

1 **ExoRNAi, a new tool to probe plant gene function exposes contrasting roles** 2 **for sugar exudation in host-finding by plant pathogens**

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11

12 **Abstract**

13 Here we present a simple and rapid new method for RNAi-induced knockdown of genes in
14 tomato seedlings, through treatment with an aqueous solution of double-stranded RNA
15 (exoRNAi). The exoRNAi method is used to assess the involvement of tomato Sugar
16 Transporter Protein genes, *stp1* and *stp2* on the root exudation of glucose, fructose and
17 xylose; monosaccharide constituents of tomato root exudate. Plant parasitic nematodes
18 (PPNs) are responsible for an estimated 12.3% loss in crop production globally, which
19 equates to financial losses of approximately £100 billion annually. Our data show that
20 infective juveniles of the promiscuous PPN, *Meloidogyne incognita* are attracted to glucose
21 and fructose, but not xylose. Glucose and fructose also agonise serotonergic stylet thrusting
22 in *M. incognita* infective juveniles; a key parasitic behaviour necessary for invasion and
23 parasitism of host plants. In contrast, infective juveniles of the selective Solanaceae PPN,
24 *Globodera pallida* are not attracted to tested monosaccharides, nor do the
25 monosaccharides stimulate stylet thrusting. We demonstrate that knockdown of both
26 *SISTP1* and *SISTP2* in tomato seedlings by the exoRNAi method is robust and specific, and
27 that corresponding reductions of glucose and fructose, but not xylose, in collected exudate,
28 correlate directly with reduced infectivity and stylet thrusting of *M. incognita*. Knockdown
29 of *SISTP1* or *SISTP2* have no impact on the infectivity or stylet thrusting of *G. pallida*. The
30 exoRNAi platform can underpin future efforts to understand the early stages of plant-
31 pathogen interactions in tomato, and potentially other crop plants.

32

33 **Introduction**

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

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

53 Plant root exudate comprises a complex mixture of compounds including volatile
54 and soluble chemicals which may derive from intact or damaged root cells, or sloughed-off
55 root border cells (Dakora and Phillips, 2002). It has been estimated that 11% of
56 photosynthetically-assimilated carbon is released as root exudate (Jones *et al.*, 2009). The
57 monosaccharides glucose, fructose and xylose represent the major sugar component of
58 tomato root exudates (Kamilova *et al.*, 2006). Plant parasitic nematodes (PPNs) are
59 responsible for an estimated 12.3% loss in crop production globally each year (Sasser and
60 Freckman, 1987), and are attracted to host plants by components of plant root exudate.
61 Here we purposed to assess the chemosensory response of root knot nematode,
62 *Meloidogyne incognita* (a promiscuous pathogen of flowering plants), and potato cyst
63 nematode, *Globodera pallida* (a selective pathogen of Solanaceae plants) to each of the
64 three major monosaccharide sugars of tomato plant root exudate, and to assess the efficacy
65 of exoRNAi against *SISTP1* and *SISTP2*, known transporters of monosaccharide sugars in
66 tomato (Gear *et al.*, 2000).

