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Camargo RA, Barbosa GO, Possignolo IP, Peres LEP, Lam E, Lima JE, Figueira A, Marques-Souza H. 2016. RNA interference as a gene silencing tool to control *Tuta absoluta* in tomato (*Solanum lycopersicum*) PeerJ 4:e2673 <https://doi.org/10.7717/peerj.2673>

# RNA interference as a gene silencing tool to control *Tuta absoluta* in tomato (*Solanum lycopersicum*)

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RNA interference (RNAi), a gene-silencing mechanism that involves providing double-stranded RNA molecules that match a specific target gene sequence, is now widely used in functional genetic studies. The potential application of RNAi-mediated control of agricultural insect pests has rapidly become evident. The production of transgenic plants expressing dsRNA molecules that target essential insect genes could provide a means of specific gene silencing in larvae that feed on these plants, resulting in larval phenotypes that range from loss of appetite to death. In this report, we show that the tomato leafminer (*Tuta absoluta*), a major threat to commercial tomato production, can be targeted by RNAi. We selected two target genes [Vacuolar ATPase-A and Arginine kinase] based on the RNAi response reported for these genes in other pest species. In view of the lack of an artificial diet for *T. absoluta*, we used two approaches to deliver dsRNA into tomato leaflets. The first approach was based on the uptake of dsRNA by leaflets and the second was based on "in planta-induced transient gene silencing" (PITGS), a well-established method for silencing plant genes, used here for the first time to deliver in planta-transcribed dsRNA to target insect genes. *Tuta absoluta* larvae that fed on leaves containing dsRNA of the target genes showed an ~60% reduction in target gene transcript accumulation, an increase in larval mortality and less leaf damage. We then generated transgenic 'Micro-Tom' tomato plants that expressed hairpin sequences for both genes and observed a reduction in foliar damage by *T. absoluta* in these plants. Our results demonstrate the feasibility of RNAi as an alternative method for controlling this critical tomato pest.

1 **RNA interference as a gene silencing tool to control *Tuta absoluta* in tomato (*Solanum***  
2 ***lycopersicum*)**

3

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19

20 **Abstract**

21 RNA interference (RNAi), a gene-silencing mechanism that involves providing double-stranded  
22 RNA molecules that match a specific target gene sequence, is now widely used in functional genetic  
23 studies. The potential application of RNAi-mediated control of agricultural insect pests has rapidly  
24 become evident. The production of transgenic plants expressing dsRNA molecules that target  
25 essential insect genes could provide a means of specific gene silencing in larvae that feed on these  
26 plants, resulting in larval phenotypes that range from loss of appetite to death. In this report, we  
27 show that the tomato leafminer (*Tuta absoluta*), a major threat to commercial tomato production,  
28 can be targeted by RNAi. We selected two target genes [*Vacuolar ATPase-A* and *Arginine kinase*]  
29 based on the RNAi response reported for these genes in other pest species. In view of the lack of an  
30 artificial diet for *T. absoluta*, we used two approaches to deliver dsRNA into tomato leaflets. The  
31 first approach was based on the uptake of dsRNA by leaflets and the second was based on “*in*  
32 *planta*-induced transient gene silencing” (PITGS), a well-established method for silencing plant  
33 genes, used here for the first time to deliver *in planta*-transcribed dsRNA to target insect genes.  
34 *Tuta absoluta* larvae that fed on leaves containing dsRNA of the target genes showed an ~60%  
35 reduction in target gene transcript accumulation, an increase in larval mortality and less leaf  
36 damage. We then generated transgenic ‘Micro-Tom’ tomato plants that expressed hairpin sequences  
37 for both genes and observed a reduction in foliar damage by *T. absoluta* in these plants. Our results  
38 demonstrate the feasibility of RNAi as an alternative method for controlling this critical tomato  
39 pest.

40

41 **Keywords:** agro-infiltration, dsRNA delivery, dsRNA uptake, Micro-Tom, pest control, PITGS,  
42 RNAi.

43

## 44 Introduction

45 The mechanism of RNA interference (RNAi), in which small RNAs can rapidly cause post-  
46 transcriptional specific gene silencing, has become a powerful tool for analysing gene function in a  
47 variety of organisms (Hannon, 2002). The mediators of sequence-specific mRNA degradation are  
48 ~21 nucleotide-long short-interfering RNA molecules (siRNA) generated from Dicer cleavage of  
49 longer double-stranded RNA (dsRNA) (Zamore et al., 2000). Gene silencing by introducing dsRNA  
50 into organisms has proven to be an excellent strategy for reducing specific gene expression in  
51 several insect orders, including Diptera, Coleoptera, Hymenoptera, Orthoptera, Blattodea,  
52 Lepidoptera and Isoptera (Katoch et al., 2013). The potential application of RNAi-mediated control  
53 of agricultural insect pests has rapidly become evident (Gordon & Waterhouse, 2007), but a major  
54 challenge has been the development of an easy, reliable method for dsRNA production and  
55 delivery. The original RNAi studies used microinjection to deliver dsRNA into insects; ‘soaking’  
56 has also been used as a delivery method to target cell cultures or individual larvae (Price &  
57 Gatehouse, 2008). The demonstration that dsRNA uptake through ingestion was sufficient to reduce  
58 target gene expression allowed the possibility of applying RNAi on a larger scale (Koch & Kogel,  
59 2014). Transgenic plants engineered to express insect dsRNAs emerged as a potential technology  
60 after two independent groups proved the concept of applying RNAi to control agricultural insect  
61 pests (Baum et al., 2007; Mao et al., 2007). This approach has been developed to control  
62 lepidopteran, coleopteran and hemipteran agricultural pests (Katoch et al., 2013), including  
63 *Helicoverpa armigera* in cotton (Mao et al., 2011) and tobacco (Zhu et al., 2012; Xiong et al.,  
64 2013), *Diabrotica virgifera virgifera* in maize (Baum et al., 2007), *Nilaparvata lugens* in rice (Zha  
65 et al., 2011), *Myzus persicae* in *Nicotiana benthamiana* and *Arabidopsis thaliana* (Pitino et al.,  
66 2011) and *Sitobion avenae* in wheat (Xu et al., 2013). However, the availability of methods that  
67 allow the screening and evaluation of candidate RNAi targets is a critical requisite for developing  
68 specific and efficient RNAi-based pest control.

69           The tomato leafminer, *Tuta absoluta* (Meyrick), is a small neotropical oligophagous  
70 lepidopteran that attacks many solanaceous species, particularly *Solanum lycopersicum* (tomato) and  
71 other species of economic importance (Cifuentes et al., 2011; Desneux et al., 2010). The tomato  
72 leafminer is a multivoltine species whose young larvae can damage tomato plants during all  
73 developmental stages by forming large galleries in the leaves and burrowing into stalks, shoot apex,  
74 and green and ripe fruits. *Tuta absoluta*, which can cause yield losses of up to 100% in various  
75 regions and under diverse cultivation systems, has become the major tomato pest in South America  
76 (Desneux et al., 2010; Urbaneja et al., 2013). This insect invaded Europe in 2006 and spread to  
77 northern Africa in 2007 (Urbaneja et al., 2013), where it caused extensive economic losses to  
78 growers; multiple efforts have since been made to control this pest (Desneux et al., 2011). To  
79 aggravate this situation, resistance to insecticides has been reported for *T. absoluta*, making the  
80 development of alternative means of controlling this pest even more urgent (Urbaneja et al., 2013;  
81 Campos et al., 2014).

82           To provide an alternative method of control based on RNAi we first investigated two  
83 approaches for delivering dsRNA to *T. absoluta* larvae, a critical requisite for efficiently screening  
84 effective target genes before developing transgenic plants. Traditionally, target genes are screened  
85 and evaluated by adding dsRNA to artificial diets offered to insect larvae (Terenius et al., 2011;  
86 Zhang, Li & Miao, 2012), but in the case of *T. absoluta*, there is no readily available artificial diet  
87 (Urbaneja et al., 2013). We therefore investigated two delivery approaches in which *T. absoluta*  
88 larvae feed on tomato leaflets containing dsRNA. In the first approach, tomato leaflets were allowed  
89 to uptake dsRNA from an aqueous solution, in a manner similar to that described for the sap-  
90 sucking *Bemisia tabaci* (Luan et al., 2013). The second approach was based on the transient  
91 transcription of dsRNA by the host plant after the infiltration of *Agrobacterium* cells carrying  
92 binary plasmids that expressed hairpin versions of the target gene sequences ('agro-infiltration').  
93 Agro-infiltration was originally developed to investigate plant-virus interactions (Bendahmane et  
94 al., 2000) but was later adapted to functional analyses of plant genes, e.g., for assessing gene over-

95 expression or silencing and screening for insect resistance genes (Grimsley et al., 1987; Leckie &  
96 Stewart, 2010). A method designated as “*in planta*-induced transient gene silencing” (PITGS)  
97 successfully delivered hairpin-silencing constructs in wheat to determine the pathogenicity-related  
98 gene functions of *Puccinia triticina* (leaf rust) (Panwar, McCallum & Bakkeren, 2012). Here, we  
99 investigated whether the same rationale could be used to silence insect genes.

100 The target genes selected for this study were the *Vacuolar ATPase catalytic subunit A* gene  
101 (*V-ATPase*) and *Arginine kinase* (*AK*). The H<sup>+</sup>-ATPase vacuolar pump, one of the most essential  
102 enzymes present in almost all eukaryotic cells, is responsible for generating energy gradients in  
103 many membranes and organelles (Nelson et al., 2000). Arginine kinase belongs to a transferase  
104 protein family that catalyzes the transfer of a high-energy phosphate group from ATP to L-arginine  
105 to yield phosphoarginine that is used for energy storage (Bragg et al., 2012). These genes have been  
106 previously used as RNAi targets in exploratory studies in many different insects and crop plants  
107 (Baum et al., 2007, Thakur et al., 2014); however, this is the first report of silencing the *V-ATPase*  
108 *subunit A* and *Arginine kinase* genes in a lepidopteran species.

