Engineering host resistance against parasitic weeds with RNA interference

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Abstract

Host genetic resistance is a key component of integrated pest management. The present authors and others are investigating the use of RNA interference (RNAi) as a genetic tool for engineering host resistance against parasitic weeds. The general approach is to transform a host plant with a plasmid encoding a double stranded hairpin RNA (hpRNA) targeted against one or more vital parasite genes. When the hpRNAs are specifically designed against parasite gene sequences, the hpRNA should have no phenotypic effect on the host. They will, however, have a dramatic effect on the parasites that have taken up the parasite-specific RNAi from the host via the haustorium. The current status of using RNAi technology for controlling parasitic weeds is reviewed. A key component to success with RNAi technology is identifying the best parasite genes to silence. Some of the criteria for RNAi targets are discussed, the existing status of parasitic plant sequence databases is described and internet access points to the parasite genome data are highlighted. Sequence information obtained from different parasite species can be used to clone the homologous gene from a particular pest or can be directly transformed into crop plants.

Keywords: Triphysaria; engineered resistance; parasitic plant; genetic resistance

1 TRIPHYSA SPECIES

The family Orobanchaceae has been recently revised to include Striga and other hemiparasitic genera previously grouped with Scrophulariaceae. These genera have been notoriously difficult to group because of the accelerated rates of morphological evolution in parasitic lineages, presumably resulting from the redundancy of photosynthesis in heterotrophic plants. Molecular phylogeny using both plastid and nuclear markers establishes the Orobanchaceae as a single monophyletic clade of parasites whose nearest non-parasitic neighbor is Lindenbergia philippinensis (Cham) Benth. What is particularly interesting about the Orobanchaceae family from an evolutionary point of view is that all trophic levels of plant parasitism, including autotroph, facultative hemiparasite, obligate hemiparasite and holoparasite, are represented by extant species. The single evolutionary event that distinguishes parasitic Orobanchaceae from their autotrophic relatives is their ability to develop root haustoria in response to chemical and tactile signals from host plants.

Triphysaria species were formerly grouped taxonomically within the genus Orthocarpus, the subject of pioneering parasitic plant investigations in the 1960s and early 1970s. Five Triphysaria species were elevated to their own genus in 1991 on the basis of their ability to cross-hybridize. Triphysaria are facultative parasites that grow to maturity without host plants. Their natural habitats are annual grasslands distributed throughout the Pacific Coast from Canada to Baja. The host plants of Triphysaria are typically annuals, so new parasite–host associations need to be re-established every year. Triphysaria is well adapted to a changing host environment because Triphysaria, like other facultative hemiparasites, has a broad host range. Triphysaria can associate with 27 different families of monocots and dicots in the field. In pot cultures and laboratory studies, Triphysaria will infest a large number of hosts, including Arabidopsis, rice, Medicago and maize.

Four of the five Triphysaria species have a gametophytic self-incompatibility system that precludes self-pollination. Triphysaria pusilla is the sole self-pollinator in this group, indicating that a mutation from self-incompatibility to self-compatibility occurred since the elaboration of the clade. Triphysaria flowers are amenable to classical genetic manipulations, and interspecific crosses can be readily made. Unlike Striga and Orobanche, Triphysaria is not a weedy problem in either managed or unmanaged ecosystems, and, in fact, Triphysaria, commonly called Johnny Tuck, is a popular springtime wildflower. The lack of weediness in Triphysaria is an important characteristic for laboratories, like that of the present authors, that are located in agricultural regions without ongoing Striga or Orobanche infestations.

As a facultative hemiparasite, Triphysaria represents the evolutionarily earliest form of plant parasitism, an autotrophic plant capable of developing invasive haustoria. Later mutational events in the evolution of parasitic plants result in an increased specificity and requirement for host resources, as seen with obligate hemiparasites such as Striga, or the complete loss of photosynthesis, as in the achlorotic Orobanche. While Striga and Orobanche are morphologically distinct from Triphysaria, the fundamental genetic mechanisms regulating early parasitic events are likely conserved in all species. The authors predict that the genes con-
2 TRANSLATION OF INFORMATIONAL MACROMOLECULES ACROSS THE HAUSTORIUM