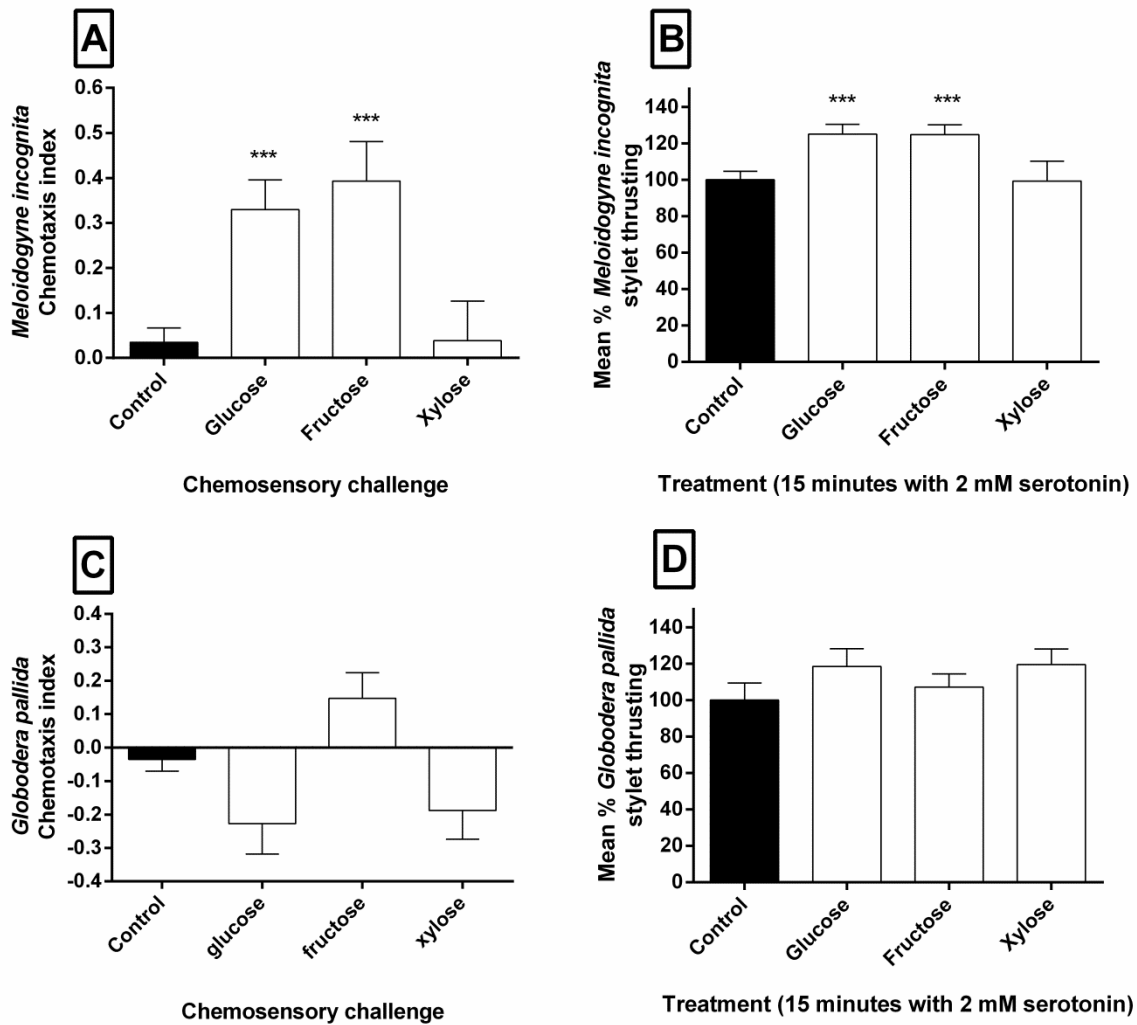
67

68 **Results**

69 *Nematode chemosensory and stylet thrusting response to sugars*

70 *Meloidogyne incognita* infective stage juveniles were attracted to glucose (CI: 0.33 ± 0.07 ;
71 $P < 0.01$) and fructose (CI: 0.39 ± 0.09 ; $P < 0.01$), but not xylose (CI: 0.04 ± 0.09 ; $P > 0.05$) as
72 compared to control treated worms (Fig 1A). Glucose ($125.1\% \pm 5.5$; $P < 0.001$) and fructose
73 ($124.8\% \pm 5.4$; $P < 0.001$) also triggered an elevated level of serotonin-triggered stylet
74 thrusting in treated juveniles; xylose failed to trigger any significant response (99.36%
75 ± 10.87 ; $P > 0.05$) when compared to control treatments (Fig 1B). *Globodera pallida* infective
76 stage juveniles were mildly repelled by glucose (CI: -0.23 ± 0.09 ; $P > 0.05$), and did not
77 appreciably respond to fructose (CI: 0.15 ± 0.08 ; $P > 0.05$), or xylose (CI: -0.19 ± 0.09 ; $P > 0.05$)
78 as compared to control treated worms (Fig 1C). Glucose ($118.6\% \pm 9.7$; $P > 0.05$), fructose
79 ($107.2\% \pm 7.3$; $P > 0.05$), or xylose ($119.6\% \pm 8.6$; $P > 0.05$) had no significant impact on the
80 frequency of serotonin-triggered stylet thrusting in *G. pallida* infective juveniles when
81 compared to control treatments (Fig 1D).