109 Our results demonstrate the viability of using RNAi to control *T. absoluta* with delivery  
110 approaches based either on dsRNA uptake by detached tomato leaflets or through transient  
111 expression after leaf agro-infiltration, followed by infestation with pest larvae. Silencing of the  
112 insect *V-ATPase* and *AK* genes after treatment with dsRNA resulted in increased larval mortality.  
113 Further, the development of transgenic tomato lines expressing the hairpin version of these genes  
114 demonstrated that this approach can adversely affect insect development and/or viability by  
115 reducing the expression of insect target genes.

116

## 117 **Methods**

### 118 **Biological material**

119 The colony of *T. absoluta* was originally established from two populations provided by the  
120 Insect Biology Laboratory of the Department of Entomology at ESALQ/USP (Piracicaba, SP,  
121 Brazil) and Bayer Crop Science (Uberlândia, MG, Brazil). This colony was reared for many  
122 generations in tomato plants or leaves under laboratory conditions ( $25 \pm 2$  °C;  $60 \pm 10\%$  relative  
123 humidity; 14 h photoperiod). ‘Santa-Clara’ or ‘Cherry’ tomato cultivars were used for *T. absoluta*  
124 maintenance and in dsRNA uptake experiments. The ‘Micro-Tom’ cultivar was used for genetic  
125 transformation.

126

### 127 **Target gene selection**

128 Genes encoding a vacuolar ATPase catalytic subunit A (*V-ATPase*) and arginine kinase  
129 (*AK*) were chosen based on previous successful reports of RNAi used for insect control (Baum et  
130 al., 2007; Zhao et al., 2008). Since no sequences were available for *T. absoluta* genes, degenerated  
131 primers (Table S1) were developed based on conserved amino acid sequence regions from aligned  
132 homologs of *Aedes aegypti* (GenBank accession *V-ATPase*: XP\_001659520.1), *Bombyx mori* (*V-*  
133 *ATPase*: NP\_001091829.1; *AK*: NP\_001037402.1), *Drosophila melanogaster* (*V-ATPase*:  
134 NP\_652004.2; *AK*: AAA68172.1), *Helicoverpa armigera* (*AK*: ADD22718.1), *Heliothis virescens*  
135 (*AK*: ADE27964.1), *Homalodisca vitripennis* (*AK*: AAT01074.1), *Manduca sexta* (*V-ATPase*:  
136 P31400.1), *Spodoptera litura* (*AK*: ADW94627.1) and *Tribolium castaneum* (*V-ATPase*  
137 XP\_976188.1; *AK*: EFA11419.1). Based on these orthologous genes, the complete *V-ATPase A*  
138 coding sequence was estimated to be around 1850 bp, while the *AK* coding sequence ranged from  
139 1062 to 1476 bp.

140

### 141 ***Tuta absoluta* RNA extraction and cDNA synthesis**

142 Total RNA was extracted from 100 mg of *T. absoluta* larvae at the four instar stages using  
143 TRIzol (Invitrogen; Carlsbad, CA, USA). RNA was quantified spectrophotometrically and analyzed  
144 by electrophoresis in 1% denaturing agarose gels in MOPS buffer. Around 1 µg of total RNA was



145 treated with DNase I and 20 U of Ribolock (Fermentas; Burlington, Canada) at 37 °C for 30 min,  
146 with the reaction stopped by adding EDTA (50 mM) and heating to 65 °C for 10 min. One  
147 microgram of DNase-treated RNA samples was reversed transcribed in a total reaction volume of  
148 20 µL containing 500 µM of each dNTP, 2.5 µM oligo dT, 5 mM DTT and 200 U SuperScript III  
149 (Invitrogen) in appropriate buffer at 50 °C for 60 min, followed by enzyme inactivation at 70 °C for  
150 15 min.

151

### 152 **Target gene amplification and cloning**

153 Target genes were amplified from cDNA using a nested PCR-based method with  
154 degenerate primer pairs (Table S1) in a 20 µL reaction volume containing ~100 ng of cDNA, 3 mM  
155 MgCl<sub>2</sub>, 100 µM of each dNTP, 1 µM of each primer and 2 U of High Fidelity *Taq* DNA polymerase  
156 (Invitrogen) in the appropriate buffer. Amplifications were done in a Veriti thermocycler (Applied  
157 Biosystems, Foster City, CA, USA) programmed to cycle at 94 °C for 5 min, followed by 35 cycles  
158 of 94 °C for 40 s, 45 °C for 60 s, 72 °C for 60 s and a final cycle at 72 °C for 10 min. The second  
159 reaction was run under the same conditions as the first reaction using 1 µL from the latter.  
160 Amplification products were analyzed by gel electrophoresis and target fragments were excised,  
161 purified using a PureLink Quick gel extraction kit (Invitrogen) and cloned into pGEM-T Easy  
162 vector (Promega; Madison, WI, USA) using standard procedures. Identity was confirmed by  
163 sequencing three clones from each target gene in an ABI PRISM 3130 (Applied Biosystems).

164

### 165 **Cloning the target gene fragments as hairpins in the RNAi silencing vector**

166 To clone the target gene fragments in the binary silencing vector pK7GWIWG2(I) (Karimi,  
167 Inze, Depicker, 2002) primers were synthesized to amplify fragments flanked by the recombination  
168 sequences *attL1* and *attL2* (Table S2). The amplification reactions contained 10 ng of pGEM-T-  
169 cloned DNA, 200 µM of each dNTP, 0.4 µM of each primer and 2 U of Phusion DNA polymerase  
170 (New England Biolabs; Ipswich, MA, USA) in a final reaction volume of 50 µL. Temperature

171 cycling for amplification was programmed to start at 98 °C for 2 min, followed by 35 cycles at 98  
172 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s, with a final cycle at 72 °C for 5 min. The amplified  
173 products were purified from agarose gels using QIAEX II gel extraction kits (Qiagen; Hilden,  
174 Germany), quantified, recombined into pK7GWIWG2(I) using LR clonase (Invitrogen) according  
175 to the manufacturer's instructions and transformed into TOP10 *Escherichia coli* cells. The presence  
176 of the insert was confirmed by amplification and the direction of insertion was verified by digestion  
177 and sequencing. Successful constructs were then transformed by heat shock into *Agrobacterium*  
178 *tumefaciens* GV3101/MP90 cells and confirmed by PCR.

179

### 180 **dsRNA synthesis**

181 pGEM-T Easy clones containing *T. absoluta V-ATPase* and *AK* gene fragments were used  
182 as a template for transcription *in vitro* to produce dsRNA using T7 RNA polymerase (MegaScript  
183 T7; Life Technologies, Carlsbad, CA, USA). Target sequences cloned into pGEM-T Easy were  
184 amplified with a T7 primer and a SP6 primer fused to a T7 sequence  
185 (TAATACGACTCACTATAGGGATTTAGGTGACACTATAG) to operate as a T7 promoter for  
186 bidirectional *in vitro* transcription. The amplification reactions contained 10 ng of plasmid DNA,  
187 1.5 mM MgCl<sub>2</sub>, 200 μM of each dNTP, 0.5 μM of each primer and 1.0 U of *Taq* polymerase in a  
188 final reaction volume of 20 μL. Temperature cycling for amplification started at 95 °C for 2 min,  
189 followed by 35 cycles of 15 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C, with a final extension at 72 °C  
190 for 5 min. Amplified fragments were run on and purified from 1% agarose gels as described above.  
191 Purified products were quantified by fluorimetry and used for *in vitro* transcription reactions  
192 containing 100 ng of target DNA, 7.5 mM of each ribonucleotide and 200 U of MegaScript T7 in  
193 appropriate buffer in a final volume of 20 μL. The reactions were run at 37 °C for 16 h, followed by  
194 the addition of 2 U of DNase for 15 min at 37 °C. Double-stranded RNA (dsRNA) was purified by  
195 precipitation with 7.5 M LiCl (30 μL) at -20 °C for 1 h followed by centrifugation (12,000 g, 15  
196 min, 4 °C). The RNA pellet was washed with 70% ethanol and resuspended in DEPC-treated water.

197 The green fluorescent protein (GFP) gene was used as a negative control. The vector  
198 pCAMBIA1302 was used as a template to amplify a negative control *GFP* gene fragment (276 bp)  
199 with the specific primers GFP-F (5'-TAATACGACTCACTATAGGGCAGTGGAGAGGGTGAA)  
200 and GFP-R (5'-TAATACGACTCACTATAGGGTTGACGAGGGTGTCTC), both containing  
201 additional T7 sequences (underlined). Similar transcription *in vitro* was done with this template.

202

### 203 **Labeling dsRNA to follow uptake by tomato leaflets and ingestion by *T. absoluta***

204 *In vitro* transcription of dsRNA was done as described above, except that 2  $\mu\text{L}$  of Cy3-  
205 labelled riboCTP was added (Zhang et al., 2013). Fluorescently-labelled dsRNA was purified using  
206 a MEGAclear kit (Ambion; Carlsbad, CA, USA) and provided in solution to tomato leaflets at 500  
207  $\mu\text{g mL}^{-1}$ . Larvae ( $n=15$ ) were placed on the treated leaflets and collected 6 h or 24 h later for  
208 observation by confocal fluorescent microscopy with an AxioVision Zeiss LSM780-NLO  
209 microscope (Carl Zeiss AG).

