Trans-Specific Gene Silencing of Acetyl-CoA Carboxylase in a Root-Parasitic Plant

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Parasitic species of the family Orobanchaceae are devastating agricultural pests in many parts of the world. The control of weedy Orobanchaceae spp. is challenging, particularly due to the highly coordinated life cycles of the parasite and host plants. Although host genetic resistance often provides the foundation of plant pathogen management, few genes that confer resistance to root parasites have been identified and incorporated into crop species. Members of the family Orobanchaceae acquire water, nutrients, macromolecules, and oligonucleotides from host plants through haustoria that connect parasite and host plant roots. We are evaluating a resistance strategy based on using interfering RNA (RNAi) that is made in the host but inhibitory in the parasite as a parasite-derived oligonucleotide toxin. Sequences from the cytosolic acetyl-CoA carboxylase (ACCase) gene from *Triphysaria versicolor* were cloned in hairpin conformation and introduced into *Medicago truncatula* roots by *Agrobacterium rhizogenes* transformation. Transgenic roots were recovered for four of five ACCase constructions and infected with *T. versicolor* against parasitic weeds. In all cases, *Triphysaria* root viability was reduced up to 80% when parasitizing a host root bearing the hairpin ACCase. *Triphysaria* root growth was recovered by exogenous application of malonate. Reverse-transcriptase polymerase chain reaction (RT-PCR) showed that ACCase transcript levels were dramatically decreased in *Triphysaria* spp. parasitizing transgenic *Medicago* roots. Northern blot analysis identified a 21-nucleotide, ACCase-specific RNA in transgenic *M. truncatula* and in *T. versicolor* attached to them. One hairpin ACCase construction was lethal to *Medicago* spp. unless grown in media supplemented with malonate. Quantitative RT-PCR showed that the *Medicago* ACCase was inhibited by the *Triphysaria* ACCase RNAi. This work shows that ACCase is an effective target for inactivation in parasitic plants by trans-specific gene silencing.

Parasitic weeds, particularly root parasites in the family Orobanchaceae, are notorious agricultural weeds in many parts of the world (Parker and Riches 1993). Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are economically destructive parasitic weeds, particularly in lesser

developed countries, and threaten the lives of over 100 million African, Asian, and European people (Parker 2012; Scholes and Press 2008). These root parasites are the most destructive before they emerge from the ground; therefore, the majority of crop loss occurs before the infection is diagnosed. A variety of physical, cultural, chemical, and biological control methods have been developed (Ejeta and Gressel 2007; Hearne 2009; Ransom et al. 2012; Rubiales et al. 2009). However, host genetic resistance against parasitic weeds, typically considered a cornerstone of integrated pest management systems, has not been well exploited in maize. Therefore, biotechnological approaches should be explored.

Pest control strategies based on RNA interference (RNAi) have recently proven effective against a range of viral, microbial, and multicellular pests. RNAi is a general phenomenon whereby short, double-stranded regions of RNA (dsRNA) elicit a defense reaction that inhibits the transcription of sequences homologous to the dsRNA. Plants can be engineered to express dsRNA by transforming with vectors containing target sequences cloned in inverted orientation, such that the RNA transcribed forms hairpin (hp) structures with localized regions of dsRNA. The dsRNA is cleaved by the plant DICER enzymes, resulting in small interfering RNAs (siRNAs) that direct the RNA-induced silencing complex to inhibit endogenous gene expression by transcriptional or post-transcriptional means (Baulcombe 2004; Eamens et al. 2008; Ghildiyal and Zamore 2009). RNA interference can be targeted against plant pests that take up the RNAi molecules during feeding.

The strategy of engineering host resistance by transforming crops with modified versions of pathogen-derived genes was proposed almost 30 years ago (Sanford and Johnston 1985). The first demonstration of pathogen-derived resistance was in *Tobacco mosaic virus* (TMV)-resistant plants made by transforming the TMV coat protein gene in tobacco (Abel et al. 1986). What was originally thought to be resistance associated with the viral coat protein was later shown to be mediated by RNAi (Ratcliff et al. 1997). Pathogen-derived resistance has been useful for engineering resistance against several viruses, including *Cucumber mosaic virus*, *Barley yellow dwarf virus*, and *Papaya ringspot virus*, the latter being responsible for the recovering of the papaya industry in Hawaii (Ferreira et al. 2002; Gonsalves 2006; Tricoli et al. 1995; Wang et al. 2000). Other pests that have been targeted for trans-specific RNAi are tomato crown gall disease, powdery mildew, cyst and root-knot nematodes, and herbivorous insects (Baum et al. 2007; Escobar et al. 2001; Huang et al. 2006; Ibrahim et al. 2011; Mao et al. 2011; Nowara et al. 2010).

Parasitic plants directly invade their hosts through haustoria, parasitic organs responsible for host attachment, penetration, and resource acquisition (Kuijt 1969; Riopel and Timko 1995).

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The parasite obtains several types of resources from the host, including water, nutrients, proteins, and oligonucleotides (Aly et al. 2011; Irving and Cameron 2009; Westwood et al. 2009). The movement of informational oligonucleotides from host to parasites opens up the potential of using the RNAi approach to develop parasite resistance (Runo et al. 2011; Yoder et al. 2009). Trans-specific RNAi silencing in a parasitic plant root was visualized first by transforming roots of the hemiparasite *Triphysaria versicolor* with the β -glucuronidase (GUS) reporter gene and then allowing the transgenic *Triphysaria* spp. to parasitize host roots expressing an RNAi hairpin construction of GUS (hpGUS). Histochemical staining and semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) demonstrated that GUS expression was reduced in *Triphysaria* roots parasitizing lettuce roots which were transgenic for hpGUS (Tomilov et al. 2008). The bidirectional movement of RNAi across the haustorium was observed by parasitizing a single, nontransgenic *T. versicolor* onto roots of two *Arabidopsis* plants: one transgenic for hpGUS and one transgenic for GUS. GUS activity was reduced near the haustorium connection with the second plant, indicating that the RNAi silencing factor moved from one host to a second via the parasite bridge.