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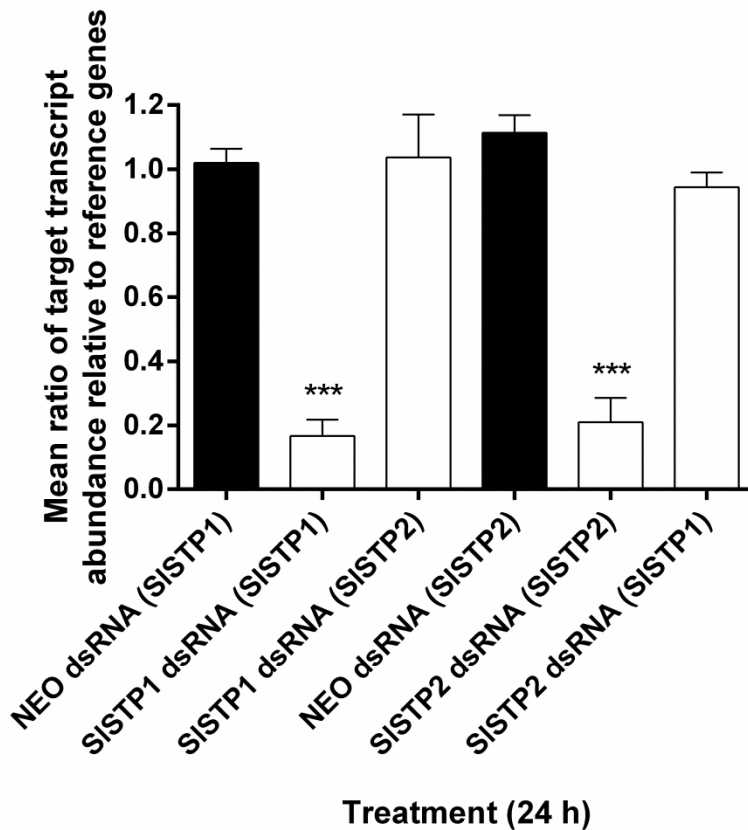
84 **Figure 1. Glucose and fructose stimulate different chemotaxis and stylet thrusting**

85 **responses in *M. incognita* and *G. pallida*.** (A) Chemosensory response (chemosensory
 86 index) of *M. incognita* infective juveniles to glucose, fructose, xylose and control (water)
 87 assay challenge. Each data point represents the mean (\pm SEM) of 10 assays of 100 infective
 88 juveniles each. (B) Mean percentage (\pm SEM) stylet thrusting of glucose, fructose and xylose
 89 treated *M. incognita* infective stage juveniles ($n=100$) relative to control (2 mM serotonin in
 90 water). (C) Chemosensory response of *G. pallida* infective juveniles to glucose, fructose,
 91 xylose and control (water) assay challenge. (D) Mean percentage (\pm SEM) stylet thrusting of
 92 glucose, fructose and xylose treated *G. pallida* infective stage juveniles ($n=100$) relative to
 93 control (2 mM serotonin in water). ***, $P<0.001$.

94

95 *exoRNAi* impact on tomato gene expression,

96 Treatment of tomato seedlings with *SISTP1* dsRNA triggered a significant reduction in *SISTP1*
 97 transcript abundance (0.17 ± 0.05 ; $P<0.001$), yet had no impact on *SISTP2* abundance (1.037
 98 ± 0.13 ; $P>0.05$) relative to neomycin transferase (NEO) dsRNA treatment. Likewise, *SISTP2*
 99 dsRNA induced significant reductions in *SISTP2* transcript abundance (0.21 ± 0.06 ; $P<0.001$),
 100 but not *SISTP1* (0.94 ± 0.05 ; $P>0.05$) relative to NEO dsRNA treatments (Fig 2).