210

### 211 **dsRNA delivery to *T. absoluta* larvae via feeding**

212 dsRNA was delivered into tomato leaves by two methods and the effects were evaluated  
213 based on gene expression analysis in fed larvae, larval mortality or tomato tissue damage. In the  
214 first method, detached leaflets from 'Santa-Clara' tomatoes had their petioles immersed in 200  $\mu\text{L}$   
215 of water containing either 5  $\mu\text{g}$  of dsRNA from each target gene or a *GFP* control, in triplicate.  
216 Uptake of the dsRNA solution by the tomato leaflets took 3-4 h. Immediately after uptake, first  
217 instar larvae ( $n=50-100$ ) were gently placed onto leaflets for feeding and individuals were sampled  
218 24 h, 48 h and 72 h after initiation of treatment. Negative controls with dsRNA from the *GFP* gene  
219 sequence were run in parallel. The effects of RNAi on the larvae were evaluated by quantitative  
220 amplification of reversed transcripts (RT-qPCR) of each target gene compared to the control.

221 The second delivery method used 'Santa Clara' tomato leaflets infiltrated with  
222 *Agrobacterium* cells containing hairpin target gene fragments cloned into the pK7GWIWG2(I)

223 vector or a similar hairpin expression construct for the *GFP* gene as a transient assay. Initially, to  
224 validate the potential of gene silencing in a transient assay, leaves were either infiltrated only with  
225 *Agrobacterium* GV3101/pMP90 cells containing an expression construct for enhanced GFP (*eGFP*)  
226 to visualize the GFP transient expression or in combination with another *Agrobacterium* line  
227 containing a GFP silencing construct (*GFPi*). The *Agrobacterium* suspensions were infiltrated into  
228 the abaxial side of the tomato leaves using a microsyringe and the treated area was marked with a  
229 permanent marker. Leaf tissues that had been agro-infiltrated with the *eGFP* or *eGFP* plus *GFPi*  
230 constructs were examined two days after treatment using a confocal fluorescent microscope  
231 (AxioVision Zeiss LSM780-NLO, Carl Zeiss AG, Germany) to monitor the degree of gene  
232 silencing based on *eGFP* expression.

233 In subsequent feeding assays, tomato leaflets were infiltrated with *Agrobacterium* cells  
234 carrying the *V-ATPase* or *AK* hairpin constructs or with *Agrobacterium* cells carrying the *GFPi*  
235 construct as a negative control, in triplicate. The *Agrobacterium* cells were grown on LB medium  
236 containing gentamycin ( $25 \mu\text{g mL}^{-1}$ ) and spectinomycin ( $100 \mu\text{g mL}^{-1}$ ) for 12 h, centrifuged at  
237  $3,000 g$  for 5 min and resuspended in water to an  $\text{OD}_{600\text{nm}} = 0.5$ . After 24 h, first instar *T. absoluta*  
238 larvae were placed on the treated leaf areas and 24 h, 48 h and 72 h later treated larvae and their  
239 respective controls were sampled for analysis of *V-ATPase* and *AK* expression by RT-qPCR.

240 Feeding assays were also done to estimate larval mortality. Detached ‘Santa Clara’ leaflets  
241 had their petioles immersed in an aqueous solution containing  $1 \mu\text{g}$  of dsRNA of each target gene  
242 (*V-ATPase* or *AK*, plus *GFP* control), a procedure that was repeated daily for 10 days, with a total  
243 of  $10 \mu\text{g}$  being provided to each leaflet. A total of 10 first instar larvae were placed to feed on these  
244 leaflets (in triplicate) and larvae mortality was estimated after 5, 7, 10 and 24 days of treatment.  
245 Under the conditions used here, the feeding cycle of *T. absoluta* lasted 10-12 days from larval  
246 emergence to pupae, and a total of *ca.* 20-24 days for adults to emerge. An additional assay was  
247 done using ‘Santa Clara’ tomato leaflets with the petiole immersed in increasing amounts of dsRNA  
248 (total: 500, 1000 or 5000 ng) of the target genes (*V-ATPase* or *AK*) or *GFP* negative control (in

249 duplicate). Five larvae were placed on each leaflet and leaflets were photographed for 11 days to  
250 visually assess the extent of damage.

251

### 252 **Quantitative amplification of reversed transcripts (RT-qPCR)**

253 For gene expression analysis of treated *T. absoluta* larvae, cDNA was synthesized using a  
254 High Capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's  
255 instructions, using 1 µg of DNase-treated total RNA, random primers and 50 U of MultiScribe  
256 reverse transcriptase in 20 µL. RT-qPCR reactions contained ~40 ng of larval cDNA, 5 µL of Fast  
257 SYBR Green Master (Invitrogen) and 0.2 µM of each gene-specific primer (Table S4) in a total  
258 volume of 10 µL. Amplifications were done starting at 50 °C for 10 min and 95 °C for 2 min,  
259 followed by 40 three-step cycles of 95 °C for 15 s, 60-61 °C for 25 s and 72 °C for 30 s in a Qiagen  
260 RotorGene-6000 (Qiagen). After amplification, melting curves were determined between 72 °C and  
261 95 °C. Reactions were done with biological replicates, technical triplicates and non-template  
262 controls. Primer efficiency was determined using a pool of cDNA in serial dilutions (10, 10<sup>-1</sup>, 10<sup>-2</sup>  
263 and 10<sup>-3</sup>). C<sub>Q</sub> values were used to determine differences in expression based on Livak &  
264 Schmittgen (2001). Reference genes were *RpL 5* (large subunit 5 ribosomal protein), *RpL23A* (large  
265 subunit 23A ribosomal protein) and *rRNA* (Table S4). Negative controls were larvae fed on *GFP*  
266 dsRNA.

267

### 268 **'Micro-Tom' genetic transformation with the RNAi silencing constructs**

269 The *ATPase* and *AK* silencing constructs were used to transform 'Micro-Tom' based on  
270 published protocols (Pino et al., 2010). Cotyledon explants were obtained from 8-day old seedlings  
271 cultivated on MS medium supplemented with sucrose (30 g L<sup>-1</sup>), B5 vitamins and 0.4 µM  
272 naphthalene acetic acid (NAA) (Pino et al., 2007). A single colony of *Agrobacterium*  
273 GV3101/MP90 (pK7GWIWG2(I)::*ATPase-esaPTA* or pK7GWIWG2(I)::*AK-KA*) grown for two  
274 days on 3 mL of LB medium with spectinomycin (100 mg L<sup>-1</sup>), rifampicin (50 mg L<sup>-1</sup>) and

275 gentamycin (25 mg L<sup>-1</sup>) was inoculated into 50 mL of LB medium with the same antibiotics and  
276 incubated overnight at 120 rpm and 28 °C. The suspensions were then centrifuged (1,000 g, 15 min,  
277 20 °C) and the pellet was resuspended in liquid MS medium containing sucrose (30 g L<sup>-1</sup>), with the  
278 OD<sub>600nm</sub> adjusted to 0.2-0.3. Acetosyringone (100 µM) was added to the suspensions 10 min before  
279 co-cultivation, which was done on the same semi-solid MS medium for two days in the dark at 25  
280 °C. Explants were then transferred to fresh MS medium supplemented with B5 vitamins, sucrose  
281 (30 g L<sup>-1</sup>), 5 µM benzylamino purine (BAP), kanamycin (100 mg L<sup>-1</sup>) and timetin (300 mg L<sup>-1</sup>) and  
282 maintained on a 16 h photoperiod at 25 °C for three weeks. Subsequently, adventitious shoots >5  
283 mm long were transferred to identical medium until roots developed and the plantlets were  
284 hardened (~two weeks), after which they were moved to a greenhouse.

285

#### 286 **Genetic analysis of transgenic plants**

287 Total DNA and RNA were extracted from putative transgenic plants using Trizol.  
288 Confirmation of transgenesis was done by PCR using a 35S promoter sense primer  
289 (GCACAATCCCACTATCCTTC) together with a target gene (*ATPase* and *AK*)-specific reverse  
290 primer (Table S4). Reactions (final volume of 25 µL) contained 100 ng of genomic DNA, 1.5 mM  
291 MgCl<sub>2</sub>, 0.2 µM of each dNTP, 0.1 µM of each primer and 1.5 U of *Taq* DNA polymerase in  
292 appropriate buffer (Fermentas). Amplification started at 95 °C for 2 min, followed by 35 cycles of  
293 15 s at 95 °C, 25 s at 60 °C and 40 s at 72 °C, with a final extension at 72 °C for 5 min. The products  
294 were analyzed in 1% agarose gels. To confirm transcript expression in transgenic plants, RT-PCR  
295 reactions were done using gene specific primers for *ATPase* or *AK* (Table S4) together with primers  
296 for a tomato ubiquitin gene as an endogenous reference gene. Reverse transcription was done with 1  
297 µg of DNase I-treated total RNA, 0.75 µM of gene-specific primers (*V-ATPase* or *AK*), 0.75 µM of  
298 ubiquitin primers, 0.5 mM of each dNTP and 200 U of Revertaid (Fermentas) in a final volume of  
299 20 µL. For RT-PCR, 1 µL of cDNA, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.1 µM of each primer  
300 and 1.5 U of *Taq* DNA polymerase (Fermentas) were mixed in a final volume of 25 µL and

301 amplified at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for  
302 20 s. Amplification products were examined by gel electrophoresis.

