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Trans-specific gene silencing between host and parasitic plants

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Summary

Species of Orobanchaceae parasitize the roots of nearby host plants to rob them of water and other nutrients. Parasitism can be debilitating to the host plant, and some of the world's most pernicious agricultural pests are parasitic weeds. We demonstrate here that interfering hairpin constructs transformed into host plants can silence expression of the targeted genes in the parasite. Transgenic roots of the hemi-parasitic plant *Triphysaria versicolor* expressing the GUS reporter gene were allowed to parasitize transgenic lettuce roots expressing a hairpin RNA containing a fragment of the GUS gene (hpGUS). When stained for GUS activity, *Triphysaria* roots attached to non-transgenic lettuce showed full GUS activity, but those parasitizing transgenic hpGUS lettuce lacked activity in root tissues distal to the haustorium. Transcript quantification indicated a reduction in the steady-state level of GUS mRNA in *Triphysaria* when they were attached to hpGUS lettuce. These results demonstrate that the GUS silencing signal generated by the host roots was translocated across the haustorium interface and was functional in the parasite. Movement across the haustorium was bi-directional, as demonstrated in double-junction experiments in which non-transgenic *Triphysaria* concomitantly parasitized two hosts, one transgenic for hpGUS and the other transgenic for a functional GUS gene. Observation of GUS silencing in the second host demonstrated that the silencing trigger could be moved from one host to another using the parasite as a physiological bridge. Silencing of parasite genes by generating siRNAs in the host provides a novel strategy for controlling parasitic weeds.

Keywords: *Triphysaria versicolor*, post-transcriptional gene silencing, haustorium, interfering hairpin RNA.

Introduction

Parasitic angiosperms directly invade host plants in order to rob them of water, minerals and other vital nutrients (Kuijt, 1969; Nickrent, 2007). Parasitic plants attach to and invade host tissues via specialized structures called haustoria that also function as physiological bridges through which host resources are translocated (Kuijt, 1977; Riopel and Timko, 1995). The Orobanchaceae is a family of parasitic plants, recently revised to include parasitic species of Scrophulariaceae, that develop haustoria on their roots in response to host contact and host root factors (Keyes *et al.*, 2000; Musselman, 1980; Tank *et al.*, 2006). The degree to which Orobanchaceae require host resources varies dramatically between species, and they range from non-photosynthetic holoparasites that require host resources throughout development to facultative hemiparasites that are photosynthetically competent and able to survive to maturity without parasitizing a host (Press, 1995). The negative effect of plant parasitism on host plants is dramatic, and some of the world's worst agricultural pests are parasitic weeds (Parker and Riches, 1993).

The translocation of water, minerals, secondary metabolites and carbohydrates across haustoria from host to parasitic plant has been well documented (Press and Graves, 1995). More recently, movement of proteins and nucleic acids between hosts and parasites has been observed. For example, GFP was observed in *Cuscuta reflexa* after parasitism of transgenic tobacco plants expressing the GFP reporter gene (Haupt *et al.*, 2001). mRNA movement between host and parasite was detected by identifying host-specific sequences in *Cuscuta pentagona* after infection

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(Roney *et al.*, 2007). Nucleic acid movement between host and parasitic plants has also been implied by evolutionary studies. The discordant phylogenetic placement of the mitochondrial gene *nad1B-C* of *Rafflesia* sp. into a group closely related to its host *Tetrastigma* suggests that horizontal gene transfer occurred from host to parasite (Davis and Wurdack, 2004). In another example, three species of *Plantago* were found to contain a duplicate pseudogene of the mitochondrial gene *atp1* that phylogenetically clusters with the *atp1* homolog found in *Cuscuta* sp., a distantly related parasite of *Plantago* (Mower *et al.*, 2004). In the latter case, the movement of nucleic acid was from the parasite to the host plant. A similar conclusion can be drawn from the transmission of viral and phytoplasma diseases between two host plants simultaneously parasitized by *Cuscuta*: the parasite can both take up and deliver macromolecules through haustorial connections (Hosford, 1967; Marcone *et al.*, 1999).

RNA interference (RNAi), or post-transcriptional gene silencing (PTGS), is a conserved mechanism in eukaryotes by which double-stranded RNA molecules (dsRNA), formed either by complementary base pairing of transgenic sequences or by fold-back of endogenous non-coding sequences, are processed by Dicer-like nucleases into short 21–24 nt interfering RNAs (siRNA) or micro-RNAs (miRNAs). These small RNAs are then incorporated, along with Argonaute-like proteins, into RNA-induced silencing complexes that direct the degradation of endogenous RNAs that are homologous to the siRNAs (Bartel, 2004; Baulcombe, 2004; Voinnet, 2002). When siRNAs are introduced into specific tissues of a plant by biolistics or agroinfection, siRNA moves through plasmodesmata into other tissues in a non-cell-autonomous fashion (Voinnet, 2005). RNA-dependent RNA polymerase amplifies the primary siRNA, allowing further spread of the silencing signal (Himber *et al.*, 2003).