Water, minerals and carbohydrates, which are required by parasitic plants to varying degrees, are translocated across haustoria from host to parasitic plants.13 The earliest evidence of protein and nucleic acid movement across a haustorial boundary was the transmission of viral diseases between two host plants simultaneously parasitized by Cuscuta.14 Movement of the non-viral green fluorescence protein (GFP) into Cuscuta reflexa Roxb. (dodder) was observed following its parasitism of transgenic tobacco plants expressing GFP.15 GFP was also used fluorescently to tag the tobacco mosaic virus movement protein and visualize its transfer into C. reflexa.16 When both a 27 kDa GFP and a 35 kDa GFP–ubiquitin fusion protein were expressed directly in companion cells using the At SUC2 promoter, the smaller GFP protein was translocated from Arabidopsis into Cuscuta, but the larger protein was not, thereby defining a size or conformational restriction for the transfer of this protein.17 The shorter GFP protein could be transferred between two Arabidopsis plants if they were both host to the same dodder.17 Together with the viral studies, these studies demonstrated that the movement of biologically active proteins and nucleic acids across haustoria can be bidirectional, with the parasite both picking up and delivering macromolecules through haustorial connections.

Two recent sets of experiments identified host (tomato or pumpkin) RNA transcripts in Cuscuta pentagona Engelm. after infection on the respective hosts.18 Host transcripts are sufficiently stable and mobile in the parasite to be detected by in situ RT-PCR up to 30 cm from the haustoria, indicating that host transcripts can move long distances in the dodder.19 The transfer of nucleic acids across the haustorial interface can also be inferred from gene phylogeny studies. Nucleic acid movement between host and parasitic plants was implicated by the discordant placement of mitochondrial gene sequences from the parasite Rafflesia into a clade closely related to its host.20 In a second example, the direction of putative horizontal gene transfer was from parasite to host. Three species of Plantago were found to contain a duplication of the mitochondrial gene atp1 that phylogenetically clusters with the atp1 homolog in Cuscuta, a parasite of Plantago.21 So, over an evolutionary timescale there is evidence of nucleic acid movement from host to parasite as well as from parasite to host.

The movement of proteins between host and parasite has been used to develop a toxic protein approach to Orobanche control.22,23 The flesh fly Sarcophaga peregrina (Rob.–Des.) produces an antimicrobial toxin, sarcotoxin IA, that integrates with the bacterial cell membrane, causing loss of its membrane electrochemical potential.24 The protein confers resistance against bacterial and fungal pathogens when transformed into tobacco.25 When the sarcotoxin IA gene was transformed into tomato under the regulation of the root specific tob promoter, there was roughly a 50% reduction in the number of Orobanche aegyptiaca (Pers.) emerging from pots containing transgenic as opposed to non-transgenic tomatoes.22 Interestingly, the authors reported a 3–5-fold increase in fruit yield from the transgenic plants in the presence of Orobanche, suggesting that the sarcotoxin was not significantly toxic to the tomato roots.22 The mechanism by which sarcotoxin preferentially is toxic to Orobanche but not Solanaceae roots is not understood.

3 RNA INTERFERENCE

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 nucleases into short 21–24-nucleotide interfering RNAs (siRNA) or micro-RNAs (miRNAs).26,27 These small RNAs are then incorporated together with proteins into RNA-induced silencing complexes that direct the degradation of endogenous RNAs that are homologous to the siRNAs.

Agrobacterium-based vectors have been developed for delivering siRNA precursors into plants selectively to target endogenous genes for inactivation. These vectors are designed so that the target RNA forms self-complementary hairpin structures (hpRNA) resulting in localized dsRNA regions that are cleaved into siRNA molecules by nucleases.28 Mechanistically, RNAi is similar to virus-induced gene silencing (VIGS), the difference being that in VIGS a recombinant virus is used to deliver the target sequence rather than an hpRNA plasmid.29