101

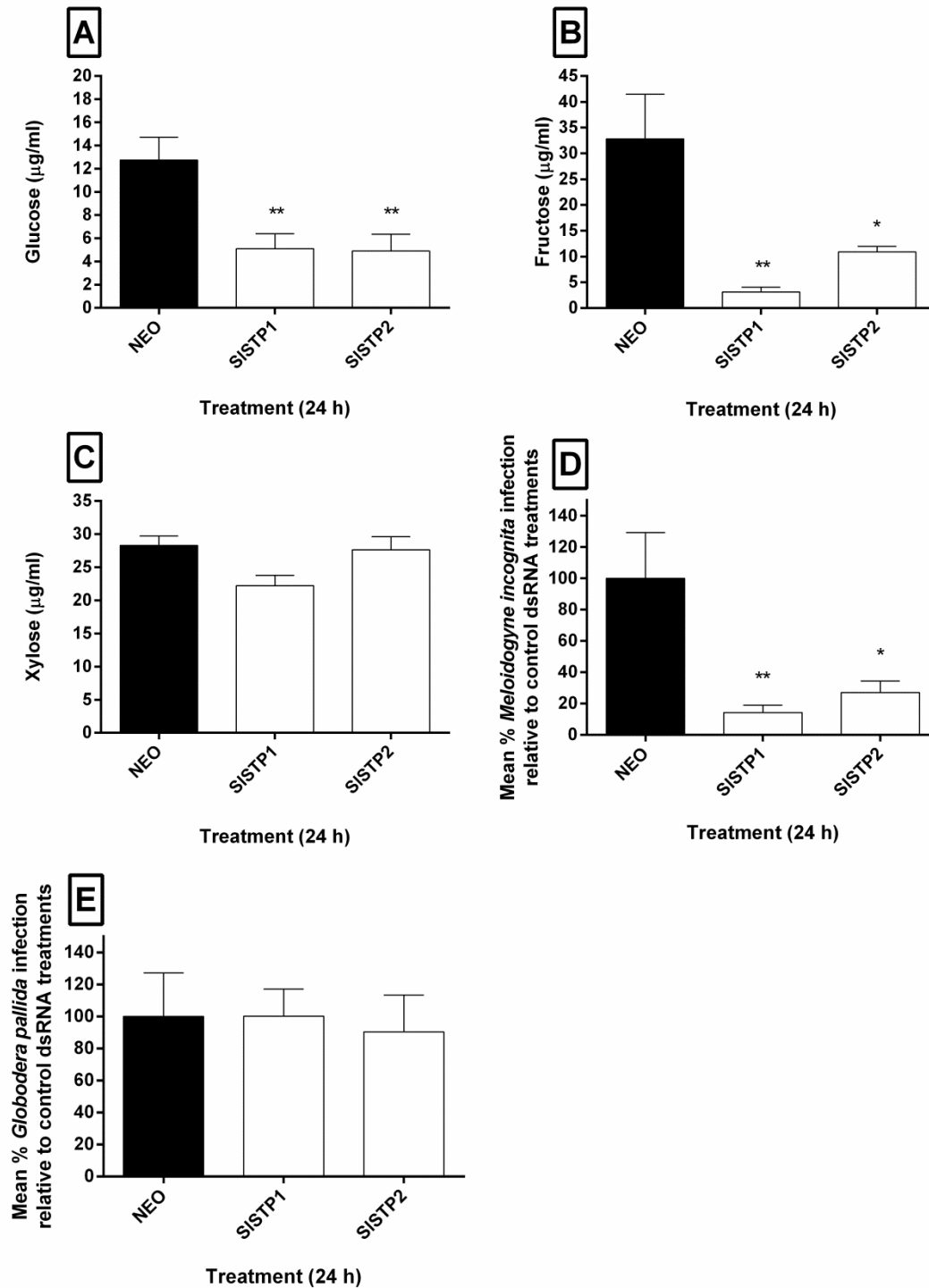
102 **Figure 2. ExoRNAi induces target-specific silencing of tomato plant genes.** Mean ratio of
103 target transcript (in parentheses) abundance relative to three endogenous reference genes.
104 Each data point represents the mean (\pm SEM) of three replicates of five seedlings each. ***,
105 $P < 0.001$.