303 To specifically detect siRNA expressed in transgenic plants, a stem-loop pulsed RT-PCR  
304 was done using the protocol described by Varkonyi-Gasic et al. (2007). Initially, the introduced  
305 gene fragment sequences were analyzed with a software for virtual prediction of siRNA (Rice,  
306 Longden & Bleasby, 2000). Stem-loop primers specific for two virtually predicted siRNA for each  
307 gene, gene-specific primers and a universal primer (Varkonyi-Gasic et al., 2007) were obtained  
308 (Table S5). As an endogenous control, primers for microRNA156 (*MIR156*) were also developed  
309 based on sequences available at miRBASe (Kozomara & Griffiths-Jones, 2011). Reverse  
310 transcription reactions used 0.05 µM of stem-loop primers and 1 µg of DNase-treated total RNA  
311 that were heated to 70 °C for 10 min and chilled to 4 °C for 10 min. Subsequently, 3 mM MgCl<sub>2</sub>,  
312 0.25 mM of each dNTP and 200 U of Revertaid (Fermentas) in appropriate buffer were added to a  
313 final volume of 20 µL. The reaction was run at 16 °C for 30 min, followed by 60 cycles at 30 °C for  
314 30 s, at 42 °C for 30 s and 50 °C for 1 s, with a final step at 85 °C for 5 min to inactivate the  
315 enzyme. Amplification was then done in reactions consisting of 1 µL of cDNA, 1.5 mM MgCl<sub>2</sub>, 0.2  
316 mM of each dNTP, 0.2 µM of each primer and 1.5 U of *Taq* DNA polymerase in appropriate buffer  
317 (Fermentas). Temperature cycling started at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 s  
318 and 60 °C for 1 min. The products were examined after separation on 3% agarose gels.

319

### 320 **Feeding assay using transgenic plants**

321 Leaves from ‘Micro-Tom’ plants (T<sub>0</sub>) transgenic lines for *V-ATPase* and *AK* and their  
322 respective controls had their petioles immersed in water. Ten recently hatched larvae were then  
323 allowed to feed on the leaves, which were photographed for seven days to monitor leaf tissue  
324 damage. Pupae were collected at the end of the larval cycle for counting and weighing.

325

326

## 327 **Results**

### 328 **Target gene isolation from *T. absoluta* and dsRNA transcription *in vitro***

329           Since little genomic information is available for *T. absoluta*, we conducted target gene  
330 fragment cloning using degenerated primers for both target genes (estimated coding sequence of  
331 ~1,850 bp for *V-ATPase* and ~1,065 bp for *AK*; unpublished data) by using nested PCR (Table S1).  
332 The final amplification products were run on agarose gels and fragments for both genes were  
333 purified and cloned. Three positive clones were sequenced in both directions for each target gene.  
334 The consensus sequence assembled from the three clones contained 285 bp for *V-ATPase*  
335 [GenBank: KM591219] and 262 bp for *AK* [GenBank: KM591220]. The sequences were  
336 conceptually translated and aligned to homologs from other species (Fig. S1); both sequences  
337 displayed conserved domains (GenBank:cd01134 for *V-ATPase* and GenBank:cd07932 for *AK*).

338           In all RNAi assays, the same regions of the target and green fluorescent protein (GFP)  
339 control genes were used, either by *in vitro* transcription of the dsRNA or by transient/stable  
340 transgenesis.

341

### 342 **dsRNA delivery methods for *T. absoluta* in tomato**

343           To overcome the lack of a suitable artificial diet for *T. absoluta*, we used alternative  
344 methods to deliver dsRNA to the insect larvae in order to facilitate the rapid screening of candidate  
345 target genes. The first approach involved supplying dsRNA transcribed *in vitro* to tomato leaflets by  
346 petiole uptake. The second approach was based on “*in planta*-induced transient gene silencing”  
347 (PITGS), an established method for silencing plant genes (Panwar, McCallum & Bakkeren, 2012)  
348 used here for the first time to deliver *in planta*-transcribed dsRNA to insect-specific genes. In this  
349 approach (‘agro-infiltration’), an *Agrobacterium* strain containing a hairpin version of the target  
350 gene was infiltrated into tomato leaflets. Fluorescent molecules were used (1) to track Cy3-labeled  
351 RNA molecules from the petioles to the larval gut (first approach) and (2) to monitor dsRNA gene



352 silencing of transiently expressed GFP in *Agrobacterium*-infiltrated tomato leaves (second  
353 approach).

354

### 355 **Leaf uptake of dsRNA and larval ingestion**

356 Detached tomato leaves absorbed a solution of dsRNA transcribed *in vitro* by the petioles  
357 and first instar larvae subsequently fed on these treated leaves. To determine whether the dsRNA  
358 could be successfully absorbed and transported to the leaf laminae and then be ingested by the  
359 insect to reach its digestive tract, Cy3-labeled (red fluorescence) dsRNA fragments of *V-ATPase*  
360 transcribed *in vitro* were provided in solution to detached tomato leaflets. The treated leaves and the  
361 feeding larvae were imaged by confocal microscopy 6 h or 24 h after treatment (Fig. 1 and Fig. S2).  
362 Labelled dsRNA species were already strongly detected in the leaflet petiole and blade (mid-rib and  
363 lateral veins) of the leaflets 6 h after treatment (Fig. 1Ab). After 24 h, Cy3-labeled RNA molecules  
364 were detected throughout the leaf blade (Fig. 1Ad and Fig. S2). With time, Cy3-labeled RNA  
365 molecules accumulated at the leaf margin until saturation was reached in certain areas (Fig. 1Ad).

366 We then imaged larvae fed on treated or untreated leaflets using the 488 channel (green  
367 fluorescence) to detect chlorophyll auto-fluorescence, indicative of plant tissue ingestion by the  
368 larvae, and the 555 channel (red fluorescence) to detect Cy3 fluorescence (Fig. 1B). In both  
369 treatments, green fluorescence was detected throughout the larval digestive tract (Fig. 1Bb),  
370 indicating that the larvae fed normally under both circumstances. However, larvae fed on dsRNA-  
371 treated leaflets showed a strong Cy3 signal in the digestive tract, indicating the presence of leaflet-  
372 absorbed Cy3-labeled RNA molecules in the gastric caeca of the midgut (Fig. 1Bc).

373

### 374 **PITGS dsRNA delivery: *in planta* dsRNA transcription and gene silencing assay**

375 The delivery approach based on the transient expression by *Agrobacterium* infiltration was  
376 chosen in an attempt to mimic stable transgenesis in which plant cells expressing hairpin versions of  
377 the target gene transcribe the dsRNA. We infiltrated 'Santa Clara' tomato leaves with two

378 *Agrobacterium* strains: one containing an expression cassette for enhanced GFP expression (*eGFP*  
379 strain) and another in which a 400 bp fragment of *eGFP* was cloned into a binary expression vector  
380 as inverted repeats in order to transcribe a hairpin version (dsRNA) of the *eGFP* gene (*GFPi* strain).  
381 Confocal fluorescence microscopy showed that agro-infiltrated leaves with the *eGFP* line displayed  
382 GFP (green) fluorescence as sparse cells on the leaf blade (Fig. S3a). When both *Agrobacterium*  
383 strains (*eGFP* and *GFPi*) were co-infiltrated, there was a drastic reduction in the number and  
384 intensity of cells with GFP fluorescence (Fig. S3b); this fluorescence was similar to that of leaf  
385 regions without agro-infiltration (Fig. S3c).

386 Together, these results indicated that both approaches were suitable for delivering dsRNA  
387 into tomato leaves. We next used both delivery methods to evaluate the effectiveness of RNAi in  
388 silencing specific target genes in *T. absoluta* larvae.

389

#### 390 **Effect of RNAi on target gene expression**

391 For both RNAi delivery methods, larvae were allowed to feed exclusively on RNAi-treated  
392 (dsRNA uptake or agro-infiltration) leaflets and collected 24 h, 48 h and 72 h later. The relative  
393 expression of *V-ATPase* and *AK* was quantified by RT-qPCR. For the PITGS dsRNA delivery  
394 method, the target gene fragments were cloned into a binary vector as a hairpin-expressing cassette  
395 and agro-infiltrated into ‘Santa Clara’ tomato leaves. The cloned fragments were amplified with  
396 primers flanked by *attL1* and *attL2* sequences (Table S2) to enable direct recombination with the  
397 binary vector pK7GWIWG2(I) (Karimi, Inze & Depicker, 2002).

398 Larvae fed on leaflets treated by the dsRNA uptake delivery method showed a significant  
399 decrease in transcript accumulation for both genes 48 h and 72 h after treatment (~40% reduction at  
400 72 h after treatment) (Fig. 2A). Larvae fed on agro-infiltration leaflets showed a decrease in  
401 transcript accumulation at all time points, with the highest decrease occurring 72 h after treatment,  
402 (~35% reduction for *V-ATPase* and 40% reduction for *AK*) (Fig. 2B).

403           Considering that both dsRNA delivery approaches resulted in similar gene silencing effects,  
404 subsequent experiments were done using only the leaf dsRNA uptake delivery method.

405

#### 406 **Effect of RNAi on larval mortality**

407           To determine the effect of RNAi on larval mortality, *T. absoluta* larvae were allowed to  
408 feed on single leaflets ( $n=3$ ) that absorbed 10  $\mu\text{g}$  of dsRNA from *V-ATPase*, *AK* or *GFP*. Larvae  
409 were sampled after five, seven and ten days of treatment and an additional pupal sample was  
410 collected after 24 days. Larval mortality was significantly higher in larvae fed on leaflets that  
411 absorbed dsRNA of either target gene when compared to the *GFP* control at all time points, with an  
412 additional increase over time (Fig. 3). By day 24, mortality had reached an average of 50% for *V-*  
413 *ATPase* and 43% for *AK* compared to 17% for the *GFP* control (Fig. 3). Independent experiments  
414 using different total amounts of dsRNA in the leaflets yielded similar results (not shown).  
415 Evaluation of larvae after 11 days of treatment (Fig. S4a-c) and at the pupal stage (Fig. S4d-f)  
416 revealed that larvae fed on RNAi-treated leaflets displayed developmental delay, reduced body size,  
417 external morphologies of the 3<sup>rd</sup> instar stage (when 4<sup>th</sup> instar was expected) (Fig. S4a-c), failure to  
418 pupate (Fig. S4d-f) and failure to emerge as adults (data not shown).