The use of RNAi to control plant pests was suggested by the observation that, when the nematode Caenorhabditis elegans Maupas ate bacteria containing hpRNAs targeted against C. elegans genes, the endogenous nematode genes were turned off.30 This led to attempts to silence critical genes in agriculturally significant root-knot nematodes by transforming the crop host with hpRNAs targeted against the nematode genes.31 In planta expression of an hpRNA targeting a conserved root-knot nematode secretory peptide that functions as a ligand for a putative plant transcription factor and stimulates root growth in Arabidopsis resulted in resistance effective against four major root-knot nematode species.32 In another set of experiments, transgenic tobacco plants expressing hpRNAs targeting a transcription factor in Meloidogyne javanica (Treub) Chitwood resulted in a reduction in the transcription factor but no reduction in nematode growth.33

A similar RNAi strategy has been attempted at least once in transgenic maize to generate Striga resistance.34 Hairpin constructs were made that specifically targeted five Striga asiatica genes; two required for fatty acid biosynthesis, one for the synthesis of aromatic amino acids, one for the biosynthesis of adenosine monophosphate and a fifth gene controlling vacuole morphogenesis. These were transformed into maize plants that were subsequently challenged with germinating seeds of Striga asiatica (L.) Kunztle. While the analyses were still ongoing at the time of publication, none of the 11 transgenic maize lines tested was resistant to S. asiatica, although some differences in Striga growth rate were observed.34

4 RNAi SILENCING OF GUS IN TRANSGENIC TRIPHYSDARIA ROOTS

The authors used transgenic Triphysaria plants that were expressing the beta-glucuronidase reporter gene (GUS) in their roots to determine whether transcript levels in the parasite would be affected by expression of an hpGUS in the host. The visual assay for GUS activity provides a higher-resolution analysis of gene activity
in the parasite than could be achieved using the RNAi targets described above.34

Transgenic parasite roots expressing GUS were made using an A. rhizogenes-mediated root transformation system.35 Transgenic Triphysaria roots retain their ability to develop haustoria in response to host factors and to invade host roots.36 GUS-expressing Triphysaria roots were allowed to parasitize lettuce roots, some of which were transgenic for hpGUS while others were non-transgenic controls. The silencing activity of the hpGUS in different transgenic lettuce lines was determined by vacuum-infiltrating a constitutively expressed GUS gene into lettuce leaves expressing hpGUS.37 Lettuce lines that most strongly silenced GUS in the transient assays were used as hosts for Triphysaria. The parasitism assays were set up under aseptic conditions in petri dishes containing agar medium. Three weeks after placing GUS-expressing Triphysaria roots onto hpGUS-expressing lettuce roots, the authors stained the haustoria and attached root tissues for GUS activity using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc). The results described below have recently been published.38

GUS-expressing Triphysaria roots attached to non-transgenic lettuce uniformly stained dark blue. In contrast, when parasite roots were attached to transgenic lettuce expressing hpGUS, reductions in GUS staining were observed in about 50% of Triphysaria roots. It is not known if the non-silenced Triphysaria failed to make functional haustorial connections or if the lack of silencing resulted from a post-connection mechanism. There was considerable variability in the degree of GUS silencing in different host–parasite associations, but in the most striking cases the parasite root that developed after attachment had little or no detectable GUS activity.

GUS transcript levels in Triphysaria roots attached to hpGUS and control lettuce roots were quantified. This analysis indicated a 10–95% reduction (depending on host line) in the steady-state message level of GUS in Triphysaria attached to hpGUS lettuce compared with control lettuce. GUS transcripts were reduced in root tissues both proximal and distal to the haustorium, but GUS activity as assayed by staining was restricted to root tissues distal to haustoria. This apparent anomaly was attributed to the stability of GUS protein in root tissues that developed before haustorium establishment; these would continue to stain for GUS even if GUS transcripts were eliminated. Reduction in active GUS protein is only observed in root tissues made after the RNAi signal has been translocated across the haustorium. These experiments demonstrate that hpRNA constructs engineered into host plants can silence the expression of transgenes in root parasitic plants.

