juveniles each. *, $P < 0.05$; **, P
342 < 0.01; ***, $P < 0.001$.
343