106

107 *exoRNAi impact on sugar exudation and nematode infectivity*

108 Corresponding reductions in glucose and fructose exudate concentration were observed for
109 both *SISTP1* ($5.10 \mu\text{g/ml} \pm 1.31$; $P < 0.01$ and $3.14 \mu\text{g/ml} \pm 0.92$; $P < 0.01$, respectively) and
110 *SISTP2* ($4.90 \mu\text{g/ml} \pm 1.45$; $P < 0.01$ and $10.90 \mu\text{g/ml} \pm 1.07$; $P < 0.05$, respectively) dsRNA
111 treated seedlings. No significant changes in xylose exudate concentration were observed
112 across treatment groups (Fig 3A-C). When exoRNAi-treated seedlings were challenged by
113 *M. incognita* infection assay, significant reductions in percentage infection levels relative to
114 NEO dsRNA treatment were observed for both *SISTP1* ($14.15\% \pm 4.77$; $P < 0.01$) and *SISTP2*
115 ($27.08\% \pm 7.32$; $P < 0.05$) dsRNA treatments (Fig 3D). Knockdown of *SISTP1* ($14.15\% \pm 4.77$;
116 $P > 0.05$) or *SISTP2* ($14.15\% \pm 4.77$; $P > 0.05$) did not significantly reduce the percentage
117 infection levels of *G. pallida* relative to NEO dsRNA treatment.

118



119

120 **Figure 3. ExoRNA interference (exoRNAi) of tomato seedling sugar transporters alters**
121 **root exudate and plant nematode infection.** Exudate concentration of (A) glucose, (B)
122 fructose and (C) xylose across NEO (double stranded [ds]RNA control), *SISTP1* and *SISTP2*
123 dsRNA treated tomato seedlings. Each data point represents the mean (\pm SEM) of three
124 replicates of ten seedlings each. (D) Mean percentage *M. incognita* infection levels of
125 *SISTP1* and *SISTP2* dsRNA treated tomato seedlings normalised to NEO double stranded

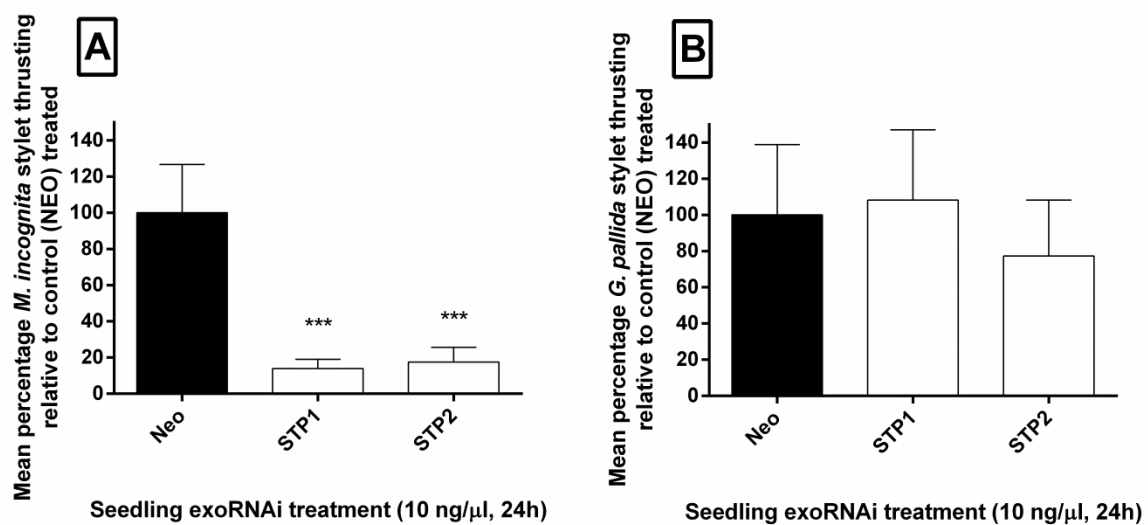
126 (ds)RNA (control) treated seedlings. (E) Mean percentage *G. pallida* infection levels of
127 *SISTP1* and *SISTP2* dsRNA treated tomato seedlings normalised to NEO double stranded
128 (ds)RNA (control) treated seedlings. Each data point represents the mean (\pm SEM) of ten
129 seedlings challenged with 500 infective stage juveniles each. *, $P<0.05$; **, $P<0.01$; ***,
130 $P<0.001$.

