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The Parasitic Plant Genome Project: New Tools for Understanding the Biology of *Orobanche* and *Striga*

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The Parasitic Plant Genome Project has sequenced transcripts from three parasitic species and a nonparasitic relative in the Orobanchaceae with the goal of understanding genetic changes associated with parasitism. The species studied span the trophic spectrum from free-living nonparasite to obligate holoparasite. Parasitic species used were *Triphysaria versicolor*, a photosynthetically competent species that opportunistically parasitizes roots of neighboring plants; *Striga hermonthica*, a hemiparasite that has an obligate need for a host; and *Orobanche aegyptiaca*, a holoparasite with absolute nutritional dependence on a host. *Lindenbergia philippensis* represents the closest nonparasite sister group to the parasitic Orobanchaceae and was included for comparative purposes. Tissues for transcriptome sequencing from each plant were gathered to identify expressed genes for key life stages from seed conditioning through anthesis. Two of the species studied, *S. hermonthica* and *O. aegyptiaca*, are economically important weeds and the data generated by this project are expected to aid in research and control of these species and their relatives. The sequences generated through this project will provide an abundant resource of molecular markers for understanding population dynamics, as well as provide insight into the biology of parasitism and advance progress toward understanding parasite virulence and host resistance mechanisms. In addition, the sequences provide important information on target sites for herbicide action or other novel control strategies such as trans-specific gene silencing.

Nomenclature: Egyptian broomrape, *Orobanche aegyptiaca* (Pers.) (Syn. *Phelipanche aegyptiaca*) ORAAE; *Lindenbergia philippensis* (Cham. & Schltdl.) Benth. LINPH; yellowbeak owl's-clover, *Triphysaria versicolor* (Fisch. & C.A. Mey) TRVEV; purple witchweed, *Striga hermonthica*, (Del.) Benth. STRHE.

Key words: EST sequencing, gene expression, haustorium, parasitic plant evolution, parasitic weeds, RNA-seq, transcriptome sequencing, weed genomics.

Parasitic plants of the Orobanchaceae are among the most devastating agricultural weeds. Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are especially destructive and affect large areas of the globe, including many developing nations, where they have potential to greatly decrease yield and quality of host crops. At present, over 50 million ha of the arable farmland under cultivation with cereals and legumes in sub-Saharan Africa are infested with one or more *Striga* species, resulting in annual yield losses that are estimated to exceed US$10 billion (Ejeta 2007; Scholes and Press 2008). The impact of *Orobanche* parasitism on yields typically ranges from 20 to 100% crop loss, depending on the infestation (Barker et al. 1996; Bernhard et al. 1998; Parker and Riches 1993; Sauerborn 1991). Although accurate numbers on infested acreage and yield losses are difficult to obtain, it is evident that despite efforts aimed at their control, both witchweed and broomrape persist and are expanding their negative impact on crop productivity (Parker 2009). As documented elsewhere in these symposium proceedings, witchweeds and broomrapes have spread well beyond their centers of origin to infest a large swath of the world's tropical and subtropical regions. Isolated infestations have occurred in the United States (Eplee 1992; Frost and Musselman 1980), and although further expansion of territory has been limited, these species remain a constant threat to agriculture.

The difficulty in controlling parasitic weeds is due in large part to the highly specialized life cycle of parasitic plants. The underground location of the parasites, their physical attachment to host roots, and their synchrony of growth with the host complicates control by conventional mechanical or chemical approaches (Goldwasser and Kleifeld 2004; Hearne 2009). The seeds may lie dormant in the soil for years, only germinating in the presence of potential host species that exude specific chemical signals. The parasite seedling must then attach to the host and form vascular connections to access water and other resources required for growth. Each of these steps involves exquisite communication between parasite and host that represent both fascinating biological adaptations and potential points of weakness that can be targeted for parasite control. However, understanding of parasite development and mechanisms of host interaction has been constrained by a lack of parasite gene sequence information that has lagged behind that of model and crop species (Torres et al. 2005; Westwood 2001). To fill this gap, the Parasitic Plant Genome Project (PPGP) was initiated with the goal of discovering as much of the transcribed gene sets as possible from three parasitic species of the Orobanchaceae, including one representative each from the witchweed and broomrape groups. In this paper, the design of the PPGP, its major outputs, and the potential of the emerging transcriptomic data to contribute to control of parasitic weeds is discussed.