The viability of *Orobanche aegyptiaca* was reduced when grown on tomato containing an RNAi targeting the tomato mannose 6-phosphate reductase (M6PR) gene that encodes a key enzyme in mannitol biosynthesis (Aly et al. 2009). The large accumulation of mannitol in *O. aegyptiaca* spp. during parasite development is thought to be an osmotic driver moving host resources into the parasite (Simier et al. 1994). When *O. aegyptiaca* parasitized transgenic tomato expressing an M6PR hairpin construct, M6PR transcript levels and the percentage of mannitol in total sugars were reduced. The authors reported that up to 60% of *O. aegyptiaca* tubercles on the transgenic host plants died over a 4-week period compared with 3% on nontransgenic lines (Aly et al. 2009). Other experimenters demonstrated trans-specific silencing of a KNOTTED-like homeobox transcription factor gene SHOOT MERISTEMLESS-like (STM) in the stem parasite dodder (*Cuscuta pentagona*). A hairpin construct of STM, driven by the vascular-specific SUCROSE PROTEIN SYMPORTER 2 promoter, was transformed into tobacco and infected with dodder. STM-specific small RNAs were detected in both the host and

attached parasite, which had aberrant haustorium development and establishment (Alakonya et al. 2012).

Although these results are encouraging, clearly, the RNAi strategy needs to be further refined for effective use against parasitic weeds. One of the critical parameters is the proper selection of the targeted gene. Inhibition of the target gene needs to be fatal for the parasite; therefore, genes encoding enzymes that are herbicide targets are good candidates. The experiments described here target the parasite gene encoding cytosolic acetyl-CoA carboxylase (ACCase; EC 6.4.1.2). ACCase catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Alban et al. 2000). Two structurally distinct forms of ACCase are present in dicots. The plastid form is a heteromeric enzyme that makes malonyl-CoA for de novo fatty acid synthesis (Schulte et al. 1997). The cytosolic form of ACCase is a homodimer that is the target of cyclohexanedione and aryloxyphenoxypropionate herbicides and the focus of the current study (Parker et al. 1990). Cytosolic malonyl-CoA is a precursor to a number of biochemical pathways, including flavonoid biosynthesis and the elongation of very-long-chain fatty acids (VLCFA) which are themselves integrated into triacylglycerides, cuticle, waxes, and sphingolipids. Malonylation of some D-amino acids, glycosides, and the ethylene precursor aminocyclopropane-1-carboxylate is also dependent on the cytosolic malonyl-CoA pool (Roesler et al. 1994). In *Arabidopsis*, the cytosolic ACCases are encoded by two genes, *ACC1* and *ACC2*. Mutants in *ACC1* are embryo lethal but can be recovered when treated with exogenous malonate; *ACC2* transcripts are barely detectable with quantitative RT-PCR and mutants have no known phenotype (Baud et al. 2003, 2004).

The experiments described here test the utility of the ACCase gene as a parasite target for RNAi-mediated trans-specific gene silencing. The parasitic plant used in these studies is *Triphysaria versicolor*, a facultative, hemiparasitic Orobanchaceae sp. with a broad host range that includes *Medicago truncatula*. As a non-weedy genus of Orobanchaceae, *Triphysaria* provides a tractable model system for investigating parasitic plants without environmental risk or quarantine restrictions. *Triphysaria* haustoria have been well characterized and the recent availability of a transformation system, in vitro assays, and emerging genomic resources further promote the use of *T. versicolor* as a model parasitic species. (Albrecht et

Fig. 1. Acetyl-CoA carboxylase (ACCase) sequences used in hairpin constructions. Thick black lines at the top represent the *Medicago* ACCase gene and the homologous region in the *Triphysaria* gene. Sequences used in hairpin constructions, ACC1 to ACC5, are shown under the thick lines. Percentages of nucleic acid identity between the *Medicago* and *Triphysaria* genes were determined by ClustalW alignments and are shown for each unique targeted region by the thin lines at the bottom.

al. 1999; Heide-Jørgensen and Kuijt 1993, 1995; Jamison and Yoder 2001; Tomilov et al. 2004; Torres et al. 2005; Westwood et al. 2012).

RESULTS

Triphysaria ACCase hairpin constructs can silence ACCase in *Medicago* roots.

The RNAi vectors pHpACC1 through pHpACC5 contain ACCase sequences from *T. versicolor* cloned in hairpin orientation into the plant transformation vector pHG8-YFP (Helliwell and Waterhouse 2005). The plasmid pHpACC1 is the largest hairpin, with two copies of a 481-nucleotide (nt) ACCase sequence; the other plasmids contain smaller fragments of ACCase that overlap with ACC1 to varying degrees (Fig. 1). All of the fragments lie within the ACCase open reading frame nearer the 3' end of the gene. Alignments made between the *Triphysaria* and *Medicago* ACCase gene sequences indicated 71 to 81% identify between the two sequences in the different hairpin constructions (Fig. 1).