131

132 *exoRNAi* impact on nematode stylet thrusting

133 Root exudates collected from tomato seedlings which had been treated with either *SISTP1*
134 or *SISTP2* dsRNA were less capable of stimulating stylet thrusting in *M. incognita* relative to
135 exudates collected from control dsRNA treated seedlings ($13.92 \pm 5.10\%$, $P<0.001$; and 17.53
136 $\pm 8.12\%$, $P<0.001$, respectively. Fig 4A). No significant difference in stylet thrusting
137 frequency was observed for *G. pallida* juveniles when exposed to root exudates from *SISTP1*
138 or *SISTP2* dsRNA-treated seedlings, relative to control treated seedlings ($108.2 \pm 38.87\%$,
139 $P>0.05$; and $77.34 \pm 30.84\%$, $P>0.05$, respectively). In general, *G. pallida* juveniles were less
140 readily activated by tomato seedling exudates, accounting for much higher variation, even
141 after extended exudate exposure times of 24 hours (Fig 4B).

142



143

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

148

149 Discussion

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

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

165 Plant root exudation is an energetically expensive process for plants (Badri and
166 Vivanco, 2009), with an estimated 11% of photosynthetically-assimilated carbon released in
167 the form of various root exudate constituents (Jones *et al.*, 2009). It is well established that
168 plant root exudates mediate both positive and negative interactions with commensal and
169 pathogenic microbes (Badri *et al.*, 2009), insects (Walker *et al.*, 2003), and other plants (Bais
170 *et al.*, 2006). Plant parasitic nematodes also respond to plant root exudates (Teillet *et al.*,
171 2013). The present study aimed to probe the involvement of monosaccharide sugars of
172 tomato root exudate for involvement in the attraction and activation of parasitic behaviours
173 in the promiscuous root knot nematode *M. incognita*, and the host-selective potato cyst
174 nematode *G. pallida*.

175 STP1 and STP2 are known transporters of monosaccharide sugars (Gear *et al.*, 2000),
176 and our data demonstrate that both play a role in regulating the level of glucose and
177 fructose (but not xylose) exudation from tomato seedling roots. exoRNAi knockdown of
178 each transporter significantly reduced the amount of glucose and fructose secreted from
179 plant roots, which corresponded with a decrease in *M. incognita* infectivity, but not *G.*
180 *pallida* infectivity. These results suggest that glucose and fructose are important chemical
181 cues which infective stage *M. incognita* use to find host plants. The role of glucose and
182 fructose in *M. incognita* host-finding was further demonstrated through *in vitro* assays
183 which validated *M. incognita* attraction towards both glucose and fructose. Glucose and
184 fructose also triggered an increase in the rate of serotonin-triggered stylet thrusting, a key
185 parasitic behaviour associated with parasite activation and plant infectivity (Teillet *et al.*,
186 2013). Xylose had no impact on chemosensory behaviour, or stylet thrusting of *M.*
187 *incognita*, and silencing of both *SISTP1* and *SISTP2* had little impact on the amount of xylose
188 secreted from the seedlings. In contrast, the selective Solanaceae PPN, *G. pallida* did not
189 utilise monosaccharide components of tomato root exudate to locate a host, nor do the
190 monosaccharides play any role in the activation or enhancement of stylet thrusting.
191 Glucose and fructose appear to trigger host-finding and activation behaviours in
192 promiscuous PPNs, as opposed to host-specific PPNs, an observation which is consistent
193 with the ubiquitous nature of monosaccharide sugars in plant root exudates (Kamilova *et*
194 *al.*, 2006), which could render these exudate components as uninformative to selective
195 pathogens. Our data suggest that manipulation of sugar exudation from crop plant roots