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.27 The extent of cell-to-cell movement is a function of the plasmodesmata aperture; in leaf tissues, siRNAs move approximately 10–15 cells from the initial silenced cells, while in embryos the distance is closer to 25–35 cells.39 Further symplastic movement of siRNAs is fairly conserved among higher plants. In order to find specific sequences that would not inhibit PDS in the host plant, 5′ and 3′ RACE were performed, and about 300–400 bps of upstream non-coding sequences together with some coding frame was cloned into a hairpin vector. Four hpPDS constructions were prepared: two were specific for the 5′ end of Triphysaria PDS and two were specific for 3′ sequences. The hairpin pairs differed in that one was driven by the SUC2 promoter expressed in companion cells,45 and the other by a CAB3 promoter expressed in the nutrient source tissues.46 These four constructions were transformed into Arabidopsis, and families of the resulting F2 generations were grown with Triphysaria and observed for bleaching. No discernable bleaching was detected in either the Arabidopsis host or the Triphysaria parasite.

There are several possible reasons why the PDS silencing may not have been observed in above-ground tissue. While it was clear from examining the roots that Triphysaria infected Arabidopsis, the Arabidopsis roots are so small and delicate that perhaps the connections did not provide sufficient quantities of RNAi signals to elicit silencing in the parasite. The authors are currently screening transgenic tomatoes bearing the same constructs to see if they

The authors investigated the reciprocity of RNAi exchange between host and parasite by using non-transgenic parasites simultaneously to bridge a lettuce plant expressing hpGUS and a second host, either lettuce or Arabidopsis, expressing GUS. In about one-half of the double-junction associations, GUS staining was reduced or eliminated in the second root near the site of haustorial attachment. In contrast, in control single-junction experiments where Triphysaria parasitized only GUS-expressing lettuce roots, the host root stained dark blue and there was no evidence of GUS silencing. The results of these experiments indicate that RNAi signals are translocated across haustorial junctions in both directions, which can result in the exchange of RNAi signals between different, non-parasitic plant families.

5 ATTEMPTS TO SILENCE AN ENDOGENOUS PARASITE GENE BY RNAI

One of the ancient functions still served by RNAi in plants is as a defense against pathogenic viruses that have a double-stranded RNA growth phase.41 A highly expressed transgene in a plant may be differentially susceptible to silencing by RNAi than an endogenous, natural gene. Therefore, the authors next targeted an endogenous gene, again one whose inactivation resulted in a visual phenotype. Phytoene desaturase (PDS) is an enzyme on the β-carotene pathway that catalyzes the desaturation of phytoene to zeta-carotene. PDS is localized in the plastid but encoded by nuclear genes. Plant tissues deficient in PDS do not make carotenoids, which normally protect the plant against photobleaching. Plant tissues that lack PDS bleach white under high light, a useful marker for monitoring RNAi movement in living plants.42 Also, while the GUS silencing showed that an RNAi signal translocated bidirectionally across the haustorium, it was of interest to ascertain whether a silencing signal delivered by the host would move systemically in the parasite or remain localized to the root. RNAi signals can enter the phloem and spread systemically throughout a plant, even across graft junctions from transgenic stocks to non-transgenic scions.43,44

In collaboration with John Bowman (University of Monash) and Elsa Tretter (UC Davis), the authors identified a Triphysaria PDS-encoding cDNA by searching the Triphysaria databases described below. The Triphysaria PDS sequence has an open reading frame fairly conserved among higher plants. In order to find Triphysaria specific sequences that would not inhibit PDS in the host plant, 5′ and 3′ RACE were preformed, and about 300–400 bps of upstream non-coding sequences together with some coding frame was cloned into a hairpin vector. Four hpPDS constructions were prepared: two were specific for the 5′ end of Triphysaria PDS and two were specific for 3′ sequences. The hairpin pairs differed in that one was driven by the SUC2 promoter expressed in companion cells, and the other by a CAB3 promoter expressed in the nutrient source tissues. These four constructions were transformed into Arabidopsis, and families of the resulting F2 generations were grown with Triphysaria and observed for bleaching. No discernable bleaching was detected in either the Arabidopsis host or the Triphysaria parasite.

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serve as a more robust host source. Another possibility is that the PDS siRNA signal is translocated to Triphysaria roots but does not move into the phloem for translocation to the above-ground parts. By expressing the hpPDS from the SUC2 promoter, it is possible to ensure that the signal gets into the host phloem, but it may not reach the parasite phloem as required to be translocated longer distances. Alternatively, PDS activity in Triphysaria tissues might not be fully suppressed, so that a bleaching phenotype is not observed. A potentially significant difference between the two systems is that the GUS is a transgene while PDS and the Striga targets in the de Framond study are endogenous genes. The question of whether transgenes are preferentially regulated by host RNAi as opposed to endogenous parasite genes is still unresolved.