419

#### 420 **Effect of RNAi on tomato leaf damage**

421           We next assessed whether the gene silencing and larval mortality observed for both RNAi  
422 target genes resulted in less herbivory by *T. absoluta* on tomato leaves. After 11 days of *T. absoluta*  
423 herbivory, leaflet blades treated with increasing amounts of dsRNA (total: 500, 1000 or 5000 ng) of  
424 the target genes (*V-ATPase* or *AK*) (Fig. 4c-f) were visibly less damaged by larval herbivory when  
425 compared to leaflets treated with GFP dsRNA (Fig. 4a,b), and the observed protective effect  
426 appeared to be dose-dependent (Fig. 4c-f). A protective effect was seen even at lower doses of  
427 dsRNA treatment, particularly for *V-ATPase* (Fig. 4d).

428

#### 429 **RNAi transgenic tomato plants**

430 To examine the effects of constitutive RNAi *in planta*, pK7GWIWG2(I) plasmids  
431 containing *V-ATPase* or *AK* employed in *Agrobacterium* transient hairpin expression were used to  
432 stably transform ‘Micro-Tom’ tomato plants. ‘Micro-Tom’ plants were successfully transformed in  
433 three independent experiments (Table S3). The efficiency of transformation ranged from 3.5% to  
434 18.9% and varied among gene constructs. To characterize the events, 13 plants derived from 11  
435 events of the *V-ATPase* construct transformation were analyzed by PCR amplification and eight  
436 events were positive for the transformation (not shown). From *AK* constructs, nine plants from six  
437 events were characterized and all were positive (not shown). The confirmed transgenic plants (eight  
438 *V-ATPase* and nine *AK*) were analyzed for the presence of the full transcript by RT-PCR; all *V-*  
439 *ATPase* plants plus six *AK* plants (#2.1, #2.2, #2.3, #3, #5 and #6) showed the presence of the  
440 respective transcript (Fig. S5A). Similarly, the plants were analyzed for the presence of a potential  
441 siRNA derived from the gene fragments inserted by stem-loop pulsed RT-PCR (Varkonyi-Gasic et  
442 al., 2007) using specific primers (Table S4), and all plants from both target genes presented the  
443 expected siRNA, except for *AK* event #4 (Fig. S5B). Absence of the transcript in *AK* #4 was also  
444 demonstrated by RT-PCR. Conversely, events *AK*#1.1 and *AK*#1.2 did not show the presence of the  
445 transcript by RT-PCR, but the stem-loop pulsed RT-PCR assay revealed the presence of the  
446 expected siRNA.

447

#### 448 **Larval survival and leaf damage in RNAi transgenic tomatoes**

449 To test whether RNAi transgenic plants would promote larval mortality and protect against  
450 herbivory, *T. absoluta* larvae were allowed to feed on T<sub>0</sub> transgenic ‘Micro-Tom’ leaves until  
451 emergence as adults. Larvae fed on these leaves were collected and larval mortality was evaluated  
452 by counting survivors and by visually detecting deleterious larval phenotypes based on larval size  
453 and weight. When fed on non-transgenic control plants, 100% of the larvae developed normally and  
454 reached the pupal stage. Conversely, feeding on RNAi transgenic plants resulted in a significant

455 increase in larval mortality that ranged from 30% in events ATPase1.1 and ATPase7 to 40% in  
456 ATPase 9 and AK 1.1 (Fig. 5A). The effect of RNAi was also assessed by comparing larval weight  
457 between treatments. Larvae fed on non-transgenic controls had a mean weight of 3.5 mg, while  
458 those fed on leaves of the different RNAi transgenic plants had a mean weight of 1.7 to 2.4 mg (Fig.  
459 5B).

460 Visual analysis of leaves from the different treatments revealed a clear protective effect  
461 against larval herbivory in RNAi transgenic tomato plants based on an analysis of the leaflets before  
462 (Fig. 5Aa-e) and after (Fig. 5Af-j) larval feeding. Whereas non-transgenic leaves had almost no  
463 undamaged leaf blade (Fig. 5Af), areas of apparently intact leaf blade were clearly seen in RNAi  
464 transgenic leaves (Fig. 5Ag-j).

465

## 466 Discussion

467 The tomato leafminer *T. absoluta* can cause up to 100% damage in tomato plants in various  
468 regions and under diverse cultivation systems (Desneux et al., 2010; Urbaneja et al., 2013).  
469 Resistance to insecticides has been reported for this pest, making the development of alternative  
470 means for control even more urgent (Urbaneja et al., 2013; Campos et al., 2014). In this work, we  
471 demonstrated that RNAi for *V-ATPase* and *AK*, known RNAi target genes among pest insects,  
472 significantly reduces target gene expression, increases larval mortality and reduces leaf damage  
473 caused by larval herbivory. Both of the genes studied here have been previously used as RNAi  
474 target genes in exploratory studies in many insects and crop plants; however, this is the first report  
475 on the silencing of the *V-ATPase subunit A* and *AK* genes in a lepidopteran species.

476 The major challenges to implementing an effective RNAi strategy for controlling  
477 agricultural pests involve the reliable delivery of dsRNA to the insects and the effectiveness of the  
478 target gene in conferring pest resistance or improved plant viability. The use of RNAi in crop-  
479 protection requires a delivery system to provide dsRNA continuously as a diet component to be  
480 ingested by the insects, either through transgenic plants expressing hairpin versions of target genes

481 or by spraying dsRNA, currently a more costly option (Katoch et al., 2013). The optimization of  
482 RNAi conditions, such as determining the specificity and effectiveness of the target gene, optimal  
483 dsRNA size and dose response or phenotypic effect, requires an efficient, high throughput delivery  
484 system. Traditionally, RNAi target genes are screened and evaluated by adding dsRNA to artificial  
485 diets offered to insect larvae (Terenius et al., 2011; Zhang, Li & Miao, 2012). However, artificial  
486 diets are not readily available for many of the important pest species, including *T. absoluta*  
487 (Urbaneja et al., 2013). Hence the need for alternative delivery methods to overcome this limitation.  
488 To date, two alternative methods for oral feeding have been used to deliver dsRNA to insects:  
489 microinjection into the hemocoel and soaking (Price & Gatehouse, 2008; Gu & Knipple, 2013).  
490 However, despite being very straightforward methods, RNAi-based pest control still relies mostly  
491 on host-insect interactions such that delivery methods based on ingestion would be more  
492 appropriate for this use of RNAi.

493 In this work, we used two RNAi delivery methods for *T. absoluta* based on larval feeding  
494 on tomato leaflets containing dsRNA transcribed *in vitro* and absorbed by the plant, or transcribed  
495 *in planta*. ‘Leaf-absorbed dsRNA delivery’ is based on the leaflet uptake of dsRNA transcribed *in*  
496 *vitro*. This method is similar to that proposed for RNAi-based gene silencing in the sap-sucking *B.*  
497 *tabaci* (Luan et al., 2013). The use of confocal microscopy allowed us to follow the trajectory of  
498 Cy3-labeled dsRNA through the leaf vasculature until it reached the larval digestive system.  
499 ‘PITGS delivery’, based on the infiltration of *Agrobacterium* cells carrying binary plasmids that  
500 express hairpin versions of the target gene sequences, is shown here, for the first time, to be useful  
501 in silencing insect pest genes. By using *Agrobacterium* cells carrying GFP-expressing and -  
502 silencing expression cassettes, we were able to show specific target gene knock-down and to  
503 determine whether the dsRNA molecules transcribed *in planta* were being processed by the plant or  
504 maintained as dsRNA until ingested by the larvae. The reduction in GFP fluorescence seen in  
505 *GFPi*-treated leaves indicated that the dsRNA was processed *in planta*, which suggested that the  
506 RNAi molecules ingested by the larvae had already been processed by the plant. Based on this

507 finding, we assumed that the RNAi mechanism that resulted in *eGFP* silencing in plant leaves  
508 would similarly process the insect-specific dsRNA into siRNA that would later be ingested by the  
509 larvae during feeding, resulting in the silencing of larval genes.

510 Both of the RNAi delivery methods used here to target *ATPase* and *AK* were successful in  
511 delivering dsRNA to larvae and resulted in gene silencing, increased larval mortality and protection  
512 against larval herbivory. These methods have an advantage over dsRNA delivery via an artificial  
513 diet since they allow the use of natural feeding material (host plant leaves) ingested during  
514 herbivory. The main advantage of the ‘Leaf-absorbed dsRNA’ method over ‘PITGS delivery’ is that  
515 it is quicker and easier to produce dsRNA when compared to the need to clone the gene fragments  
516 into specific expression vectors. However, despite being more time-consuming than the first  
517 method, PITGS-based delivery provides a more realistic trigger of the RNAi machinery in  
518 transgenic plants since the dsRNA hairpin molecule will be transcribed and processed by the plant  
519 cells, as expected in transgenic field plants. In addition, the same gene construct can subsequently  
520 be used to transform the host plant.

521 Both delivery methods resulted in enhanced RNAi effects over time. While the greatest  
522 reductions in transcript accumulation for *V-ATPase* and *AK* were observed at the latest sampling  
523 time points (Figs. 2 and 3), larval mortality also increased gradually with time (Fig. 3). This time-  
524 dependent RNAi response may reflect the systemic dissemination of RNAi or simply the prolonged  
525 effect of gene silencing on larval survival. Interestingly, insects may lack an siRNA signal  
526 amplification mechanism, as indicated by the apparent absence of a canonical RNA-dependent  
527 RNA polymerase (RdRP) in this phylum (Gordon & Waterhouse, 2007; Camargo et al., 2015), and  
528 this could significantly affect the source-to-signal ratio of the RNAi effects in pest insects. The  
529 ‘Leaf-absorbed dsRNA’ delivery method also allowed us to assess the dose dependence of the  
530 RNAi response in leaf protection, with greater protection being observed in leaflets that absorbed  
531 the highest dsRNA dose tested (5000 ng) for both target genes.