These hairpin constructions and the parental vector pHG8-YFP were transformed into *M. truncatula* roots, selected in kanamycin, and screened for expression of yellow fluorescent protein (YFP) by fluorescent microscopy. Essentially, all of the *Medicago* seedlings transformed with the pHG8-YFP parent vector or any of the hairpin ACCase vectors pHpACC2 through pHpACC5 developed kanamycin-resistant roots, approximately 80% of which expressed YFP (Table 1). In stark contrast, less than 10% of *Medicago* spp. transformed with pHpACC1 developed kanamycin-resistant roots and only one of these expressed YFP (Table 1). Furthermore, plants with

roots transformed with pHpACC1 had yellowish leaves and were smaller than plants transformed with pHG8-YFP (*data not shown*). This suggested that the *Triphysaria* ACC1 hairpin construct may be toxic to *Medicago* root development.

If the apparent toxicity of pHpACC1 was due to inhibition of *Medicago* ACCase activity, we predicted that the lethal phenotype could be complemented with malonate (Baud et al. 2004; Fatland et al. 2005). *Medicago* seedlings were transformed with either pHG8-YFP or pHpACC1 and incubated in kanamycin media for 6 weeks, at which time the plants were transferred to new plates without kanamycin but containing malonate concentrations of 0, 3, 10, or 100 mM. All seedlings treated with 100 mM malonate were dead after approximately a week; therefore, only plants treated at the lower concentrations were evaluated. The lower concentrations of malonate did not obviously affect the growth of roots transformed with pHG8-YFP but they significantly increased both the number and size of transgenic roots developing from pHpACC1 (Table 2; Fig. 2). Although there were no significant differences in the number of roots that developed with either 3 or 10 mM malonate, roots that developed in 10 mM were longer and more branched (Fig. 2). The recovery of transgenic pHpACC1 roots by complementing with exogenous malonate is consistent with pHpACC1 toxicity being caused by lack of ACCase activity in *M. truncatula*.

The complementation of transgenic pHpACC1 roots with malonate allowed us to grow roots to an appropriate size for RNA isolation. RNA was isolated from *Medicago* roots transformed with pHG8-YFP and each of the pHpACCase constructions and steady-state transcript levels of ACCase determined by quantitative RT-PCR. The relative expression levels of ACCase were at least two orders of magnitude lower in *Medicago* spp. transformed with pHpACC1 compared with any of the others (Fig. 3).

In conclusion, the hairpin construction pHpACC1, containing *Triphysaria* ACCase gene sequences, inhibited ACCase tran-

Table 1. Transgenic *Medicago* roots recovered in different transformations^a

Construct	Plants with kanamycin resistant roots (%) ^b	YFP roots per total roots (%) ^c
pHG8-YFP	100.0 ± 0 (n = 76)	81.7 ± 3.3 (n = 318)
pHpACC1	6.7 ± 2.7* (n = 76)	2.8 ± 4.8* (n = 10)
pHpACC2	97.6 ± 2.1 (n = 77)	75.8 ± 4.6 (n = 278)
pHpACC3	96.6 ± 2.4 (n = 76)	80.3 ± 1.9 (n = 321)
pHpACC4	98.8 ± 2.1 (n = 76)	83.3 ± 6.7 (n = 294)
pHpACC5	94.4 ± 9.4 (n = 77)	86.8 ± 1.4 (n = 300)

^a Plants were grown under kanamycin selection and the percentage of plants with at least one root longer than 1 cm was determined 6 weeks after inoculation. Values are means ± standard deviations with n = total number of plants or roots in all replicates. Values were determined from three independent transformation experiments per construction, each with four replicates (12 replicates total). Analysis of variance was determined with the General Linear Model procedure and mean separation with the least significance difference. Pairwise comparisons of treatments within a given measured parameter labeled with different numbers of asterisks (*) are significantly different at $\alpha = 0.05$.

^b Percentage of plants with at least one root longer than 1 cm.

^c YFP = yellow fluorescent protein.

Fig. 2. Root growth complementation of pHpACC1 *Medicago* spp. with malonate. *Triphysaria* seedlings were infected with pHpACC1 and grown with kanamycin selection for 6 weeks with and without supplemental malonate. A, Water-treated; B, 3 mM malonate; and C, 10 mM malonate.

Table 2. Recovery of transgenic *Medicago* roots with malonate^a

Malonate (mM)	Plants with roots (%) ^b		YFP roots per total roots (%) ^c	
	pHG8-YFP	pHpACC1	pHG8-YFP	pHpACC1
0	100.0 ± 0* (n = 126)	66.0 ± 26*** (n = 126)	72.1 ± 7* (n = 548)	6.3 ± 7*** (n = 163)
3	99.2 ± 4* (n = 126)	93.7 ± 8** (n = 126)	67.9 ± 8* (n = 474)	54.0 ± 11** (n = 421)
10	100.0 ± 0* (n = 126)	91.3 ± 9** (n = 126)	71.6 ± 9* (n = 461)	58.0 ± 10** (n = 459)

^a Plants were grown on kanamycin media for 6 weeks and then transferred to media without antibiotics prior to adding malonate. Data were collected 6 weeks later. Values are means ± standard deviations, with n = total numbers of plants or roots in all replicates, each having 5 to 10 replicates for a total of 21 replicates. Analysis of variance was determined with the General Linear Model procedure and mean separation with the least significance difference. Pairwise comparisons of treatments within a given measured parameter labeled with different numbers of asterisks (*) are significantly different at $\alpha = 0.05$.

^b Percentage of plants with at least one root longer than 1 cm.

^c YFP = yellow fluorescent protein.

scription in *M. truncatula* and killed transgenic roots. The four smaller hairpin constructions, pHpACC2 to pHpACC5, did not reduce ACCase transcript levels in *Medicago* roots.