Several criteria for good siRNA targets have been discussed at the meeting. The target gene needs to encode a lethal phenotype when transcription is reduced or eliminated by RNAi. Genes that encode protein target sites for herbicides are good first candidates. The disruption of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity by the herbicide glyphosate rapidly inactivates amino acid biosynthesis and kills Striga and Orobancheaceae. The Striga EPSPS gene was targeted in experiments by de Framond and colleagues and remains a worthwhile target. The same group selected siRNA constructs targeted against Striga genes predicted to encode acetyl-CoA carboxylase, enoyl-ACP reductase and adenylo-succinate synthase, three biosynthetic proteins of primary plant metabolism. There are many more biosynthetic proteins that result in lethality when inactivated than have been identified with herbicides. Genes that alter normal cell growth and development are also good candidates for RNAi. Several mutations are described in Arabidopsis that are lethal in embryos, and many of these could be effective RNAi toxins. The high-throughput root transformation transformation described for Medicago truncatula Gaertn. may be a good approach for identifying lethal RNAi targets against parasitic plants.

A related consideration is the selection of parasitic tissue to target. Host tomato mRNAs can be found in dodder plants at a considerable distance from the haustorium, suggesting transport through the phloem. However, the number of host transcripts in dodder decreases by three or four orders of magnitude within 10 cm of the haustorium, indicating a rapid decrease in transcript abundance with systemic movement. RNAi silencing of GUS in Triphysaria roots is also consistent with system movement of the silencing molecule without amplification in the parasite phloem. It seems reasonable to target parasite tissue very near or at the haustorium. By expressing the RNAi from a root-specific promoter, the silencing molecules would be made in the host tissue invaded by the haustorium. Good targets include parasite genes that are expressed at the interface between host and parasite tissues and are essential for functional haustoria to mature. The Parasitic Plant Genome Project described below is using laser microdissection to isolate mRNAs expressed at the interface, and these can be developed into RNAi constructions.

A further consideration for siRNA design is that the silencing should occur in the parasite but not the host. This can in principle be achieved by making the siRNA target sequences sufficiently distinct from those of the host gene that the host transcript is not recognized by the silencing complex. Gene silencing in plants occurs optimally when the size of the double-stranded hairpin formed by the inverted repeat of target sequences is 300–800 bps. Because siRNAs are 21–24 nucleotides long, there should be no similarly sized stretches of perfect homology between the target and the host gene sequences. If the genes are well conserved between host and parasite, it may be possible to find use sequences from 5′ or 3′ untranslated regions as siRNA targets, since these are generally less conserved than those encoding open reading frames.

## 6 GENOME RESOURCES FOR ROOT PARASITIC PLANTS

There are several cases of hpRNA constructs made against a target gene in one species being active in silencing the homolog in a second. One of the most striking examples is the VIGS silencing of the Nicotiana benthamiana Domin. PDS by a sequence homologous to the monocot Lilium longiflorum Thumb. PDS gene. A drought-induced gene from peanut (Arachis hypogaea L) led successfully silenced the homolog in tomato. Success with the RNAi resistance strategy requires an efficient means to identify parasite sequences in order to make highly specific hpRNAs that will debilitate the parasitic plant without altering growth or yield of the host. The first step in identifying gene sequences for hpRNA cloning is to search public databases for sequences predicted to be vital for parasite survival. There is a current effort funded by the US National Science Foundation (NSF) to develop just such a sequence database for Striga, Orobanche and Triphysaria (The Parasitic Plant Genome Project, J Westwood, C DePamphilis, M Timko and J Yoder). In a related project, over 100,000 ESTs from Triphysaria have been sequenced by the Joint Genome Institute of the US Department of Energy (http://www.jgi.doe.gov/) (Lucas S, Rokhsar D, Wang M, Lindquist EA, Tomilov A, Tomilova N et al, unpublished). The authors will give a brief review here of the status of the Triphysaria EST project, provide information on accessing the data and make suggestions on how the data can be useful for engineering Striga resistance.