532            Labelling dsRNA with Cy3 fluorochrome revealed a characteristic trajectory of these  
533 molecules in the leaf blade, as observed by following the dynamics of the strongest fluorescent  
534 signals over time. Upon absorption, the labelled molecules filled the mid rib (Fig. 1Ab) and then  
535 moved to lateral veins (Fig. 1Ad), as expected for soluble molecules flowing in the leaf vasculature.  
536 With the continuous absorption of labelled dsRNA over time, the fluorescent molecules started to  
537 accumulate in the veins and leaf lamina (Fig. 1Ad). Interestingly, this accumulation occurred from  
538 the marginal regions towards the blade center until the entire leaf was saturated with labelled  
539 molecules (Fig. S2). This phenomenon suggested that the RNAi molecules were initially distributed  
540 throughout the entire leaf vasculature, including the end of the vasculature tracks located at the leaf  
541 margins. Upon saturation of the vascular track, the molecules started to diffuse to the leaf lamina  
542 and until they covered the entire leaf.

543            In summary, the results of this study suggest that sufficient dsRNA accumulation is  
544 required to produce gene silencing in *T. absoluta* and that a minimum dose is required before  
545 triggering RNAi (Yu et al., 2012). The core machinery for dsRNA-mediated gene silencing in  
546 insects requires transmembrane proteins, such as SID1, involved in dsRNA uptake and systemic  
547 spreading. Our results have been corroborated by the identification of putative orthologous *Sid-1*  
548 genes in the *T. absoluta* transcriptome analyzed by RNA-seq (Camargo et al., 2015), together with  
549 other core genes, such as *Dicer-like* and *Argonaute*; however, no canonical RdRP was found, an  
550 occurrence widely described for insects (Terenius et al., 2011; Gu & Knipple, 2013; Katoch et al.,  
551 2013; Zhang et al., 2013).

552            With the increase in *T. absoluta* resistance to currently used insecticides (Urbaneja et al.,  
553 2013; Campos et al., 2014), the application of RNAi could be a promising approach for controlling  
554 this insect. Our results show that, in the absence of an artificial diet, two alternative delivery  
555 systems can be used to evaluate the effectiveness of potential gene targets. We also demonstrated  
556 that transgenesis is a potentially useful delivery method for RNAi-mediated control of *T. absoluta*.



557 This method could provide a useful starting point for the development of alternatives to  
558 conventional pesticides.

559

#### 560 **Competing interest**

561 The authors declare that they have no competing interests.

562

#### 563 **Authors' contributions**

564 RAC did the laboratory work, developed the assays and helped to draft the manuscript.  
565 GOB did the confocal analyses, and LEPP and IP were responsible for tomato transformation and  
566 some of the molecular analyses. EL helped to develop the agro-infiltration assay. AF, JEL and  
567 HMBS conceived the study, participated in its design and wrote the manuscript. All authors  
568 participated in writing and revising the manuscript and in approval of the final version.

569

#### 570 **Acknowledgments**

571 This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo  
572 (FAPESP, grant. no. 2011/12869-6) and by a fellowship to JEL (grant no. 2010/11313-1). RAC was  
573 the recipient of a CAPES fellowship, and GOB, IP, LEPP and AF were supported by Conselho  
574 Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brazilian National Research  
575 Council).

576

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703 **Figure legends**

704 Figure 1. Trajectory of Cy3-labeled dsRNA molecules through the tomato leaflets to the larval  
705 intestinal track. **(A)**. Confocal images of tomato leaflets exposed (b,d) or not (control) (a,c) to  
706 dsRNA from the *V-ATPase* gene fragment labeled with Cy3 (seen as red dye) by *in vitro*  
707 transcription, taken at 6 h (bar = 250  $\mu\text{m}$ ) (a,b) or 24 h (bar=1 mm) (c,d) after treatment.  
708 Chlorophyll is seen as green fluorescence. **(B)**. Confocal images of first instar *T. absoluta* larvae  
709 fed on tomato leaflets exposed (larva on the right hand side) or not (control – larva on the left  
710 hand side) to dsRNA from the *V-ATPase* gene fragment labelled with Cy3 by *in vitro*  
711 transcription, taken 24 h after treatment (bar in d = 250  $\mu\text{m}$  and applies to other panels). Larval  
712 images were taken with bright light (a), 488 (b), 555 (c) channels and images a, b and c were  
713 merged in d.

714

715 Figure 2. Effect of the dsRNA uptake and PITGS dsRNA delivery methods on the relative  
716 expression of target genes in *T. absoluta* larvae. Relative expression of the target genes *V-*  
717 *ATPase* and *AK* in *T. absoluta* larvae fed on tomato leaflets that absorbed a dsRNA solution (25  
718  $\mu\text{g mL}^{-1}$ ) (A) and tomato leaflets that were infiltrated with *Agrobacterium* cells containing  
719 constructs that transcribed inverted repeats of the target gene fragments *V-ATPase* and *AK* (B).  
720 The larvae in A and B were sampled 24 h, 48 h and 72 h after the initiation of feeding. Gene  
721 expression was normalized to positive controls that were exposed to *GFP* dsRNA ( $n= 3$ ). The  
722 *Rpl 5* gene was used as a reference gene ( $n= 3$ ). The columns represent the mean  $\pm$  SE. \* $P<0.05$   
723 and \*\* $P<0.01$  (Student's *t*-test).

724

725 Figure 3. Effect of RNAi on larval mortality. The mortality of *T. absoluta* larvae was assessed after  
726 feeding on tomato leaves treated with dsRNA from *V-ATPase*, *AK* or *GFP* control for various  
727 intervals ( $n=10$ ). Tomato leaflets were provided with 1  $\mu\text{g}$  of each dsRNA (*V-ATPase* or *AK*,

728 plus *GFP* control) per leaflet per day for 10 days (total of 10  $\mu\text{g}$ ). The columns represent the  
729 mean  $\pm$  SE.

730

731 Figure 4. Effect of RNAi on leaf damage caused by larval herbivory. Tomato leaflets that absorbed  
732 increasing amounts (500, 1000 or 5000 ng) of *GFP* (a, b), *V-ATPase* (c, d) and *AK* (e, f) dsRNA  
733 were exposed to herbivory by *T. absoluta* larvae for 11 days. Two replications (column 1 and 2)  
734 are shown for each treatment.

735

736 Figure 5. RNAi transgenic plants. (A). Transgenic ‘Micro-Tom’ tomato leaflets after one (a-e) and  
737 seven days (f-j) of herbivory by *T. absoluta* larvae. Pupae (k-o) obtained from the respective  
738 treatments. (B) Average pupal weight obtained from larvae fed on the various transgenic leaflets.  
739 The columns represent the mean  $\pm$  SEM ( $n= 6-10$ ).

740

#### 741 **Supplementary Figures**

742 Figure S1. Multiple alignment of translated cloned sequences from *Tuta absoluta* and homologs  
743 from other insect species. For Vacuolar ATPase subunit-A: *Aedes aegypti* (XP\_001659520.1),  
744 *Drosophila melanogaster* (NP\_652004.2), *Tribolium castaneum* (XP\_976188.1), *Manduca sexta*  
745 (P31400.1), *Bombyx mori* (NP\_001091829.1) and *Tuta absoluta* (KM591219). Arginine kinase:  
746 *Spodoptera litura* (ADW94627.1), *Helicoverpa armigera* (ADD22718.1), *Bombyx mori*  
747 (NP\_001037402.1), *Tribolium castaneum* (EFA11419.1), *Homalodisca vitripennis*  
748 (AAT01074.1), *Drosophila melanogaster* (AAA68172.1) and *Tuta absoluta* (KM591220). Red  
749 letters refer to perfect consensus among the seven sequences; blue partial consensus; and black no  
750 consensus.

751

752 Figure S2. High magnification of the trajectory of Cy3-labeled dsRNA molecules through tomato  
753 leaflets. Detail of various regions of treated leaves indicating the dsRNA distribution over leaf  
754 areas (bar = 200  $\mu\text{m}$ ).

755

756 Figure S3. Agroinfiltration experiments with *Agrobacterium* expressing *eGFP* or *eGFP* plus *GFPi*.

757 **(A)** Expression of *eGFP* in a tomato leaf infiltrated with *Agrobacterium* suspension containing a  
758 plasmid with *eGFP*. **(B)** Infiltration of a tomato leaf with two *Agrobacterium* lines expressing  
759 *eGFP* together with a construct expressing *GFPi*. Note the decrease in GFP expression. **(C)**  
760 Tomato leaf tissue distant from the area infiltrated with *Agrobacterium* suspension containing a  
761 plasmid with *eGFP*. All images were obtained by confocal fluorescence microscopy. Bar = 100  
762  $\mu\text{m}$  in all panels.

763

764 Figure S4. Effect of RNAi on larval development. *Tuta absoluta* individuals after feeding on tomato  
765 leaflets for 11 days and absorbing 500 ng of dsRNA of the *GFP* control (a) and the target genes  
766 *V-ATPase* (b) and *AK* (c). The total number of individuals that reached the pupal stage after  
767 feeding on the *GFP* control is shown in d and the target genes *V-ATPase* and *AK* in the larval  
768 stage are shown in e and f, respectively.

769

770 Figure S5. Detection by RT-PCR of expressed sequences (cDNA, siRNA and microRNA) from  
771 various transgenic events. **(A)** Detection of cDNA amplification products (RT-PCR) from various  
772 transgenic 'Micro-Tom' events and the non-transformed control (WT) using primers specific for  
773 insect *V-ATPase* (top panel; 139 bp) or *AK* (bottom panel; 190 bp) and tomato ubiquitin (UBI, 108  
774 bp). **(B)** Detection of amplification products for potential siRNA derived from target genes (*V-*  
775 *ATPase* or *AK*, both 60 bp), plus the *microRNA156* (MIR156; 60 bp) control. The products were  
776 obtained by stem loop pulsed RT-PCR and analysed by electrophoresis in 3% agarose gels.