***Medicago* roots transgenic for pHpACC inhibit parasitic plant root growth.**

We assayed the capacity of *T. versicolor* to parasitize *Medicago* roots in vitro by aligning the root tips of *Triphysaria* seedlings at right angles to *Medicago* roots growing on agar (Fig. 4). Under these conditions, more than 80% of *Triphysaria* roots developed haustoria within 24 h. The tips of *Triphysaria* roots attached to *Medicago* roots via haustoria were marked every day for 8 days. In all, 93% of *Triphysaria* roots attached to *Medicago* roots transformed with the parent pHG8-YFP vector continued to grow for at least 8 days after attachment (Fig. 5). In contrast, only 17 to 35% of the *Triphysaria* roots

Fig. 3. Transcript levels of MtACCase in transgenic *Medicago* roots. Steady-state transcript levels of acetyl-CoA carboxylase (ACCcase) in *Medicago* roots transformed with various pHpACC constructions were determined by quantitative polymerase chain reaction and normalized to the constitutively expressed gene MtActin. Data are means $\pm$ standard deviations of three technical replicates of three plants ($n = 9$). Expression level in pHG8-yellow fluorescent protein transgenic roots was set to 100%. Note the log-scale y axis.

Fig. 4. Haustorium assay system. In all, 15 to 20 2-week-old *Triphysaria* seedlings are arrayed in close proximity with transgenic *Medicago* roots. Haustoria develop at the junction of the *Triphysaria* and *Medicago* roots.

attached to *Medicago* roots transformed with pHpACC1 to pHpACC5 survived 8 days or more (Fig. 5). The worst host roots were transgenic for pHpACC1 (roots were obtained by growing in media containing malonate and removing the malonate prior to adding *T. versicolor*). Because *Medicago* roots transgenic for pHpACC3 or pHpACC5 also supported significantly less *Triphysaria* root growth than controls without having visible effects on the host, subsequent analyses focused primarily on these two constructions.

Triphysaria roots attached to pHpACCcase roots were stained with fluorescein diacetate (FDA) and propidium iodide (PI) and examined under a microscope to determine viability and cellular morphology. FDA exclusively stains viable cells green while PI stains damaged or dead cells red. Eight days after infection, over 90% of the *Triphysaria* roots that had attached to *Medicago* roots transgenic for pHG8-YFP stained green (Fig. 6). In contrast, when *Triphysaria* roots were attached to pHpACC3 or pHpACC5 roots, most of the roots stained red. Cells in the *Triphysaria* root tip were abnormally large and misshaped (Fig. 6). These phenotypes are consistent with the *Triphysaria* root tips attached to pHpACCcase *Medicago* roots being dead.

To determine the timeline of trans-specific silencing in *T. versicolor*, root tip growth was measured daily for 8 days after attaching to *Medicago* roots bearing pHpACC3 or pHpACC5. *Triphysaria* root growth was unaffected until 4 days after infection, when growth was significantly disrupted (Fig. 7). By 8 days after infection, only 15% of the *Triphysaria* roots attached to pHpACC3 or pHpACC5 *Medicago* roots survived and continued to grow. In contrast, 83% of the *Triphysaria* roots parasitizing pHG8-YFP roots survived (Fig. 7).

A similar assay was performed with 3 mM malonate in the media to determine whether the lethal phenotype could be complemented in the parasite. The percentage of *Triphysaria* roots surviving after 8 days attached to pHpACC3 or pHpACC5 roots was similar to that obtained on roots without HpACCcase (Fig. 8). This indicates that the reduction of *Triphysaria* root growth after attaching to pHpACC3 or pHpACC5 roots is associated with reduced ACCcase activity.

We estimated ACCcase transcript levels in *Triphysaria* roots attached to *Medicago* plants expressing pHpACC3, pHpACC5, or pHG8-YFP 8 days after attachment using limited-cycle PCR. Because high-quality RNA could not be obtained from dying *Triphysaria* roots, the assay was run in media containing

Fig. 5. *Triphysaria* root growth 8 days after infecting transgenic *Medicago* spp. Values are mean $\pm$ standard deviation with total of 90 roots in all nine replicates. Means are the averages of three independent transformation experiments. Data was analyzed using with the General Linear Model procedure and mean separation was done with the least significance difference method (Statistical Analysis Software 9.1; SAS Institute, Cary, NC, U.S.A.). Pairwise comparisons labeled with different lowercase letters are significantly different at $\alpha = 0.05$.

malonate so that RNA could be isolated from living parasite roots. To obtain sufficient amounts of RNA for analysis, 45 to 60 haustoria-forming roots tips were pooled for RNA isolation. Primers specific for the *Triphysaria* ACCase transcript were synthesized and used in PCR reactions with cDNA isolated from *T. versicolor* grown on transgenic *Medicago* roots (Fig. 9). Although ACCase transcripts were readily amplified in *Triphysaria* roots attached to nontransformed *Medicago* roots or transgenic for pHG8-YFP, few or no ACCase transcripts were observed in *T. versicolor* attached to pHpACC3 or pHpACC5 roots. PCR reactions performed using primers specific for TvQR2 or TvACTIN, two genes whose transcripts are not predicted to be influenced by hairpin ACCase, were readily amplified after attachment to transgenic *Medicago* roots. We also ran PCR reactions using primers specific for three *Medicago* genes (MtPSP, MtALPHA, and MtACTIN) to determine whether the *Triphysaria* RNA was contaminated by *Medicago* transcripts, which could happen during the isolation of *Triphysaria* tissues from the assay plates. There was a small amount of *Medicago* actin transcript detected in one of the *Triphysaria* samples, consistent with low levels of contamination of the *Triphysaria* RNA with *Medicago* material.