Rationale and Approach of the PPGP

Selection of the Focal Species. An important consideration at the start of the PPGP was selecting the specific species for

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study. In addition to the strong economic justification for studying witchweeds and broomrapes, parasites of the Orobanchaceae are excellent subjects for study because this family is unique among parasitic plant lineages in containing genera that span the full spectrum of host dependence from free-living autotrophs to obligate holoparasites (Westwood et al. 2010). This creates a comparative framework for evolutionary analysis that provides insight into mechanisms underlying the unique features of parasitism. In addition, the species chosen also have been relatively well studied and methods for their specific manipulation under controlled laboratory conditions have been developed.

The broomrapes represent the evolutionary extreme of heterotrophy, being holoparasites that lack expanded leaves and photosynthetic capacity and therefore are completely host dependent. For the broomrapes, important weedy species considered were *Orobanche crenata* (Forsk.), *O. cumana* (Wallr.), *O. minor* (Sm.), *O. aegyptiaca*, and *O. ramosa* (L.), which attack many important dicotyledonous crop plants including food legumes in North Africa, Europe, the Middle East and West Asia. *Orobanche aegyptiaca* was ultimately selected because of its broad host range that includes the model species tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), and *Arabidopsis thaliana* (L.) Heynh. (Goldwasser et al. 2002; Westwood 2000), and a large body of scientific literature. Parasite species that can be cultivated on host plants with sequenced genomes and transformation systems will speed experimental studies of host–parasite interactions, and have been the focus of our work.

The witchweeds are similar to broomrapes in being host dependent at the early stages of parasite development. Once germinated and attached to a suitable host, the developing seedlings rapidly emerge from belowground, accumulate chlorophylls, and produce leaves capable of photosynthesis. Witchweeds [*S. hermonthica*, *S. aspera* (Willd.) Benth., and *S. asiatica* (L.) Kuntze] occur mainly in sub-Saharan Africa, India, and Southeast Asia where they affect primarily cereal grass crops maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench ssp. *bicolor*], rice (*Oryza sativa* L.), and millets (*Pennisetum* spp.). Another important species, *S. gesnerioides* (Willd.) Vatke, attacks primarily leguminous crops (Mohamed et al. 2001). Although autogamous species are attractive for genomic studies because the use of inbred lines facilitates gene and genome assembly, we selected the outcrossing species *S. hermonthica* because of its greater economic impact in world agriculture. Host species used were maize and sorghum, both of which have sequenced nuclear genomes (Paterson et al. 2009; Schnable et al. 2009).

Triphysaria is a genus of five hemiparasitic species common in grasslands throughout the Pacific Coast (Chuang and Heckard 1991; Hickman 1993). *Triphysaria* was included because it is a facultative hemiparasite, able to live without a host if necessary, but possessing the specialized machinery that enables it to opportunistically parasitize roots of neighboring plants. Like some other hemiparasites, *Triphysaria* is a generalist with a very broad host range including monocots and dicots, facilitating comparisons between this plant and *Striga* and *Orobanche* growing on the same hosts. *Triphysaria versicolor,* an outcrossing species, was used in this project.

For comparison we included two nonparasitic relatives as outgroups. One is the basal Orobanchaceae species *Lindenbergia philippensis*, the other is *Mimulus guttatus* (Monkeyflower). *Mimulus* is a genus of traditional Scrophulariaceae (now Phrymaceae) that includes about 160 species with worldwide distribution (Olmstead et al. 2001). *Mimulus* is a popular genus for ecological investigations since it has a wide distribution and numerous genetic resources already available (Hall and Willis 2005), including a sequenced genome (Wu et al. 2008).

Tissue Collection Strategy. The sequencing strategy was based on next-generation sequencing of tissue-specific and whole plant transcriptomes to discover the largest set of expressed sequences for important protein coding genes. This is the most cost-effective approach for discovery and characterization of most expressed genes in plants (Wall et al. 2009). The relatively large size of the parasite genomes (Westwood et al. 2010) makes full genomic DNA sequence, assembly, and annotation a challenging task (Imelfort and Edwards 2009) but recent advances in genome sequencing and de novo assembly are making this an attainable goal. For transcriptome sequencing, messenger RNA (mRNA) was isolated and converted to complementary DNA for sequencing as described in Wickett et al. (2011).