RNAi signals can also enter the phloem and spread systemically throughout a plant, and even across graft junctions from transgenic stocks to non-transgenic scions, although the nature of the translocated molecule is not known (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997). *Agrobacterium*-based vectors have been developed to deliver siRNA precursors into plants in order to selectively target endogenous genes for inactivation. These vectors are designed so that the target RNA forms self-complementary, hairpin structures (hpRNA) that result in localized dsRNA regions that are cleaved into siRNA molecules by Dicer-like nucleases (Helliwell *et al.*, 2005; McGinnis *et al.*, 2005).

Triphysaria is a genus of five hemiparasitic species that grow as common annuals throughout the Pacific Coast of the western USA (Hickman, 1993). *Triphysaria* has a broad host range that includes maize, rice and Arabidopsis, and although *Triphysaria* is closely related to the agricultural pests *Striga* and *Orobanche*, *Triphysaria* has no agricultural significance and so can be grown without quarantine restrictions (Goldwasser *et al.*, 2002). *Triphysaria* flowers are amenable to classical genetic manipulations and genomic resources are being developed, making *Triphysaria* a useful model species for parasite studies (Torres *et al.*, 2005).

The experiments described here were designed to investigate movement of RNAi molecules between parasitic and host plants. Using an *Agrobacterium rhizogenes*-mediated transformation system, we developed root cultures of *Triphysaria* that express the GUS reporter gene (Jefferson *et al.*, 1987). These roots retain their ability to develop haustoria in response to host factors and to invade host roots. We used these GUS-expressing parasite roots to monitor GUS activity following parasitism of host plants transgenic for a GUS hairpin (hpGUS) silencing construct. We also investigated the reciprocity of signal exchange by using non-transgenic parasites to simultaneously bridge a host plant expressing hpGUS and a second plant expressing GUS. The results of these experiments indicate that RNAi signals are translocated across haustorial junctions in both directions, and mediate gene silencing in both parasite and host plants.

Results

GUS activity is reduced in parasite roots following attachment to host plants expressing hpGUS

To determine whether silencing signals move across haustoria from hosts to parasitic plants, transgenic root cultures of *Triphysaria* expressing the GUS reporter gene were allowed to parasitize transgenic roots of lettuce expressing an interfering hairpin RNA homologous to GUS (hpGUS) (Figure 1). Three weeks after initiating the assay, we stained both parasite and host roots for GUS activity with 5-bromo-4 chloro-3-indolyl-β-D-glucopyranoside (X-Glu). *Triphysaria* parasitized onto non-transgenic lettuce, or not attached to any host, typically stained a uniformly dark blue (Figure 2a). In contrast, parasite roots attached to hpGUS lettuce stained at reduced levels. Reduced GUS activity was observed in those tissues of the parasite roots distal to the haustorium, but root tissues that developed prior to parasitism had full GUS activity (Figure 2b–h). There was considerable variability in the degree of GUS silencing in different host–parasite associations. In the most striking cases, the parasite root that developed after attachment had little or no detectable GUS activity (for example Figure 2b–e). In other associations, there was patchy blue staining throughout the distal portion of root, characteristic of reduced GUS activity (Figure 2f–h). Altogether we examined 65 parasite attachments, and approximately half showed at least some level of silencing (Figure 3). In contrast, all but one of the 28 control associations with non-transgenic lettuce had full GUS activity. These phenotypes indicate that the silencing signal translocated across the haustorium from lettuce into *Triphysaria*.

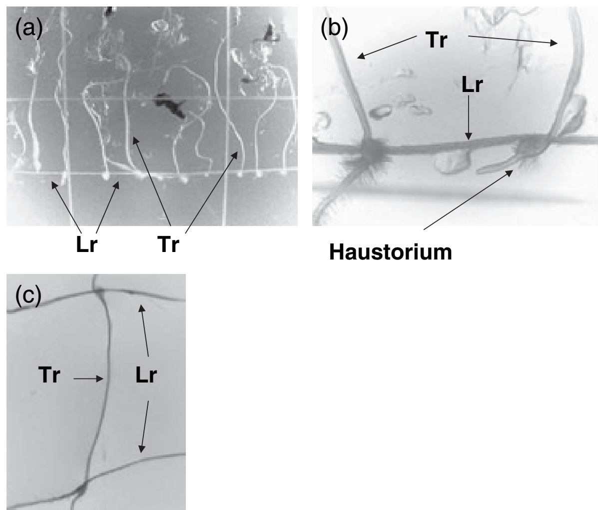

Figure 1. *In vitro* parasitism of lettuce roots.
(a) The parasite assay was initiated by placing *Triphysaria* root cultures (Tr) on the surface of agar plates perpendicular to lettuce roots (Lr), such that the tips of *Triphysaria* contacted the lettuce host.
(b) The swollen, hairy haustorial connections between *Triphysaria* (Tr) and lettuce roots (Lr) formed about 1 week after initiation. The *Triphysaria* root that develops after haustorium attachment is distal, and appears below the lettuce root.
(c) The double-junction assay in which a non-transgenic *Triphysaria* seedling was used to bridge the vasculature of a lettuce root transgenic for hpGUS and a second lettuce root transgenic for GUS.