Expressed sequence tags (ESTs) are partial, single-pass sequences from different regions of a cDNA clone. The cDNA is obtained from RNA transcripts that are present in the sampled tissue during the time of RNA isolation. ESTs can be used to identify homologous genes in other plant or animal species, with the expectation that general functions can be discerned by comparing conserved regions of the translated protein. Many ESTs can be generated from one gene, and, when their sequences overlap or align with a reference cDNA, they are aligned and assembled into tentative consensus (TC) sequences. The TC is a consensus of all overlapping EST sequences and in many cases represents full-length transcripts. Singletons are ESTs that went through the assembly process but did not meet the criteria to be assembled with other ESTs and hence are not contained in a TC.

The authors have sequenced transcripts from two species of Triphysaria: T. pusilla Benth. and T. versicolor Frisch & C.F.Mey. Triphysaria pusilla is a small (5–20 cm), decumbent, finely spreading, self-pollinating species with tiny purple cleistogamous flowers. Triphysaria versicolor is an erect, 10–60 cm, self-incompatible species with bright-yellow flowers that can be hybridized with T. pusilla pollen. The authors are mapping traits for haustorium development in the F3 generation of a cross between T. pusilla and T. versicolor, and sequencing of both species facilitates detection of polymorphisms. Because ESTs are electronically tagged as to their library source, ESTs from one or more species can be assembled into a TC without losing information about transcription.

The primary goal of this project is to identify as many different transcripts as possible that are expressed in Triphysaria roots.
during early haustorium development. The first set of RNA libraries was isolated from pools of *T. pusilla* roots that had been exposed for up to 5 h to host (*Arabidopsis*) roots, host root exudates, chemical haustorium-inducing factors and growth medium. A cDNA library was made and enriched for full-length transcripts using the BD Biosciences SMART technology. Full-length sequences are important for defining regulatory regions on haustorium genes and for determining protein function by expression in heterologous systems. They are also useful for homology comparisons between other parasitic and non-parasitic plants to identify swapped or frequently mutated domains. The cDNAs were normalized for transcript abundance to improve the frequency of novel gene recovery. The library was constructed in the pDNR-LIB vector, which allows simple transfer of sequenced insertions into Cre-Lox-based acceptor vectors (BD Biosciences). This is called the *T. pusilla* full-length library.

Additional cDNA libraries were made that were enriched by suppressive subtractive hybridization (SSH) for transcripts regulated in *T. versicolor* by haustorium-inducing treatments. This PCR-based protocol includes cDNA–cDNA hybridizations in combination with suppression PCR to enrich for differentially expressed transcripts and simultaneously normalize for transcript abundance. "Forward-subtracted" libraries are enriched by this procedure for transcripts upregulated by the treatment. "Reverse-subtracted" libraries are enriched for downregulated transcripts. cDNAs from SSH were cloned into pCR8/GW/TOPO TA, which allows rapid subcloning into Gateway-compatible vectors (Invitrogen, Carlsbad, CA).

Forward- and reverse-subtracted SSH libraries were prepared for *Triphysaria* roots exposed to five haustorium-inducing treatments. The treatment most closely mimicking a natural interaction was to overlay roots of aseptic *Arabidopsis* across those of *Triphysaria* growing in a square petri dish on the agar surface. Haustoria connecting the two hosts could be detected within 24 h of host–parasite contact, while *Triphysaria* exposed to medium without *Arabidopsis* did not develop haustoria. *Triphysaria* roots were dissected and frozen in liquid nitrogen at times ranging from immediately after host root contact to 5 h later. Untreated samples were collected from *Triphysaria* roots exposed to media but not *Arabidopsis*. The library enriched for transcripts upregulated in response to host root contact is called the host forward (HF) library, while the library enriched for transcripts downregulated following host contact is called host reverse (HR) library.