777 Numbers represent events (first number) and plants within events (second number).



778

779 **Supplementary tables**

780 Table S1. Degenerated primers used to amplify and clone respective candidate target genes for  
781 RNAi and the expected amplicon size for each reaction. Numbers refer to the primer order used  
782 in the amplification reactions.

783

784 Table S2. Primers used to amplify target gene fragments with Gateway recombination borders *attL1*  
785 and *attL2* (underlined) and the expected amplicon size.

786

787 Table S3. Summary of three transformation experiments that used constructs containing repetitive  
788 and inverted gene fragments.

789

790 Table S4. Specific primers designed for transcriptional analysis of target genes for silencing and the  
791 expected amplicon size.

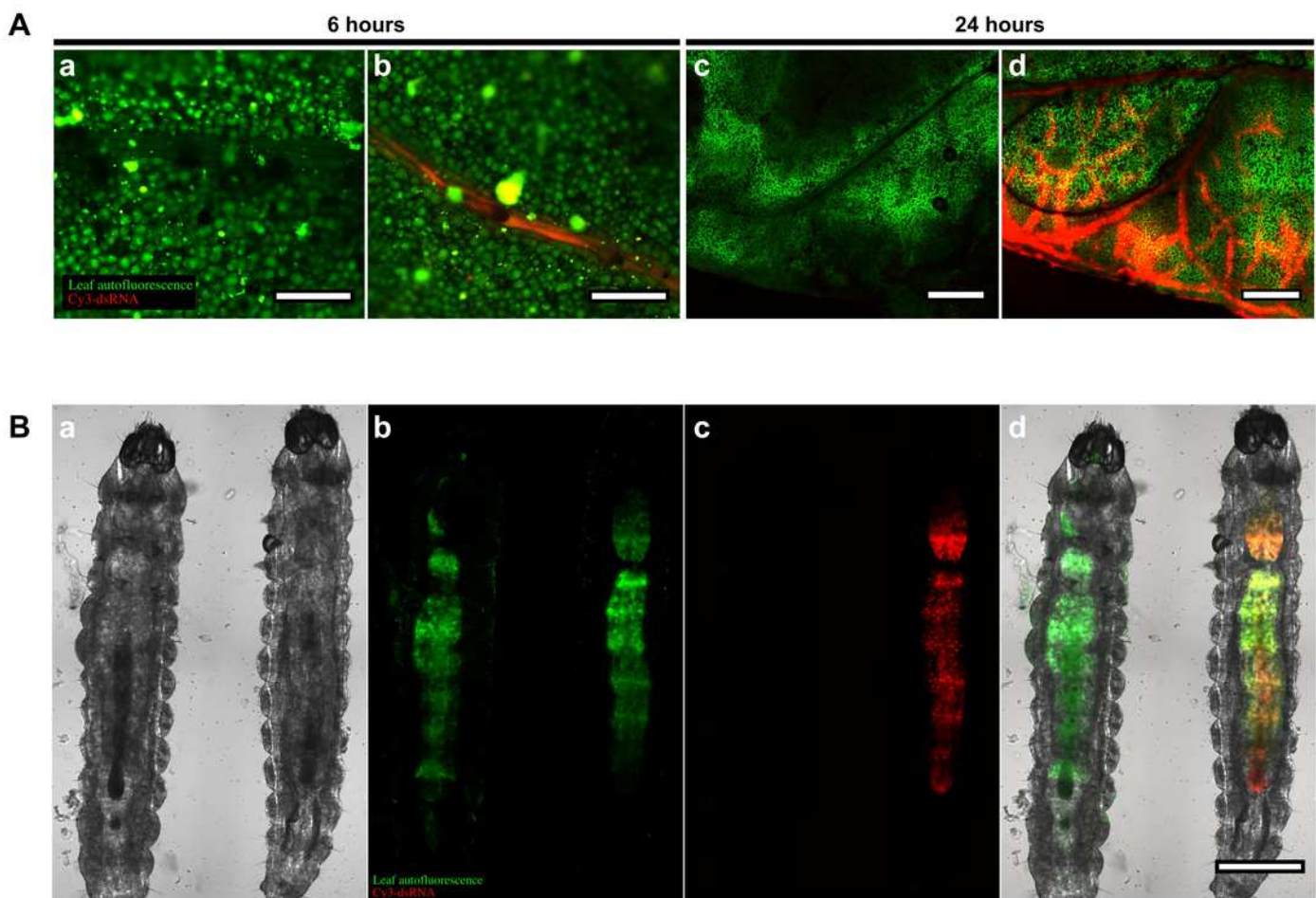
792

793 Table S5. Gene-specific and universal primer sequences used to detect the predicted small  
794 interfering RNAs (siRNA) derived from the target genes *V-ATPase* (siRNA  
795 AATACATGCGCGCTCTAGATGAC) and *AK* (siRNA AAGTATCGTCCACACTGTCTGGC)  
796 and the control *microRNA156* (UGACAGAAGAGAGUGAGCAC) in transgenic plants.

# Figure 1

Trajectory of Cy3-labeled dsRNA molecules through the tomato leaflets up to the larvae intestinal track.

**(A)** Confocal images of tomato leaflets exposed (b,d) or not (control) (a,c) to dsRNA from *V-ATPase* gene fragment labeled with Cy3 (seen as red dye) by *in vitro* transcription, taken at 6 h (bar = 250  $\mu$ m) (a,b) or 24 h (bar=1 mm) (c,d) after treatments were imposed. Chlorophyll is shown as green fluorescence. **(B)** Confocal images of 1<sup>st</sup> instar *Tuta absoluta* larva fed on tomato leaflets exposed (larva on the right hand side) or not (control - larva on the left hand side) to dsRNA from *V-ATPase* gene fragment labeled with Cy3 by *in vitro* transcription, taken 24 h after treatments were imposed (bar = 250  $\mu$ m). Larvae images were taken with bright light (a), 488 (b), 555 (c) channels and images a, b and c were merged in d.

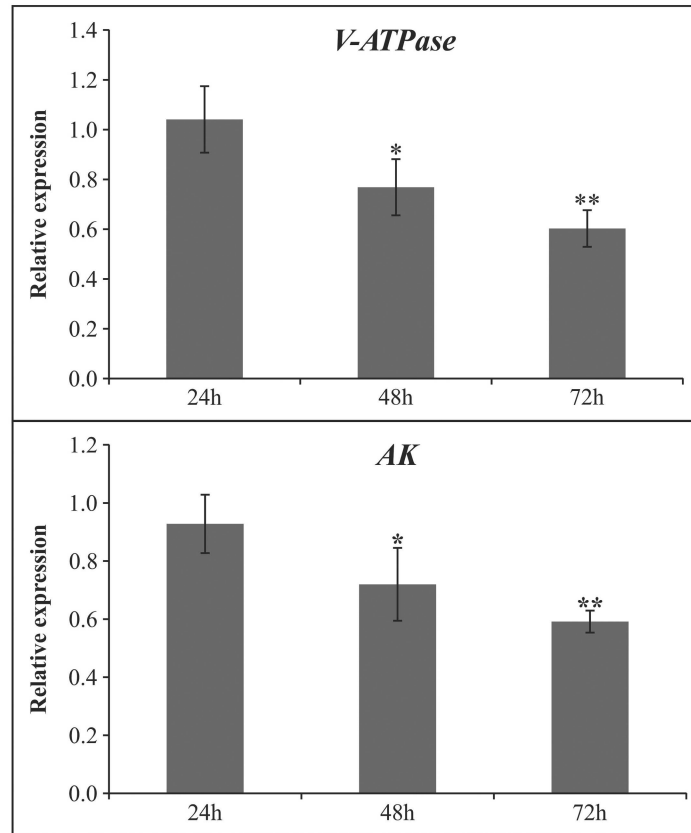


**Figure 2**(on next page)

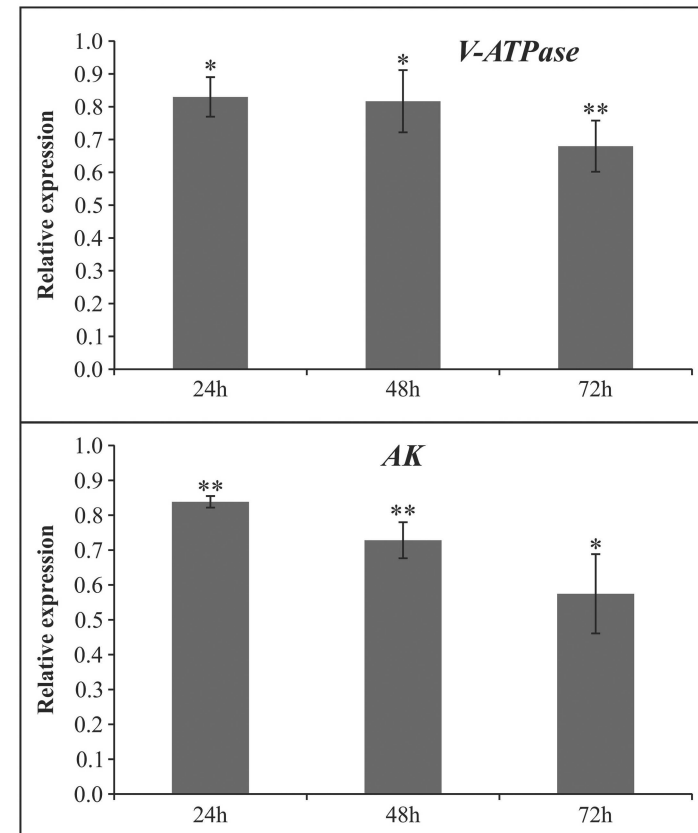
Effect of the dsRNA uptake delivery method on the relative expression of target genes in *T. absoluta* larvae.