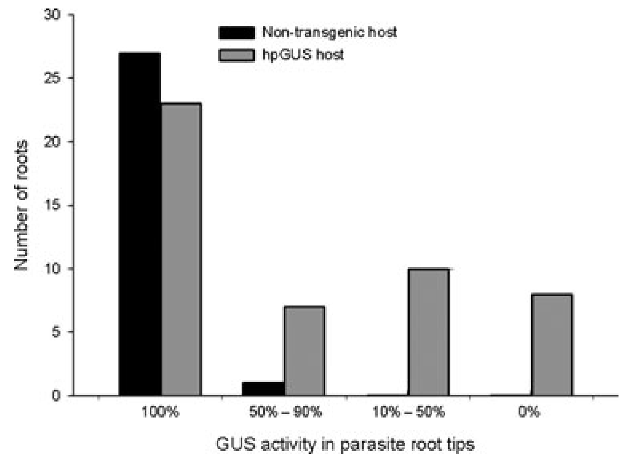

Figure 3. Variation of GUS staining in parasite roots.
Triphysaria roots attached to either hpGUS transgenic or non-transgenic lettuce roots were stained with X-Glu, and the numbers of root tips with full, partial or no GUS activity are shown.

Reduced GUS activity corresponds to reduced level of mRNA

Steady-state levels of GUS mRNA in *Triphysaria* roots were estimated by RT-PCR. GUS-specific primers that flanked the intron were used to distinguish cDNA from genomic sequences. All *Triphysaria* roots yielded the 363 bp genomic product, indicating that these plants contain the GUS transgene (Figure 4). However, when *Triphysaria* was attached to lettuce roots expressing hpGUS, there was a marked decrease in the steady-state level of GUS transcript in the parasite root. Spectral quantification and normalization of the bands obtained from cDNA against those obtained with genomic DNA indicated that the GUS transcript levels were between 5 and 90% those observed when *Triphysaria* was attached to a non-transgenic host (Figure 5). GUS transcripts in both proximal and distal root tissues were reduced, but the reductions were more pronounced in distal tissues.

The lettuce plants used as hosts in these experiments varied with respect to the degree of silencing observed (Wroblewski *et al.*, 2007). If the silencing signal is generated in the host, this variability in silencing strength may be reflected by different degrees of silencing in the parasite. We directly measured the level of GUS silencing in various

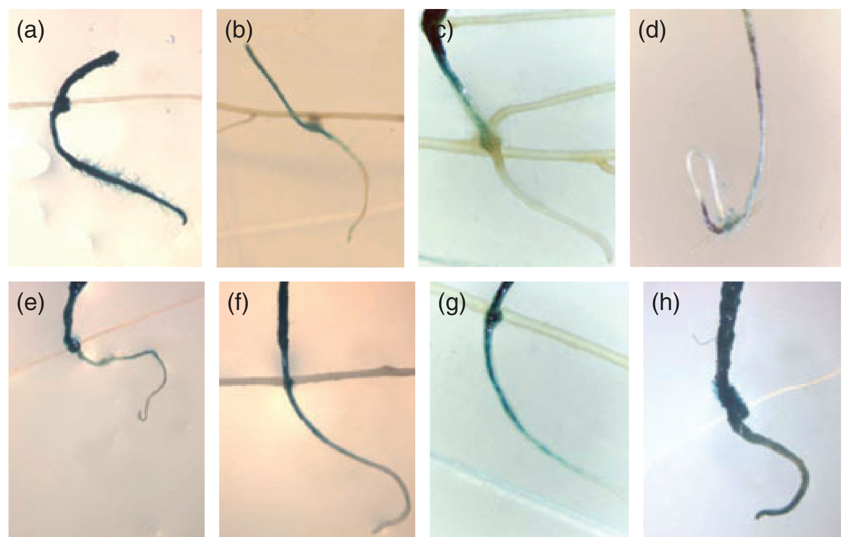

Figure 2. GUS silencing phenotypes.
Triphysaria root cultures expressing GUS parasitic on non-transgenic (a) or hpGUS-expressing lettuce roots (b–h). The root tips in (b)–(e) showed little or no GUS activity via the staining assay, that in (f) lost GUS activity in more than 50% of the root, and those in (g) and (h) show patchy GUS activity throughout the distal portion of the *Triphysaria* root.