To capture the broadest possible set of transcripts that represent the full range of gene expression in the focal species, it was necessary to include many different tissues and developmental stages. The project strategy was to concentrate most effort on collecting stages involving haustorium development and function because the haustorium is the unique organ that defines parasitic plants, and because the haustorium is a likely target for approaches to interfere with the parasite–host interaction. However, tissues from additional life stages were also included to complete the coverage of gene expression, providing valuable points of comparison to haustorial stages and because parasitic plants exhibit specialized metabolism, signaling, and development at multiple points in their life cycle (Westwood et al. 2010; Yoder 2001). Life cycles of *Triphysaria*, *Striga*, and *Orobanche* were divided into stages on the basis of the biology of parasitism that were comparable among the three species (Figure 1). Although these stages may appear to be quite specific, it is important to recognize that each stage was in turn comprised of pools of cells from different tissues at different developmental substages, again with the rationale of sequencing the maximum number of expressed genes.

The unique goal and strategy of the PPGP led to the development of a novel system for categorizing stages (Figure 1), and understanding stage composition is essential for interpreting results from the project. Stage 0 includes seeds that have been imbibed for various lengths of time, covering the period of conditioning as well as seeds that have been stimulated to germinate by treatment with a germination stimulant, GR-24 (Mangnus et al. 1992) for up to 6 h. For *S. hermonthica* and *O. aegyptiaca*, this is a key step in development because it is tied to perception of a stimulant exuded by a host root (Joel et al. 1995; Yoneyama et al. 2008). For *T. versicolor*, which does not require a germination stimulant, seeds were imbibed but not germinated. Therefore, stage 0 contains expressed sequence tags important in preparing for—and responding to—germination triggers.

Stages 1 and 2 represent radicle growth before and after the addition of a haustorial inducing factor and so encompasses the transition from nonparasitic root growth to haustorium formation. Stage 1 consists of noninduced roots for *T. versicolor* and germinated seeds with radicles for *S. hermonthica* and *O. aegyptiaca*. Haustorial growth was induced in