196 could provide a novel means of promiscuous PPN control, but not selective PPNs which
197 likely utilise more diagnostic, and less ubiquitous root exudate cues. The demonstration
198 that *SISTP1* and *SISTP2* are specifically involved in the exudation of both monosaccharides
199 from tomato roots is an important finding which can underpin future efforts to study the
200 link between plant root transporters, and chemical constituents of root exudates. These
201 data provide an important proof of concept validation of the exoRNAi approach, which
202 promises a quick and easy method for gene functional studies in tomato, and potentially
203 other crop plant species.

204

205

206 **Materials and Methods**

207 *Nematode maintenance and collection*

208 *Meloidogyne incognita* was maintained on tomato cv. Moneymaker (23°C, 18h light, 6h
209 dark). Roots were harvested from infected plants and washed thoroughly in water. Egg
210 masses were removed by treatment in 2.5% sodium hypochlorite for two minutes. Eggs
211 were collected by washing the hypochlorite-treated roots through a series of nested sieves
212 (150 µM, 53 µM and 38 µM mesh sizes respectively) with water. Eggs were hatched in
213 DEPC-treated spring water (pH 7), and hatched infective stage juveniles were used
214 immediately for downstream applications. *Globodera pallida* (pathotype Pa2/3) were
215 collected from potato cv. Cara which had been grown under glasshouse conditions.
216 Infective juveniles were hatched from cysts in potato root exudate, in complete darkness at
217 16°C, and were used immediately for downstream applications.

218

219 *Nematode stylet thrusting assay*

220 100 *M. incognita* or *G. pallida* infective stage juveniles were suspended in 20 µl of spring
221 water (autoclaved and adjusted to pH 7) containing 2 mM serotonin and 50 mM of glucose,
222 fructose or xylose (Sigma-Aldrich). Worms were incubated in this solution for 15 minutes,
223 pipetted onto a glass slide with a coverslip, and stylet thrusts were counted in randomly
224 selected infective stage juveniles for 1 minute each. In order to keep counts consistent,
225 worms were left on the slide no longer than 20 minutes. The experiment was replicated five
226 times to give stylet thrust counts for 100 infective stage juveniles per treatment. Control
227 treatments were expressed as a percentage, including technical variation, and experimental
228 treatments were normalised to control percentages across individual experiments and days.
229 We found quite a bit of inter- and intra-experimental variability in the response of *M.*
230 *incognita* infective stage juveniles to serotonin which may be linked to the age of juvenile
231 nematodes, the density of nematodes per hatching plate, or the time of day. This variability
232 was not so prominent in *G. pallida* tests. Data were analysed by One-way ANOVA and
233 Tukey's Honestly Significant Difference test using Graphpad Prism 6. Probabilities of less
234 than 5% ($P < 0.05$) were deemed statistically significant. Nematode stylet thrusting was also
235 assessed in response to seedling exudates (without the addition of exogenous serotonin).
236 Exudates were collected as below, and *M. incognita* juveniles were exposed to the exudate