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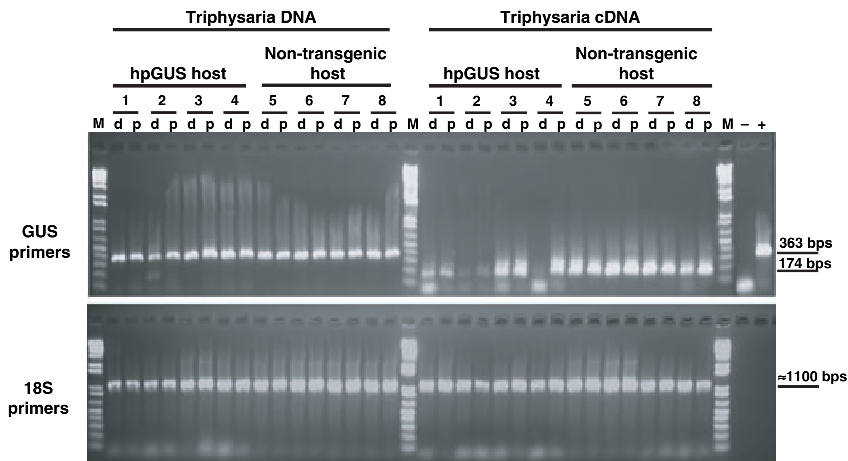

Figure 4. GUS transcript abundance following *Triphysaria* attachment onto hpGUS or non-transgenic lettuce.
Triphysaria roots were harvested after parasitism of four hpGUS transformants (1–4) or four non-transgenic (5–8) lettuce seedlings. mRNA and genomic DNA were isolated from pooled *Triphysaria* roots either distal (d) or proximal (p) to the haustorium. The DNA and cDNA pools were PCR-amplified using GUS-specific primers (top photo) or 18S primers (bottom photo). Amplification of the unspliced GUS gene resulted in a 363 bp fragment, and the spliced GUS transcript yielded a 174 bp fragment. PCR controls included genomic DNA from non-transgenic *Triphysaria* root culture (–) and the pCAMBIA1305.2 plasmid (+), which includes the GUS intron. The size markers (M) are a 1 kb DNA ladder (Invitrogen).

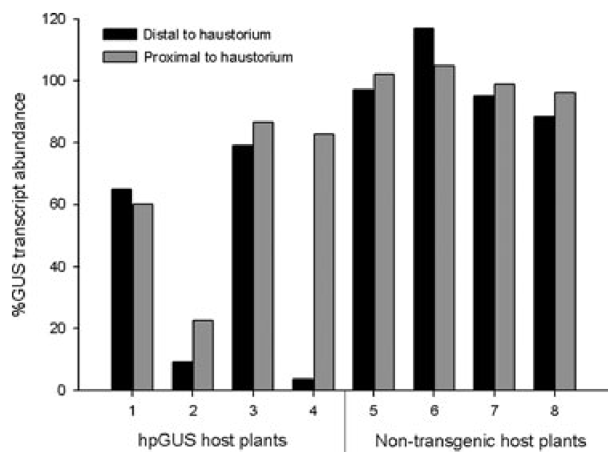

Figure 5. Quantification of GUS transcripts in parasite roots attached to lettuce.
For each tissue analyzed in Figure 4, the intensities of bands obtained using GUS primers were normalized to the intensity of bands obtained using 18S primers. The normalized GUS levels are plotted as a percentage of the mean transcript level when *Triphysaria* parasitized non-transgenic hosts.

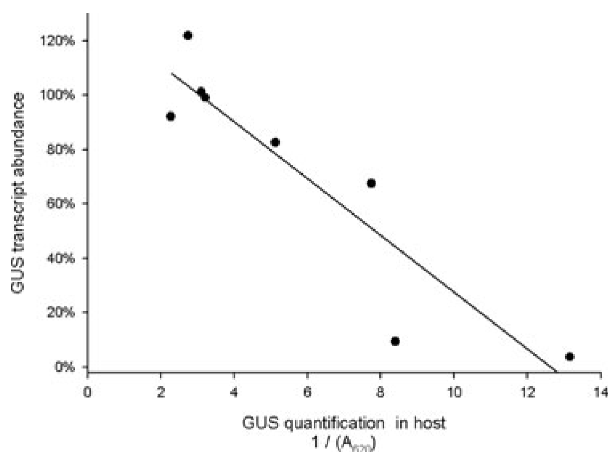

Figure 6. Regression of GUS transcripts in parasites against GUS expression in host.
The abundance of the GUS transcript in parasite roots is plotted against the amount of GUS precipitate extracted from the host roots following vacuum infiltration with pTFS40. The line is a simple linear regression, $y = -0.1044x + 1.319$, and $r^2 = 0.834$. The probability of significance using Student's *t* test is 0.0055.

hpGUS lettuce roots by transiently transforming them with *Agrobacterium tumefaciens* containing the GUS plasmid pTFS40, staining with X-Glu, and spectrally quantifying the GUS precipitate. The absorbance at 620 nm obtained for each host root was then plotted against the GUS transcript level in *Triphysaria* parasitizing that particular host. As shown in Figure 6, there was a significant correlation between the effectiveness of GUS silencing in the host and the GUS transcript abundance in the parasite; stronger silencing in host plants resulted in greater reductions in steady-state GUS transcript in the attached parasite. These experiments demonstrated that the GUS silencing signal moved from host to parasite in a quantitative, non-selective manner.