Direct detection of small RNAs homologous to ACCase in both *Triphysaria* and *Medicago* roots.

We used Northern hybridizations to determine whether small RNAs homologous to the *Triphysaria* ACCase transcripts could be detected in either transgenic *Medicago* roots or in the *Triphysaria* roots attached to them. Small RNA was isolated from nontransformed *Medicago* roots; *M. truncatula* transformed with pHG8-YFP, pHpACC3, or pHpACC5; as well as from *Triphysaria* roots attached to each of the different *Medicago* transgenics.

ACCase-specific RNAs approximately 21 nt in length were identified in *Medicago* roots transformed with the two pHpACC constructs and in the *Triphysaria* roots attached to them. No small RNA bands were identified in pHG8-YFP or nontransformed *Medicago* roots or in the attached *Triphysaria* roots (Fig. 10). Taken together, these data suggest that small RNAs were made in *M. truncatula* and translocated through the haustorium into *T. versicolor* spp. where they silenced ACCase expression, thereby killing *Triphysaria* roots.

DISCUSSION

Trans-specific RNAi targeted against vital parasitic plant genes is a potentially powerful method for genetically limiting the agricultural devastation of parasitic weeds. By cloning multiple hairpin targets in one transformation vector, the meth-

odology provides an opportunity to inactivate different developmental pathways in the parasite simultaneously. For the RNAi strategy to be effective, the target gene must encode a lethal phenotype when transcription is reduced or eliminated

Fig. 7. *Triphysaria* root growth after infecting transgenic *Medicago* host. Percentage of *Triphysaria* roots growing over an 8-day interval after attachment to one of three transgenic *Medicago* roots is shown. Values are mean $\pm$ standard deviation with total of 70 roots in all seven replicates. Means are the averages of three independent transformation experiments. Data were analyzed with General Linear Model procedure using Statistical Analysis Software 9.1 (SAS Institute, Cary, NC, U.S.A.).

Fig. 8. *Triphysaria* root growth complementation with malonate treatment. An aqueous solution of 3.0 mM malonate was added to *Triphysaria* spp. that had attached to transgenic *Medicago* spp. and root growth was monitored. Bars show percentage of roots still growing after 8 days. Values are mean $\pm$ standard deviation with total of 90 roots in all nine replicates. Means are the averages of three independent transformation experiments.

Fig. 6. *Triphysaria* root tips after the attachment of transgenic *Medicago* roots. Viable cells showed green fluorescence due to fluorescein diacetate and nonviable cells bright red due to propidium iodide. Typical patterns from approximately 50 roots observed are shown. Assay was done 8 days after infection. Size bar = 1 mm. A–C, Attached to pHG8-YFP root; D to F, attached to pHpACC3; and G to I, attached to pHpACC5.

in the parasite, it must be specifically lethal to the parasite but not the host, and the silenced phenotype cannot be compensated by host metabolites that are available to the parasite upon infection. By these parameters, ACCase seemed a reasonable candidate target for inactivation.

Hairpin constructions of overlapping ACCase sequences in *T. versicolor* were transformed into *Medicago* roots that were then parasitized by *Triphysaria* roots. Although *Triphysaria* roots successfully parasitized nontransformed *Medicago* roots or *Medicago* roots transformed with an empty parent vector, most of the *Triphysaria* roots died within a week of attaching to *Medicago* spp. transgenic for HpACC. We also detected small RNAs of approximately 20 to 21 nt in *Triphysaria* roots attached to HpACC bearing *Medicago* roots but not the controls. The role of ACCase inhibition was substantiated by recovering *Triphysaria* roots using malonate complementation and by the low level of ACCase transcripts observed in the *Triphysaria* root tips. Our conclusion is that ACCase RNAi made in the host were translocated across the haustorium into *T. versicolor*, in which they inhibited the endogenous ACCase gene, thereby killing the *Triphysaria* root.

Enlargement of the *Triphysaria* root apex and the abnormal cell morphology of the dead *Triphysaria* roots were similar to that observed in *acc1* mutants and may be due to reduced levels of VLCFA in pHpACC roots (Faure et al. 1998; Haberer et al. 2002; Torres-Ruiz et al. 1996). VLCFA are essential regulators of cell differentiation during development because they regulate polar auxin distribution (Roudier et al. 2010).

Several factors, including the mass flow of water, osmotic gradients, and differences in water potential between host and parasite tissues, can drive movement of host resources to the parasite (Hibberd and Jeschke 2001; Robert et al. 1999; Stewart and Press 1990). *T. versicolor* is a xylem feeder and, generally, one to five xylem strands can be seen inside a haustorium, with vessel element connections between host and parasite vasculature (Heide-Jørgensen and Kuijt 1993). Based on microscopic observations, Heide-Jørgensen and Kuijt (1995) proposed a direct translocation mechanism between treachery elements of the host and parasite, with a few cell-to-cell contacts. These authors hypothesized that minerals and organic compounds are transferred directly to the xylem apoplast of the host and then

to the xylem of the haustorium at the xylem bridge. Other nutrients are transferred through the symplast of parasite interface cells. Some compounds can be metabolized and the products moved symplastically in the transfer cells (Heide-Jørgensen and Kuijt 1995). In the transfer of organic compounds such as soluble sugars and carbohydrates, parenchyma cells of the plate xylem may act as a sink and then, by symplastic transport, those compounds could reach the sieve tubes of a parasite root.