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

242

243 *Nematode Chemosensory Assay*

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

263

264 *Double-stranded RNA synthesis and tomato seedling treatment*

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

276

277

278 *RNA extraction, cDNA synthesis and qRT-PCR*

279 Five seedlings were snap frozen in liquid nitrogen per biological replicate, and total RNA
280 isolated using Trizol reagent. Total RNA was treated with the Turbo DNase free kit (Life
281 Technologies), and cDNA was synthesised using the High-capacity RNA-to-cDNA kit (Applied
282 Biosciences) according to manufacturer's instructions, and using the maximum input
283 concentration of RNA. Three biological replicates were performed for each treatment.
284 Primer sets to be used for qPCR were optimised for working concentration, annealing
285 temperature and analysed by dissociation curve for contamination or non-specific
286 amplification by primer-dimer as standard (Table 1). Each individual reaction comprised 5
287 μ l Faststart SYBR Green mastermix (Roche Applied Science), 1 μ l each of the forward and
288 reverse primers (10 mM), 1 μ l water, 2 μ l cDNA. PCR reactions were conducted in triplicate
289 for each individual cDNA using a Rotorgene Q thermal cycler according to the following
290 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
291 efficiency of each specific amplicon was calculated using the Rotorgene Q software, and
292 quantification of each target amplicon calculated by an augmented comparative Ct method
293 (Pfaffl, 2001), relative to the geometric mean of three endogenous reference genes
294 (Vandesompele *et al.*, 2002) (see Table 1). Ratio-changes in transcript abundance were
295 calculated relative to control dsRNA treated seedlings in each case, and data were analysed
296 by ANOVA and Tukey's Honestly Significant Difference test using Graphpad Prism 6.
297 Probabilities of less than 5% ($P < 0.05$) were deemed statistically significant.

298

299 *Exudate collection and sugar quantification*

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

308

309 *Nematode infection assays*

310 Agar slurry was prepared by autoclaving a 0.55% agar solution with Spring water which had
311 been autoclaved and adjusted to pH 7. The agar was agitated for six hours at room
312 temperature, until it had a smooth consistency. 500 *M. incognita* or *G. pallida* infective
313 stage juveniles were added to each well of a 6 well plate (SPL Lifesciences) with one
314 exoRNAi treated seedling embedded within 3 ml of agar slurry. Plates were sealed with
315 parafilm, covered above and below with a sheet of tin foil and incubated for 24 hours at
316 23°C. Seedlings were subsequently removed from the slurry, gently washed several times by
317 immersion in deionised water, and stained using acid fuchsin (Bybd *et al.*, 1983). The
318 number of invading PPN juveniles was counted for each seedling using a light microscope.

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

321

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328

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377 **Table 1. Primer sequences.**

Primer Designation	Sequence
<i>SISTP1F</i>	CTGCTGTGATCACTGGTGGGA
<i>SISTP1R</i>	ATCCCTGGAGTTCCATT
<i>SISTP1Ft7</i>	TAATACGACTCACTATAGGCTGCTGTGATCACTGGTGGGA
<i>SISTP1Rt7</i>	TAATACGACTCACTATAGGATTCCCCTGGAGTTCCATT
<i>qSISTP1F</i>	ATGTTGCTGGATTGCTTGGTC
<i>qSISTP1R</i>	TGTGCAGCTGATCGAATTTCCAG
<i>SISTP2F</i>	ACGTTCTCTCCACCGTTGTC
<i>SISTP2R</i>	CTACGAAGATTCCCCAACCA
<i>SISTP2Ft7</i>	TAATACGACTCACTATAGGACGTTCTCTCCACCGTTGTC
<i>SISTP2Rt7</i>	TAATACGACTCACTATAGGCTACGAAGATTCCCCAACCA
<i>qSISTP2F</i>	ATTATGGCTGCTACCGGAGGTC
<i>qSISTP2R</i>	TGTAACACCACAGAACTCCAAC
<i>qSITIP41F</i>	ATGGAGTTTTGAGTCTTCTGC
<i>qSITIP41R</i>	GCTGCGTTTCTGGCTTAGG
<i>qSIEFαF</i>	TACTGGTGGTTTTGAAGCTG
<i>qSIEFαR</i>	AACTTCCTCACGATTTTCATCATA
<i>qSISANDF</i>	TTGCTTGGAGGAACAGACG
<i>qSISANDR</i>	GCAAACAGAACCCCTGAATC

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