Movement of GUS silencing signal between distinct host roots using Triphysaria *as a physiological bridge*

We next addressed the question of whether the GUS silencing signal could also move from parasite to host. This was done by using non-transgenic *Triphysaria* seedlings as

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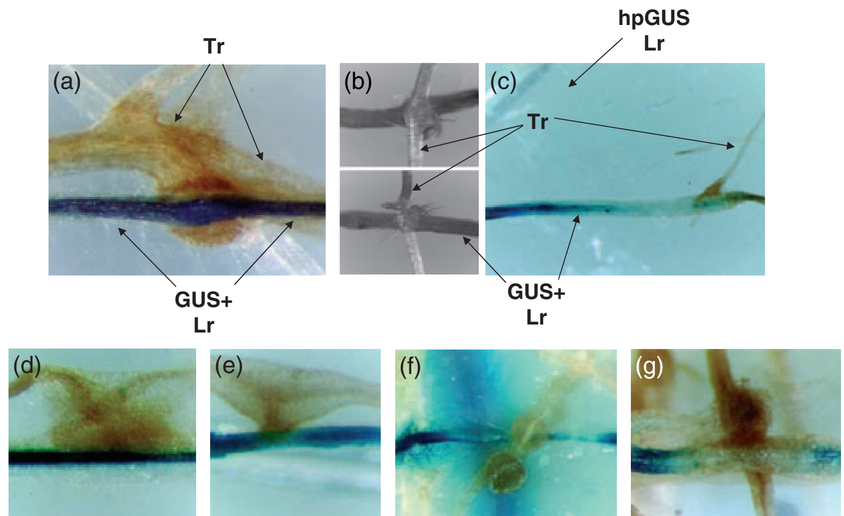

Figure 7. Bridge silencing.
(a) GUS staining of *Triphysaria* root (Tr) attached to GUS-expressing lettuce root (Lr) in a single-junction experiment.
(b) A GUS-expressing lettuce root (bottom) parasitized by a *Triphysaria* root that has also parasitized a hpGUS lettuce root (top).
(c) GUS activity in the GUS-expressing lettuce host is reduced near the site of attachment when the *Triphysaria* is also connected to an hpGUS lettuce.
(d) *Triphysaria* parasitized onto a GUS-expressing Arabidopsis root in a single-junction experiment. Note the complete GUS staining near the site of haustorial attachment.
(e–g) A GUS-expressing Arabidopsis root parasitized by a *Triphysaria* seedling that is concomitantly parasitizing an hpGUS lettuce. The three images highlight the degree of silencing variability seen in different junction experiments.

bridges connecting lettuce roots plants expressing hpGUS with those expressing GUS (Figure 1c).

In many of the double-junction associations, GUS staining was reduced or eliminated in the roots of the second lettuce plant near the site of haustorial attachment (Figure 7c,d). We examined approximately 100 double-junction associations, and GUS activity was reduced near the haustorial site in the GUS-containing host in over half of these associations (Table 1). In contrast, in control single-junction experiments in which *Triphysaria* parasitized only GUS-expressing lettuce roots, the host root stained dark blue and there was no evidence of GUS silencing (Figure 7a,b).

A similar set of experiments was conducted in which *Triphysaria* linked the hpGUS lettuce roots with GUS-expressing Arabidopsis. Again, over half of the associations showed reductions in GUS activity in the Arabidopsis root near the point of haustorium attachment. This indicates that the silencing signal had moved between hosts of different families (Figure 7f–h and Table 1).

The size of the silenced sector varied between associations. In some, the loss of staining was observed only immediately around the haustoria, but in others silencing extended to 1 mm or more on either side. Interestingly, the degree of silencing was correlated with the length of *Triphysaria* root between the two haustorial sites (Figure 8). The shorter the *Triphysaria* root separating the two haustorial junctions, the more extensive the silencing in the second host.

Table 1 Inter-family silencing across a parasite bridge

Donor host	Recipient host	No. recipients without silencing	No. recipients with silencing	Total roots
hpGUS lettuce 1	GUS+ lettuce	12	12	24
hpGUS lettuce 2	GUS+ lettuce	20	22	42
hpGUS lettuce 3	GUS+ lettuce	3	11	14
hpGUS lettuce 4	GUS+ lettuce	4	8	12
hpGUS lettuce 5	GUS+ lettuce	2	7	9
None	GUS+ lettuce	20	0	20
None	GUS+ lettuce	12	0	12
hpGUS lettuce 6	GUS+ Arabidopsis	3	8	11
hpGUS lettuce 7	GUS+ Arabidopsis	0	2	2
hpGUS lettuce 8	GUS+ Arabidopsis	1	3	4
None	GUS+ Arabidopsis	4	0	4
None	GUS+ Arabidopsis	6	0	6
None	GUS+ Arabidopsis	7	0	7

The number of recipient tissues that display GUS silencing at the haustoria junctions is shown. GUS+, GUS-expressing plants.