Lethality was only observed in root tips a few millimeters distal to the haustorium. Root tissues that developed before or shortly after the haustoria connected with *Medicago* roots, remained alive. These observations are consistent with the silencing of GUS in transgenic *Triphysaria* roots (Tomilov et al. 2008). When GUS-expressing *Triphysaria* roots parasitized lettuce roots bearing an RNAi targeting GUS, there was a reduction in GUS transcript and enzymatic activity several centimeters distal to the haustorial junction. This was interpreted as the RNAi signal requiring time after translocating across the haustorium before parasite genes are silenced.

Another consideration for siRNA design is that silencing needs to be parasite specific. In principle, this can be achieved by selecting RNAi target sequences that are sufficiently distinct from those of the host gene to avoid the host transcripts from being recognized by the silencing complex. For successful post-transcriptional gene silencing in plants, a dsRNA fragment of 300 to 500 bp with at least one stretch of 23 nt with 100% identity to the targeted transgene mRNA was thought to be essential (Thomas et al. 2001). Taking that into consideration, we tried to avoid long stretches where there were perfect homologies between the *Triphysaria* and *Medicago* gene sequences. Nevertheless, as demonstrated by RT-PCR and by the recovery of *Medicago* roots in malonate, the MtACCase gene was silenced.

None of the four smaller RNAi constructs designed against the *Triphysaria* ACCase gene were toxic to *Medicago* roots. Surprisingly, the sequences associated with inhibition of ACCase in *Medicago* roots had less identity with the *Triphysaria* sequence than regions that were not inhibitory. There was a 38-bp region present only in pHpACC1 with only two mismatches between *Triphysaria* and *Medicago* sequences. If these two mismatches are accepted by the RNAi machinery, it provides 38-bp region of identity between the hairpin construction and the *Medicago* gene (Supplementary Fig. S1).

These results agree with the finding that a 21-nt stretch of 100% identity between the heterologous and endogenous gene sequences is not absolutely required for gene silencing

Fig. 9. Gene expression assayed with semi-quantitative polymerase chain reaction (PCR). PCR was run on *Triphysaria* root tips that had attached to transgenic *Medicago* roots expressing pHpACC3, pHpACC5, or pHG8-YFP. Three sets of *Triphysaria* primers were used to detect transcripts of TvACCcase, low-abundance transcript TvQR2, and high-abundance transcript TvActin. Primers for three *Medicago* genes (MtEPSP, MtALPHA, and MtACTIN) were included to determine the degree to which *Medicago* tissues or RNA contaminated the *Triphysaria* preparations. Tv = *Triphysaria versicolor* and Mt = *Medicago truncatula*. Three biological replicates are shown for each plasmid.

Fig. 10. Detection of acetyl-CoA carboxylase (ACCase)-specific small interfering RNA. Small RNA was extracted from *Triphysaria* root tips attached to *Medicago* spp. transgenic for pHpACC3, pHpACC5, or pHG8-YFP or nontransgenic *Medicago* spp. RNA was size fractionated, transferred to a nylon membrane, and probed with negative-sense TvACCcase transcripts labeled with 32 P-UTP. Tv = *Triphysaria versicolor* and Mt = *Medicago truncatula*. Size markers as shown in the first lane.

(Senthil-Kumar et al. 2007). We consider the alternative possibility, that the ACCase RNAi is acting off target and silencing other vital parasite gene, unlikely because of the associated reduction in ACCase transcripts and complementation by malonate (Jackson et al. 2003).

In theory, silencing the *ACC1* gene should not affect the growth of nontransgenic roots. However, when pHpACC1 plants were kept in kanamycin for 6 weeks and transferred to antibiotic-free medium, only approximately 66% of the plants were able to develop roots. In nontransgenic cells that compose part of the callus, the RNAi may prevent them from forming the roots. Because siRNAs move from their site of production through plasmodesmata (Dunoyer et al. 2005), this could be due to cell-to-cell movement of the siRNA signal leading to silencing *ACC1* gene silencing in the callus-like structure from which roots develop.

This work shows that cytosolic ACCase is a potential RNAi target for controlling parasitic weeds. Although we don't yet know whether the hairpin constructions are functional against other Orobanchaceae genera, homology between Orobanchaceae spp. is more extensive than between the family Orobanchaceae and its host *Medicago* spp. (Wickett et al. 2011); therefore, RNAi molecules designed against *Triphysaria* genes would be expected to silence orthologous Orobanchaceae genes. However, silencing ACCase in maize did not confer resistance against *Striga* spp. (de Framond et al. 2007). The lateral haustoria made by *Triphysaria* spp. and the terminal, primary haustoria made by *Striga* spp. are structurally different and originated at different times in Orobanchaceae evolution. *Striga* spp. are more host specific than *Triphysaria* spp. and it's possible that *Striga* haustoria are more restrictive to translocating host siRNAs than those from *Triphysaria* spp. The disparate results in *Triphysaria* and *Striga* spp. may also reflect differences in phenotypes resulting from ACCase silencing in the two parasites. The efficacy of RNAi-mediated silencing of ACCase as a control strategy against parasitic weeds still needs to be determined in the field.

MATERIALS AND METHODS

Seed.

Seed of the hemiparasite *T. versicolor* (Fischer & C. Meyers) were collected from an open pollinated population near Napa, California. Seed of *M. truncatula* ('Jemalong') A17 were provided by D. R. Cook (University of California–Davis).

Hairpin plasmid constructions.