Relative expression of target genes *V-ATPase* and *AK* in larvae of *T. absoluta* fed on tomato leaflets that absorbed dsRNA solution ( $25 \mu\text{g mL}^{-1}$ ), sampled at 24, 48 and 72 h after initiation of treatments normalized to positive controls that were exposed to *GFP* dsRNA ( $n= 3$ ). The *Rpl 5* gene was used as gene reference ( $n= 3$ ). The asterisk indicates significant differences (*t*-test; "\*" at  $P<0.05$ ; "\*\*" at  $P<0.01$ ).

A



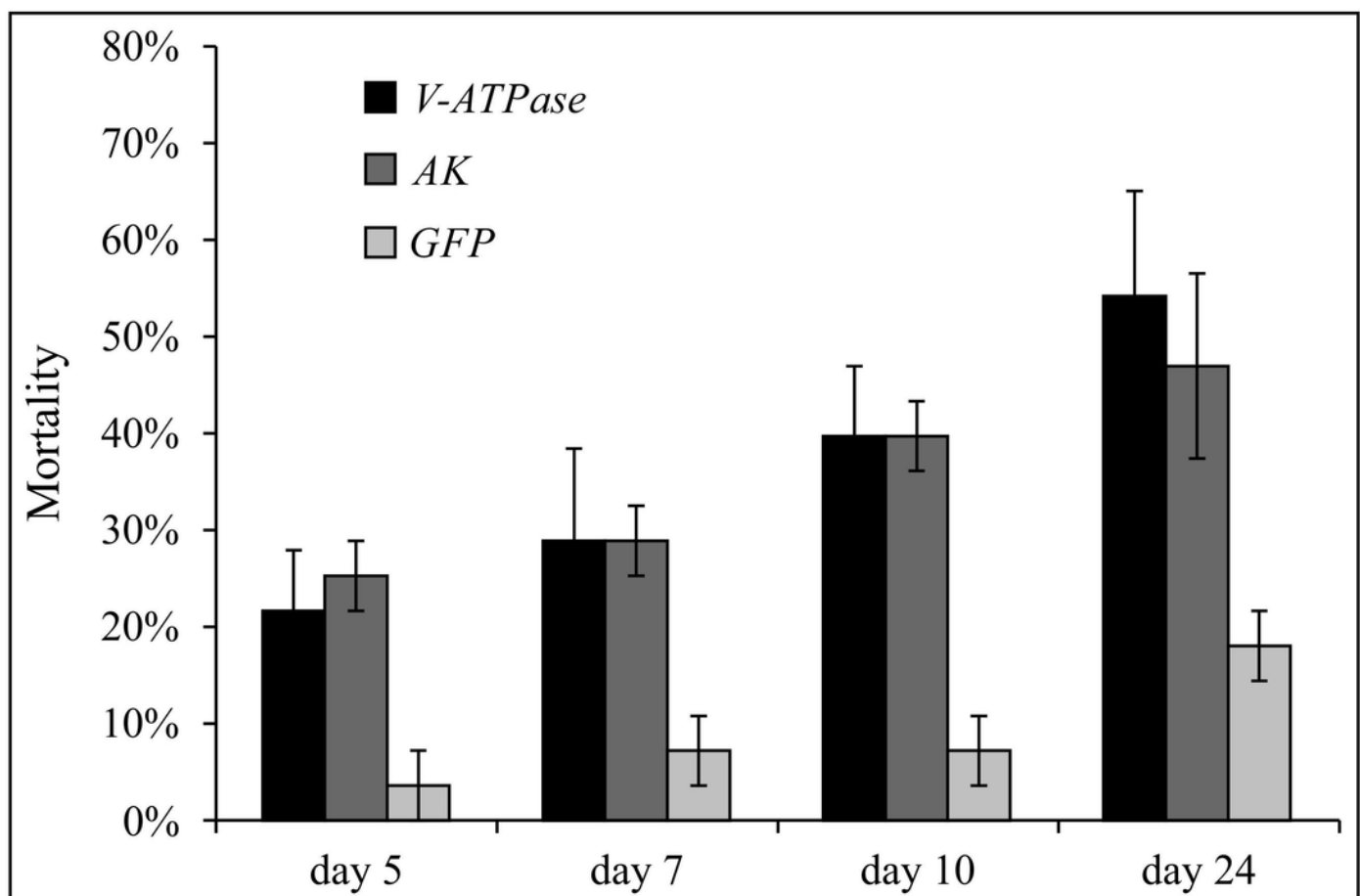
B



## Figure 3

RNAi effects on larval mortality.

Mortality of *Tuta absoluta* larvae ( $n=10$ ) after feeding on tomato leaf treated with dsRNA from *V-ATPase*, *AK* or *GFP* control for 24 days. Tomato leaflets were provided with one  $\mu\text{g}$  of each dsRNA (*V-ATPase* or *AK*, plus *GFP* control) a day per leaflet for 10 days in a total 10  $\mu\text{g}$ .

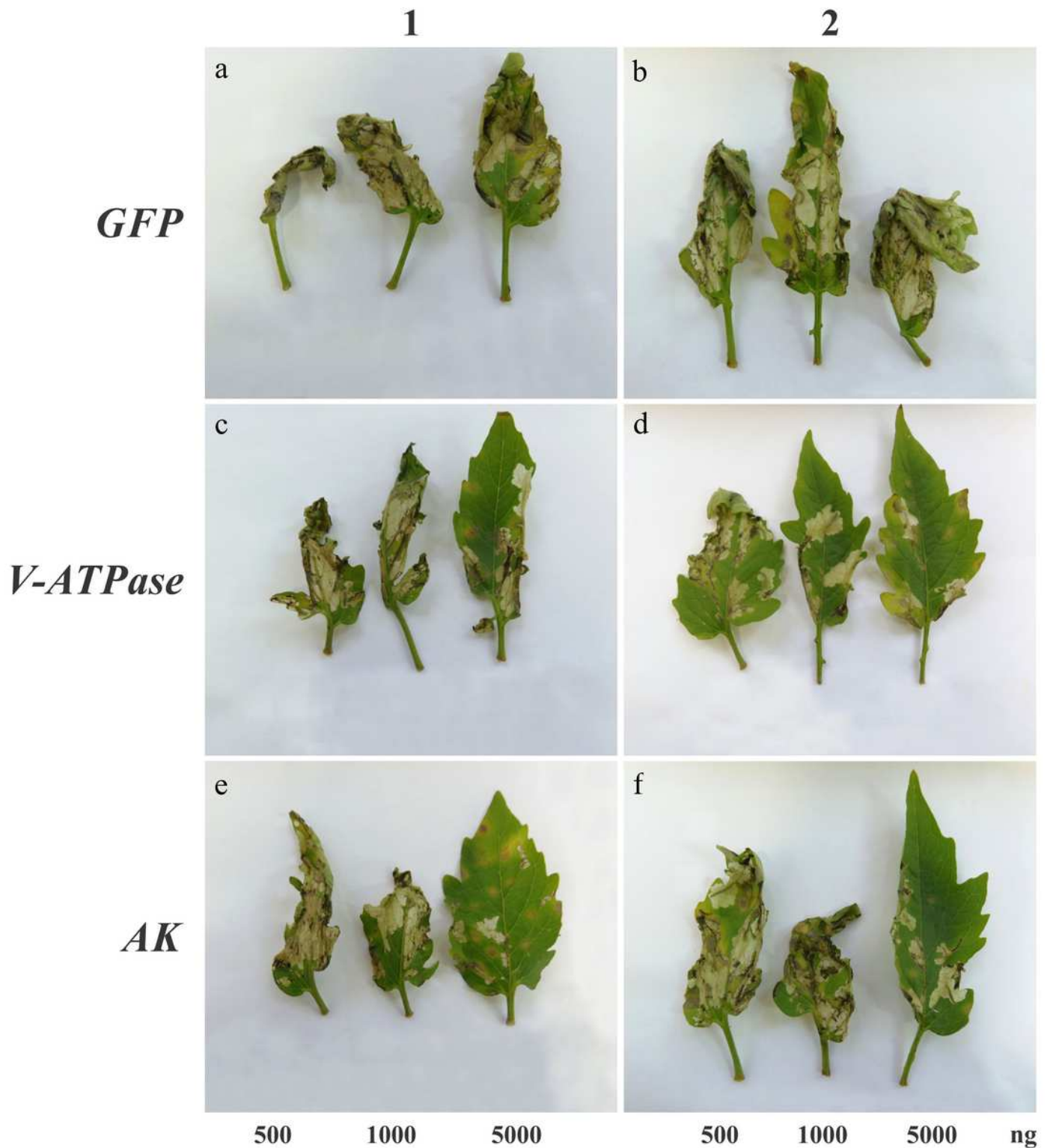


## Figure 4

RNAi effects on leaf damage caused by larval herbivory.

Tomato leaflets that absorbed increasing amounts of dsRNA (500, 1000 or 5000 ng) of GFP (a, b), *V-ATPase* (c, d) and *AK*(e, f), submitted to *T. absoluta* larvae herbivory for 11 days.

Day 11



## Figure 5

RNAi transgenic plants.

**(A)**. Transgenic 'Micro-tom' tomato leaflet at one (a-e) and seven days after (f-j) *T. absoluta* larva herbivory. Pupae (k-o) obtained from the respective treatments. **(B)** Average pupae weight obtained from larvae fed on the various transgenic events, with standard error ( $n= 6-10$ ).