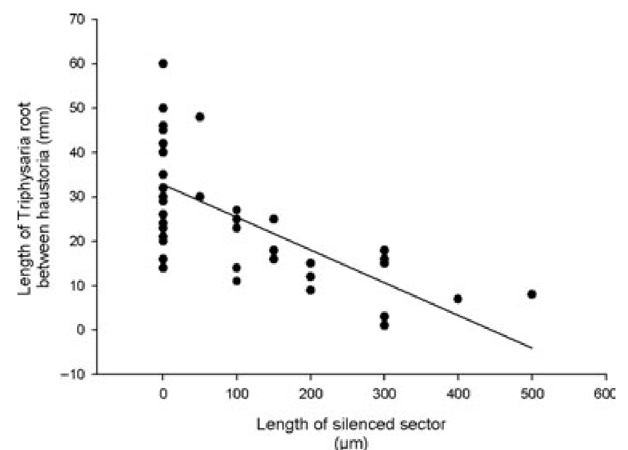

Figure 8. Silencing phenotypes in host roots depend on the distance to the junction with the hpGUS-producing host.
The length of unstained lettuce root is plotted as a function of the distance between the haustoria on each of the lettuce partners (hpGUS and GUS). Each point on the graph represents one distance measurement. The slope is significantly different from 0 ($y = 0.074x + 32.85$, $r^2 = 0.465$) with $P \leq 0.001$.

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Discussion

Our experiments demonstrate that hpRNA constructs engineered into host plants can silence the expression of transgenes in root parasitic plants. This was observed as reductions in GUS staining as well as reductions in GUS transcript levels in about 50% of *Triphysaria* roots attached to lettuce roots expressing hpGUS. It is not clear whether the non-silenced *Triphysaria* failed to make functional haustorial connections or whether the lack of silencing resulted from a post-connection mechanism. In those plants where silencing was observed, there was considerable variability in the degree of GUS silencing in different host–parasite associations; in the most extreme case, transcript levels were reduced by approximately 95%. The degree of silencing observed in parasite roots correlated with the degree of silencing in host roots. Because the efficiency of silencing is proportional to the number of silencing molecules in the cells (Dunoyer *et al.*, 2005), this correlation suggests that the number of silencing molecules transported across haustoria is a function of the number of molecules in the host, and that there is no preferential selection for or against specific molecules. The observation that parasites have the same levels of quinolizidine alkaloids and phenylpropanoid glucosides, secondary metabolites not naturally found in Orobanchaceae, as their hosts suggest a non-selective transfer of molecules between host parasite (Boros *et al.*, 1991; Marko and Stermitz, 1997; Stermitz and Harris, 1987). However, specificity for RNA uptake may occur, because only a subset of phloem-mobilized mRNAs from host plants were identified in parasitizing dodder (Roney *et al.*, 2007). It is possible that certain types or sequences of molecules are selectively transported, while transport of others is a function of the concentration in the host.

The reduction in transcript abundance occurred in parasite tissues both proximal and distal to the haustorium, suggesting the movement of silencing signal in both directions after translocation across the haustorium. However, the reduction in GUS staining was observed only in root tissues distal to the haustorium. GUS is a relatively stable enzyme and its translation in parasite cells prior to haustorium invasion accounts for the GUS staining in proximal root tissues (Jefferson *et al.*, 1987). We do not know the molecular nature of the silencing signal that passes through the haustorium. It is possible that they are short dsRNAs produced in lettuce through the action of Dicer-like enzymes that cleave the double-stranded regions of the hairpin. An alternative explanation is that hpGUS is transcribed in lettuce and translocated across the haustorium as intact mRNA and then digested into siRNAs in *Triphysaria*.

siRNAs move from their site of production to other cells through plasmodesmata (Dunoyer *et al.*, 2005). The extent of cell-to-cell movement is a function of the plasmodesmata aperture; siRNAs move approximately 10–15 cells from the initial silenced cells in leaf tissues, but the distance in embryo roots is 25–35 cells (Kobayashi and Zambryski, 2007). The silencing observed in *Triphysaria* extends over several millimetres of root, a distance that probably requires amplification of the host-encoded signal. In Arabidopsis, RNA-dependent RNA polymerases use endogenous transcripts to transcribe secondary siRNAs homologous to the original target sequence but not necessarily to the primary siRNA. These secondary siRNAs can then undergo further cell-to-cell movement (Himber *et al.*, 2003). Transitive amplification and symplastic movement of siRNAs would account for the reduction in transgene mRNA levels observed in *Triphysaria* root tips.

Triphysaria both acquired and delivered silencing signals from host plants. Non-transgenic *Triphysaria* were used in these experiments, so the GUS silencing signal must have derived from the first host. In Arabidopsis, transitive amplification of siRNAs requires an endogenous template that is lacking in these *Triphysaria*. The degree of silencing in the second host was a function of the distance between haustorial junctions, suggesting that the movement occurred without amplification of signal. Silencing molecules can move throughout the plant once they have entered the phloem stream, and it is possible that the molecules translocated from the host have entered the *Triphysaria* vascular system (Brosnan *et al.*, 2007; Voinnet, 2005). There is an obvious xylem bridge across the *Triphysaria* haustorium connecting the host and parasite stele, but phloem is not observed within the haustorium, only at the periphery of the stelar xylem. There is an abundance of transfer cells at the host–parasite interface that are thought to mediate the symplastic transport of host resources into the parasite (Heide-Jørgensen and Kuijt, 1993, 1995). The translocation of a silencing factor may utilize both short-range cell-to-cell movement as well as long-range, phloem-associated mechanisms (Himber *et al.*, 2003; Voinnet and Baulcombe, 1997).