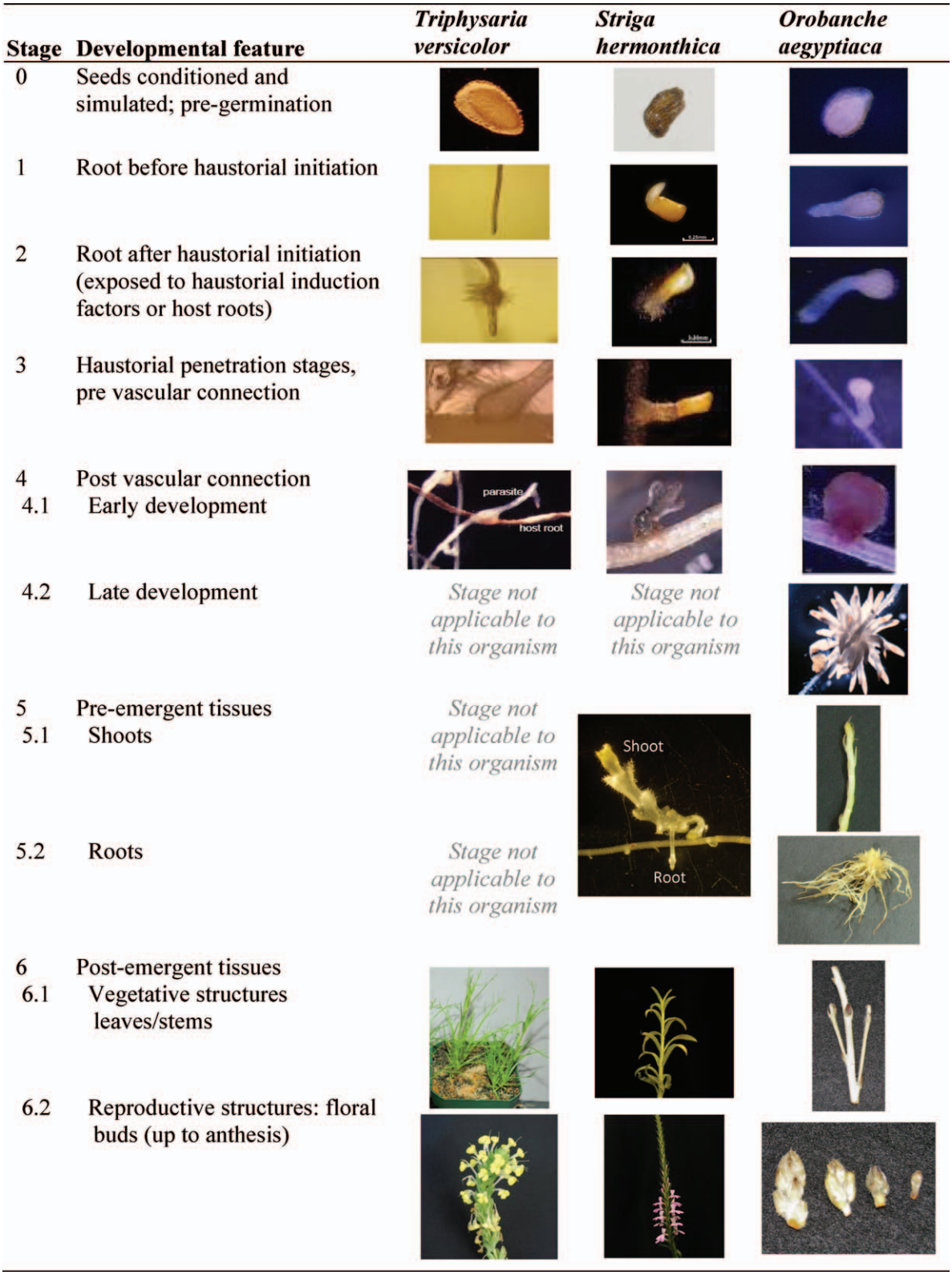

Figure 1. Comparative illustration of the key life stages targeted for sequencing in the three parasitic species, *Triphysaria versicolor*, *Striga hermonthica*, and *Orobanche aegyptiaca*.

vitro by addition of 2,6-dimethoxybenzoquinone (DMBQ) for *T. versicolor* and *S. hermonthica*, as these species are well known to respond to this chemical by initiating formation of a morphologically distinctive haustorium (Yoder 2001). *O. aegyptiaca*, on the other hand, does not develop the swollen, hairy haustorium typical of the other species, and the sensitivity of *Orobanche* to DMBQ is unclear. To ensure inclusion of stages encompassing haustorium formation, *O. aegyptiaca* seedlings (stage 1) were placed for 6, 12, and 24 h on roots of aseptically grown *Arabidopsis*. The resulting

haustorial-induced *O. aegyptiaca* seedlings were collected by rinsing them from the *Arabidopsis* roots with water before they had firmly adhered to or penetrated the host roots.

Stages 3 and 4 cover the period of development related to haustorial penetration and maturation. Stage 3 parasites had firmly adhered to the host root and were in various stages of invasion, but had not formed a vascular connection to the host. In *S. hermonthica*, the hallmark of a successful connection is the unfolding of the cotyledons (Hood et al. 1998), and this was used as a marker for stage 4. For *O. aegyptiaca*, the indicator of vascular connection was the initiation of swelling of the tubercle, and this occurred approximately 8 to 10 d after addition of GR-24. Stage 4 in *O. aegyptiaca* was subdivided to capture the complexity of development in this parasite, with stage 4.1 representing early growth and typified by expansion of the tubercle before development of secondary roots. The transition to stage 4.2 occurs at about 12 d after the addition of GR-24 and is marked by the development of roots that lead this stage to be termed the ''spider'' stage (Fig. 1, *O. aegyptiaca* stage 4.2). This subdivision of stage 4 distinguishes gene expression in the tubercle body itself from that of root growth that involves processes aimed at forming new host attachments via secondary roots (Westwood et al. 2010).