Four other sets of forward and reverse libraries, enriched for genes upregulated or downregulated respectively, were made under other haustorium-inducing treatments. One treatment was exposure to host root exudates, a 1000-fold concentrate of the water and exudates that leach from the bottom of the growth pot when the autotrophically grown plants are watered. Another treatment used *Triphysaria* root extracts because the authors had observed that, while *Triphysaria* root exudates do not induce haustoria, extractions of those same roots do. Two SSH libraries were made from roots treated with either peonidin or DMBQ (2,6-dimethoxybenzoquinone). Peonidin is an anthocyanidin that induces haustorium development at concentrations between 1 and 1000 µM. Peonidin is an antioxidant and is not phytotoxic to roots even at high concentrations. In contrast, DMBQ is an active factor between 1 and 50 µM, but at higher concentrations it is phytotoxic. About 60 000 cDNA clones from the *T. pusilla* full transcript and *T. versicolor* SSH libraries were bidirectionally sequenced, and, after base call, trimming, masking and removal of sequences of less than 100 bps, a total of 107 968 EST sequences were obtained.

Over 90 000 of the EST sequences assembled into 17 442 TCs, about a third of which were over 1 kb. There were also 17 043 singleton sequences that did not align with any other sequences in the libraries.

7 ACCESSIBILITY TO SEQUENCE DATABASES

The primary archive for sequence data is GenBank, an annotated collection of all publicly available DNA sequences. The *Triphysaria* sequences are deposited with GenBank and can be retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) by setting the nucleotide search filter for "Triphysaria". All *Triphysaria* sequences can be downloaded from NCBI.

There are some more specialized databases that provide alternative access points and analysis tools to the parasite sequence data. The Gene Index Project provides an analysis of EST sequence data to identify and annotate transcripts from all eukaryotic sources http://compbio.dfci.harvard.edu/tgi/plant.html. The wide range of organisms in this database makes it a useful source for comparing parasite genomes with highly divergent organisms, such as fungi and protists. The site allows web access to the TC and ESTs sequences, which can be easily downloaded. The Gene Index provides a keyword search of predicted functions and direct links of the TC assembles to Gene Ontogeny, a shared cross-kingdom gene nomenclature, and a visualization of the putative functions on a metabolic pathway map.

Another entry to the parasite data can be found at the Plant Genome Database http://www.plantgdb.org/. In addition to EST and TC access, this site provides tools and datasets for making comparison between different plant genomes. The tools on this site may be useful, for example, for identifying genes or gene expression patterns that are specific for parasitic plants.

8 CONCLUSIONS

One defensive strategy against herbivorous insect pests has been to transform crop plants with genes that encode insecticidal proteins specifically toxic to the insect. The limitation to applying a similar strategy against *Striga* is the lack of toxic proteins that differentially target *Striga* rather than the host. RNA interference (RNAi) uses a short gene sequence as a species-specific toxin that, like insecticidal proteins, is delivered to the pest as part of its meal. Early work shows that RNAi signals can be relayed across haustoria and retain functionality in parasites. It remains to be shown that critical parasite genes can be turned off by delivering RNAi through the haustorium. The apparent success of RNAi approaches in controlling root-knot nematodes provides support further to explore its utility against parasitic plants.

One of the recurring problems with host genetic resistance against pests is the inevitable evolution of a pest that overcomes the host resistance. For this reason it may be desirable to include multiple hRNA molecules on a single transformation vector so that multiple parasite genes, preferably in distinct pathways, are targeted simultaneously. The simultaneous silencing of multiple loci is technically feasible by making chimeric constructions, and there are many potential parasite genes to target. Resistant pests will evolve with this technology if they become mutated in a factor effecting all RNAi recognition or response, perhaps a mutation in dicer or Argonaut protein recognition. However the antiquity and
conservation of RNAi mechanisms across kingdoms makes it likely to be a difficult pathway for a pest to overcome.

Genomic resources are being developed for representative species of Orobanchaceae in order to facilitate RNAi and other biotechnological approaches to parasitic weed management. The existing database of Triphysaria EST sequences should prove valuable for identifying vital parasite genes to target via RNAi. Alternatively, a Triphysaria gene may be used directly in crops. There are several cases of hpRNA constructs made against a target gene in one species being active in silencing the homolog in a second.51 One of the most striking examples is the VIGS silencing of the Nicotiana benthamiana PDS by a sequence homologous to the monocot Lilium longiflorum PDS gene.52 It seems likely that parasite-associated genes will be sufficiently conserved in Orobanchaceae to allow the same hpRNA construction to be used against multiple species. In short, RNAi provides an alternative genetic tool in the ongoing task of developing pest-resistant crops.

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