The degree to which host attachment enhances parasite fitness is often correlated with host species (Atsatt and Strong, 1970; Gibson and Watkinson, 1989; Matthies, 1998; Seel and Press, 1993). The quality of host exudates with regard to mineral and nutrient status will significantly affect the growth and development of the parasite. Translocated secondary metabolites can have a dramatic effect on parasite ecology, as demonstrated by the decreased susceptibility of parasites to insect herbivores when the parasites are attached to alkaloid-producing lupins (Adler, 2002; Loveys *et al.*, 2001; Marvier, 1996). The experiments described here expand the spectrum of macromolecules known to be transported from hosts into parasitic plant to include siRNAs. The degree to which these types of informational macromolecules affect parasite biology remains an open question.

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The mass flow of water, osmotic gradients, and differences in water potential between host and parasite drive movement of host resources in the direction from host to parasite (Hibberd and Jeschke, 2001; Robert *et al.*, 1999; Stewart and Press, 1990). There are, however, examples of resource flow from parasites into host plants. Dodder is known to transmit viral diseases between host plants (Hosford, 1967; Marcone *et al.*, 1999). *Striga* reduces the growth of host plants more than can be accounted for by resource losses alone, suggesting the movement of a cytotoxic or pathogenic factor from parasite to host (Rank *et al.*, 2004). This factor is capable of long-distance transport in the host, as its leaves are affected while *Striga* is still underground. Dwarf mistletoe, *Arceuthobium*, also translocates parasitic factors into its hosts, altering the growth patterns of the conifers on which they grow (Hawksworth and Wiens, 1996). It may be that parasitic plants use siRNAs to modify the physiology of the host to best service the parasite.

Parasitic weeds are amongst the worlds most pernicious agricultural pests (Parker and Riches, 1993). *Striga* infests over 60% of the cultivated land in sub-Saharan Africa, where it can cause complete yield losses of maize, sorghum, cowpeas, and other staple crops (Musselman *et al.*, 2001; Scholes and Press, 2008). While host genetic resistance is generally the cornerstone in plant pest management, the search for genetic loci conferring resistance against parasitic weeds has been difficult. The observation that RNA silencing molecules produced in a host functions in a parasite suggests a new strategy for engineering parasitic plant resistance into crops, as has been done against root-knot nematodes (Tabara *et al.*, 1998; Timmons and Fire, 1998; Urwin *et al.*, 2002). Once genes critical for parasite growth and development have been identified, parasite-specific sequences can be cloned into hairpin vectors and transformed into crop plants. By designing the hairpin target sequences to be specific for the parasite, the transgenes should have no effect on the host. The parasite gene would be attenuated upon functional invasion of the host.

Experimental procedures

Transgenic Triphysaria *roots*

Triphysaria root cultures expressing GUS were obtained by inoculating wounded *Triphysaria* seedlings with *Agrobacterium rhizogenes* MSU440 containing pCAMBIA 1305.2, a T-DNA-based vector carrying the GUSPlus gene under the control of a CaMV 35S promoter (http://www.bioforge.net/forge/index.jspa). The GUSPlus reporter contains a castor bean catalase intron that interrupts the GUS reading frame so that GUS expression only occurs after expression and intron splicing in plant cells. The GUSPlus reporter also contains a glycine-rich protein secretion signal sequence that facilitates movement of the protein to the apoplastic space. Inoculation and selection of GUS-expressing *Triphysaria* roots were performed as described previously (Tomilov *et al.*, 2006).

Transgenic lettuce

Production of transgenic lettuce cv. Mariska expressing an interfering hairpin RNA homologous to the GUS reporter gene has been described previously (Wroblewski *et al.*, 2007). Briefly, a 451 bp fragment of the GUS gene was fused to a 459 bp fragment of the *RGC2B* gene coding for resistance to the lettuce downy mildew pathogen *Bremia lactucae*. This chimeric fragment was used to create an inverted repeat structure in pGSA1165, a binary vector for plant transformation (http://www.chromdb.org). The two arms of the inverted repeat were separated by the 788 bp intron 3 of the *pdk* gene from *Flaveria trinervia*. Lettuce transformations were performed as described previously (Michelmore *et al.*, 1987). Strongly silenced transgenic plants (T_1) were identified based on the loss of *RGC2*-encoded resistance and the lack of GUS expression in *Agrobacterium*-mediated transient assays (Wroblewski *et al.*, 2005). Disease resistance and GUS silencing segregated in T_2 progeny consistent with a single-locus insertion of the T-DNA containing hpGUS. T_2 progeny of one strongly silenced T_1 plant were used in the experiments described here. T_2 progeny containing the transgene were identified by plating on kanamycin-containing medium, and the presence of the transgene was confirmed by PCR. Pools of homozygous and heterozygous lines were used in these experiments. Non-transgenic plants of cv. Mariska were used as controls.