The *A. thaliana* *ACC1* coding sequence (AT1G36160) was used in tBLASTx searches of *Triphysaria* cDNAs at the PlantGDB database. These sequences were obtained from Sanger sequencing of normalized cDNA libraries from parasite roots (Torres et al. 2005). The contig PUT-162b-Triphysaria_pusilla-26270 (1,503 nucleotides [nt]) was identified and predicted to encode a polypeptide with 77% identity to the *Arabidopsis* protein over approximately 1,000 amino acid residues. The *Triphysaria* PUT-162b-26270 sequence was used in homology searches of the nonredundant database at the National Center for Biotechnology Information using BLASTn, resulting in significant hits to genes annotated as ACCase sequences in a number of species. The most similar *M. truncatula* sequence in the Gene Index was a 918-nt contig TC125222 that maps near the 3' end of the ACCase gene (Quackenbush et al. 2001). The *Triphysaria* and *Medicago* sequences were aligned by Clustal X and five PCR primer sets were designed to amplify ACCase sequences with low similarity between *Triphysaria* and *Medicago* spp. (Fig. 1; Supplementary Table S1).

RNA was obtained from roots of *T. versicolor* by grinding in liquid nitrogen and extracting with TRIzol reagent (Invitrogen,

Carlsbad, CA, U.S.A.). RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Each of the five ACCase sequences were amplified using the primer sets described above modified to contain attB recombinase sites and cloned into pDONR221 using the BP-recombinase enzyme, following manufacturer's recommendation (Invitrogen). After confirming the integrity of the constructions by restriction digests and sequencing, the fragments were Gateway recombined into the hairpin transformation vector pHG8-YFP (Bandaranayake et al. 2010; Helliwell and Waterhouse 2005). The five hairpin ACCase vectors—pHpACC1 (481 nt), pHpACC2 (150 nt), pHpACC3 (269 nt), pHpACC4 (250 nt), and pHpACC5 (328 nt)—were confirmed by restriction digestions and transformed into *Agrobacterium rhizogenes* MSU440 by electroporation (Sonti et al. 1995) (Fig. 1).

Medicago root transformation.

Agrobacterium rhizogenes-mediated transformation of *Medicago* roots was performed as described, with some modifications (Boisson-Dernier et al. 2001). *Medicago* seed were treated with concentrated sulfuric acid for 5 min, surface sterilized with 100% commercial bleach for 3 to 4 min, and washed thoroughly with sterile distilled water. Seed were germinated in the dark at room temperature and, 30 to 40 h later, the radicles were cut approximately 3 mm from the tip and inoculated with *Agrobacterium rhizogenes* that had been grown in mannitol-glutamic acid:Luria-Bertani plates (Walkerpeach and Velten 1994) containing 400 μ M acetosyringone. Inoculated seedlings were placed on sugar-free, 0.25 $\times$ Hoagland medium at a density of 6 to 8 plants/plate. The plants were incubated vertically for 7 days in a 16°C growth room and then transferred into fresh 0.25 $\times$ Hoagland plates containing kanamycin at 25 mg/liter. Plants were incubated in a 25°C growth room with a 16-h light period at 150 μ E light intensity. Approximately 2 weeks later, transgenic roots were identified by visualization of YFP fluorescence with a Zeiss Stemi SV11 dissecting microscope equipped with a YFP filter set with excitation HQ500/20, dichroic beam splitter Q515LP, and emission HQ535/30. Non-YFP roots were removed with a scalpel, and seedlings with transgenic roots were transferred onto fresh plates containing sucrose at 7.5 g/liter and the antibiotic timentin (SmithKline Beecham Pharmaceuticals, Philadelphia) at 300 mg/liter. Six weeks after transformation, the percentage of plants with at least one root and the percentage of YFP roots were determined.

Malonate complementation.

Plants lacking ACCase activity can be recovered by complementing the deficiency with malonate (Baud et al. 2004). We used this to recover transgenic *Medicago* roots containing pHpACC1 and to recover *Triphysaria* roots that had attached to *Medicago* roots bearing ACCase-silencing constructions. Six weeks after transformation of *M. truncatula* and selection in kanamycin, plantlets were transferred onto new plates without kanamycin but containing 0, 3, 10, or 100 mM malonate. Every 2 days, 3 ml of either water, 3 mM malonate, 10 mM malonate, or 100 mM malonate (pH 5.3) was applied to each plate. After 6 weeks of malonate treatment, the percentage of plants with at least one root and percentage of YFP roots per plant were determined. *Triphysaria* roots that had attached to *Medicago* roots expressing either pHpACC3 or pHpACC5 were similarly recovered by applying 3 ml of 3 mM malonate to each plate every 2 days.

Haustrorium assays.

Triphysaria seed were surface sterilized in 70% ethanol for 10 min followed by 50% (vol/vol) bleach (sodium hypochlorite, 2.13% [wt/vol] final) and 0.1% (vol/vol) Triton X-100

(Sigma-Aldrich, St. Louis, MO) for 20 min. After thoroughly rinsing in sterile deionized water, seed were plated in agar containing 0.25× Hoagland's medium, vernalized for 2 to 3 days at 4°C, and germinated at 16°C under a 12-h light regime (Jamison and Yoder 2001). In all, 15 to 20 2-week-old *Triphysaria* seedlings were then placed in close proximity to YFP-expressing *Medicago* roots growing on the surface of agar in plates oriented vertically in a culture room at 25°C with a 16-h light regime (150 μ E) (Fig. 4). The growth of *Triphysaria* roots after attaching to transgenic *Medicago* roots was monitored daily by marking the root tips with a pen and measuring daily growth with a light microscope equipped with a measuring reticule. The percentage of *Triphysaria* roots that continue to grow 8 days after attachment was determined. For those transgenics on which a significant portion of *Triphysaria* roots stop growing 3 to 4 days after attachment, the roots were treated with 3 mM malonate to recover sufficient material for RNA extraction. Three batches of *Triphysaria* roots were sequentially infected onto the same *Medicago* root to increase efficiency.

Cell viability assay.

Root tip viability was assayed by staining with FDA (Sigma-Aldrich) and PI (Sigma-Aldrich) (Ruzin 1999). Eight days after attachment, *Triphysaria* roots were stained with FDA at 5 μ g/ml for 3 min followed by PI at 3 μ g/ml for 10 min. The double-stained root tips were observed under a Zeiss Stemi fluorescence microscope equipped with epi-illumination, band pass 450- to 490-nm blue exciter filter, 510-nm chromatic beam splitter, and long-pass 520-nm barrier filter. With this filter combination, both green and red fluorescing cells could be visualized simultaneously and the percentages of living (green) and dead roots (red) determined.

Transcriptional analyses.

For quantitate RT-PCR, total RNA was isolated from *Medicago* roots using the TRIzol reagent (Invitrogen), treated with DNaseI, and further purified using RNeasy Mini Spin columns (Qiagen, Hilden, Germany). RNA (1 μ g) from each sample was converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The reverse-transcription reactions were diluted 20-fold and 2 μ l was used for SYBR green-based quantitative PCR assays with primers shown in Supplementary Table S1 using an ABI 7300 quantitative PCR system (Applied Biosystems, Foster City, CA, U.S.A.). The cycle conditions were 25°C for 2 min for initial activation, 95°C for polymerase activation, followed by 40 cycles of 95°C for 15 s for melting and 60°C for 1 min for primer annealing and extension. Melting curves of PCR products were obtained by gradually heating to 95°C, and only those producing a single melting peak were considered in the analysis. Target gene expression was measured relative to the constitutively expressed gene MtACTIN. The data were analyzed with the SDS Software using the $\Delta\Delta$ Ct (cycle threshold) ratio real-time PCR method (relative quantification), which determines the Δ Ct of the gene of interest and endogenous control, and subtracts this from the analogous measurement obtained from the internal control (the $\Delta\Delta$ Ct).

For semi-quantitative PCR, cDNA was synthesized using the high molecular weight RNA fraction described below. PCR reactions were performed in an MJ Research PTC-200 Peltier PCR machine programmed for an initial hot start at 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. ACCase gene expression was compared by comparing the intensity of PCR product across samples using expression from TvACTIN and TvQR2 genes as loading controls. Three sets of *Medicago*

primers (MtACTIN, MtEPSP, and MtALPHA) were included in the analysis to check the degree of host contamination. This analysis was done using the three biological replicates used for the small RNA Northern blots.

Small RNA Northern blots.

Triphysaria roots were infected onto *Medicago* roots that had been transformed with the empty vector donor plasmid, pHpACC3, or pHpACC5. Seven days later, 45 to 60 *Triphysaria* root tips were harvested from plates supplemented with malonate and flash frozen in liquid nitrogen. Transgenic *Medicago* roots as well as nontransformed *Medicago* and *Triphysaria* roots were similarly flash frozen. Total RNA was extracted from approximately 100 mg of tissue using the Trizol reagent (Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's recommendations but without the final ethanol wash. Small RNAs were enriched by precipitating high molecular weight RNA with 50% polyethylene glycol (PEG 8000) and 5 M NaCl. This high molecular weight fraction was used for semi-quantitative PCR analysis described above.

Approximately 0.5 μ g of small RNA from each sample was separated on a 15% polyacrylamide 8 M urea gel and transferred onto a Hybond-NX+ membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) using a Bio-Rad semidry transfer apparatus. Three biological replicates for each construct were blotted onto three separate membranes. MicroRNA Marker (catalog number N2102S; New England Biolabs, Beverly, MA, U.S.A.) was loaded into each gel as a size standard. The membrane was UV cross-linked (1,200 J and then another 600 J), and was prehybridized for 3 h at 65°C with 10 ml of ULTRA hyb-oligo prehybridization buffer (catalog number AM8663; Ambion, Austin, TX, U.S.A.), 5× Denhardt reagent, 7% sodium dodecyl sulfate, and sheared salmon sperm DNA at 100 μ g/ml.

The pCR8 plasmid containing ACC1 sequence was linearized with restriction endonuclease ApaI and used as template for in vitro transcription reactions using the T7 Maxi Script kit (catalog number AM1320; Ambion) and ³²P-UTP. After cleaning the transcripts with a NucAway (catalog number AM10070; Ambion) column, transcripts were fragmented in a solution of 120 mM Na₂CO₃ and 80 mM NaHCO₃ at 65°C for 30 min. The radiolabeled probe was added directly to the membrane in prehybridization buffer and incubated at 42°C overnight. The membrane was washed twice in NorthernMax Low Stringency Wash Buffer (catalog number AM8673; Ambion) at 42°C for 15 min and drained. Blots were then wrapped in SaranWrap and exposed to X-ray film at -80°C for 120 h.

Statistical analysis.

All the experiments were arranged in a complete randomized design with 5 to 10 technical replicates, considering each square plate as one replicate. Each experiment was repeated at least three times. Analysis of variance was used to analyze data with more than three treatments while the *t* test was performed for experiments with two treatments. Least significance difference was used for mean separation. SAS statistical software (9.2) was used for all analyses.

GenBank accession numbers of the sequences used were AtACC1: AEE31850, TvACC1: JX473245.1, TvQAN8: JX473246.1, MtACC1: XM_003638746, and MtACTIN: XM_003625217.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

PlantGDB database: www.plantgdb.org
 The Gene Index Project webpage: compbio.dfci.harvard.edu/tgi/plant.html
 SAS homepage: www.sas.com/index.html