Transgenic Arabidopsis

Arabidopsis Ws-0 was transformed with *A. tumefaciens* C58 bearing pTFS40 using the floral dip method (Clough and Bent, 1998). Homozygous lines were identified by sibling selection for GUS expression and kanamycin resistance in medium supplemented with 50 mg l^{-1} kanamycin.

In vitro *parasitism of host roots*

Lettuce, Arabidopsis or *Triphysaria* seeds were surface-sterilized in 70% EtOH for 5 min, then 30% commercial clorox bleach (final sodium hypochlorite ~1%) for 15 min, and then washed extensively in sterile water. Seeds were germinated on agar plates containing 0.25× Hoagland's medium (1.25 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0.50 mM $MgSO_4$, 50 mM H_3BO_3, 9.0 mM $MnCl_2{\cdot}4\,H_2O$, 70 nM $ZnSO_4{\cdot}7\,H_2O$, 30 nM $CuSO_4{\cdot}5\,H_2O$, and 10 nM $Na_2MoO_4{\cdot}2\,H_2O$) (Hoagland and Arnon, 1938) and 1% sucrose. After 2 weeks, the host seedlings (lettuce or Arabidopsis) were transferred to 10 × 10 cm Petri dishes containing 0.25× Hoagland's medium with 1% sucrose, and incubated in a near-vertical position, causing the roots to grow down along the surface of the agar, for an additional 2 weeks. *Triphysaria* seedlings or root cultures were then placed onto the agar with their root tips adjacent to the host roots, and the plates were returned to the 25°C growth room for 3 weeks. Several *Triphysaria* roots were placed on each lettuce root. After 3 weeks, most of the *Triphysaria* roots had developed hairy, swollen haustoria that had attached to and invaded the hosts (Figure 1a,b).

Bridge connections between two host plants were created by infecting the first host as described above, and then positioning the emerging *Triphysaria* root tip adjacent to the second host root 1 week later. In these experiments, approximately 20 *Triphysaria* seedlings were infected onto each host root. After 3 weeks, the roots were removed from the plates and assayed for GUS expression.

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GUS assays

Roots were stained in 500 μg ml^{-1} of X-Glu (Gold BioTechnology, http://www.goldbio.com/) and de-stained in 70% ethanol (Jefferson *et al.*, 1987). Stained materials were examined using a Zeiss Stemi SV11 stereomicroscope (Zeiss Microimaging, http://www.zeiss.com/micro), and photographed using a Pixera professional high-resolution digital camera system (Pixera Corporation, http://www.pixera.com/).

To determine the degree of GUS silencing in lettuce roots, we cut approximately 100 sections (2 cm long) from each lettuce root, and used a syringe to vacuum infiltrate the explants with *A. tumefaciens* C58 carrying pTFS40, a T-DNA-based vector containing a GUS gene under the control of the CaMV 35S promoter and interrupted by a plant intron (Chang *et al.*, 2002). Three days after co-cultivation, root explants were stained with X-Glu, dehydrated in ethanol, and extracted with 100 μl N-dimethylformamide (DMFA) overnight at 37°C as previously described (Tomilov *et al.*, 2005). The absorbance at 620 nm was measured on a Beckman DU Series 600 spectrophotometer (Beckman Instruments, http://www.beckmancoulter.com).

RT-PCR

Three weeks after attachment to host roots, *Triphysaria* roots were dissected and frozen in liquid nitrogen. Root tissues distal and proximal to the haustorium were harvested separately. Between one and six *Triphysaria* roots were collected and pooled from each lettuce root. The tissue was ground in liquid nitrogen, and total RNA was isolated using Trizol reagent (Invitrogen, http://www.invitrogen.com). Genomic DNA was isolated using a modified hexadecyltrimethylammonium bromide (CTAB) method (Rogers and Bendish, 1988). cDNA synthesis was performed using the SuperScript first-strand synthesis system for RT-PCR as described by the manufacturer (Invitrogen).

PCR was initiated with a hot start at 94°C for 1 min, followed by 45 cycles of denaturation (94°C for 15 sec), annealing (56°C for 30 sec) and extension (72°C for 30 sec). The reaction volume was 50 μl, and contained 100 ng cDNA, 2.0 μl of each primer (10 mM), 4 μl of dNTP mix (2.5 mM each), 5 μl PCR buffer and 1 μl Taq DNA polymerase. Under these conditions, amplification of an intron-containing GUS sequence from genomic DNA resulted in a band of 363 bp that was ready distinguishable from the 174 bp fragment generated from intron-spliced cDNA. After gel electrophoresis in 2% agarose gels and staining with ethidium bromide, the bands were quantified using an AlphaImager imaging system (Alpha Innotech Corp., http://www.alphainnotech.com/).

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