Stage 5 represents underground growth and thus the continued fully heterotrophic dependence of the parasite on the host. For *S. hermonthica*, this phase includes shoot growth before emergence from the soil, and although *S. hermonthica* at this stage consists mostly of shoot, some roots develop adventitiously. Shoots and roots were collected separately and are represented by the subdivision of this stage into 5.1 (shoots) and 5.2 (roots). For *O. aegyptiaca* stage 5 is characterized by a mass of secondary roots arising from the tubercle (5.2) and a floral shoot (5.1). No equivalent to stage 5 exists for *T. versicolor* because once the haustorial connection is established, the root tip resumes normal growth and the process of detecting a new host root, developing a haustorium, and connecting to the host is repeated. The parasite in this case never experiences a belowground phase of complete host dependence.

Stage 6 encompasses all aboveground growth. Although emerged tissues lack the unique morphology of the haustorium, they provide a valuable perspective on parasite evolution through comparisons of photosynthesis-related processes that differ dramatically among the three parasite species. Specifically, whereas *T. versicolor* and *S. hermonthica* have apparently normal leaves, those of *O. aegyptiaca* exist only as reduced scale structures. These vegetative stems and leaves were designated substage 6.1 (Figure 1). The one feature in which the parasite species would not be expected to show great differences is in genes specific to floral biology, so we sampled reproductive tissues represented by buds from the time of first appearance through anthesis (stage 6.2).

All of the stages described above were sequenced from nonnormalized libraries so that it would be possible to obtain quantitative information on mRNA abundance through read counts. In addition, aliquots of each developmentally specific stage were pooled and normalized libraries were made for each parasite species. A similar normalized library of *L. philippensis* contained RNA from seeds, roots, stems, leaves, and flowers. Normalized libraries are an efficient method to obtain low-copy-number transcripts (Wall et al. 2009; Zhulidov et al. 2004) and help to provide more consistent de novo assembly across the entire transcriptome.

Transcriptome sequencing was conducted using a combination of 454 and Illumina technologies (Zhang et al. 2011). Sequencing methods have been undergoing very rapid technological evolution and the PPGP was a beneficiary of these advances. Initial plans to use traditional Sanger sequencing were replaced by 454 FLX and later by Illumina. The sequence yield (Table 1) reflects the relative efficiencies of the methods. Initial 454 FLX half-plate runs produced about 50 MB of sequence data, and subsequent use of 454 titanium increased this output to about 150 MB per run. Illumina sequencing was the most cost-effective method and generated 2,500 to 4,000 MB in a single lane. Because of these advances and associated cost savings, the PPGP was able to conduct replicate sequencing runs on most tissues and expand the number of tissues sequenced beyond those included in the original plan.

Transcriptome Assembly. The greatest challenge created by the shift to Illumina sequencing was assembling contigs efficiently and accurately for organisms that lack reference genomes. We used two approaches to assemble the ultrahigh-throughput Illumina RNA seq reads collected for this project, both described in Wickett et al. (2011). Briefly, the first approach used the CLC assembly cell (CLC bio, 10 Rogers St # 101, Cambridge, MA 02142) to perform assemblies of a single lane of Illumina sequence data, as well as to assemble multiple libraries in a single step. A second approach used the NextGENe (SoftGenetics, LLC., 100 Oakwood Ave, Suite 350, State College, PA 16803) platform, where multiple iterations of the consolidation algorithm, followed by a maximum overlap assembly, assembled the most highly represented genes in the library. The raw reads were then mapped back to the assembled contigs, followed by the removal of the matched reads from the read pool. The consolidation step was run again using the unmatched reads, assembling the next most highly represented genes in the read pool. This cycle of skimming off classes of highly represented genes from the read pool was repeated until the consolidation step produced no contigs above a specific length threshold (200 base pairs).

We have used, among others, a measure of assembly quality based on the capture and coverage of a set of putatively conserved nuclear single copy genes (Duarte et al. 2010). From the PlantTribes 2.0 10-genome scaffold (http://fgp.bio.psu.edu/tribedb/10_genomes/index.pl, see also Wall et al. 2008), we identified 285 orthogroups (orthogroups approximate orthologous gene clusters) that contain one member from each of seven sequenced angiosperm genomes (*Arabidopsis*, *Carica papaya* (L.), *Medicago truncatula* (Gaertn.), *Oryza sativa* (L.), *Populus trichocarpa* (Torr. & Gray), *Sorghum bicolor*, and *Vitis vinifera* (L.)). Each set of assembled unigenes was sorted into the PlantTribes scaffold and those unigenes that were sorted into each of the 970 putative single-copy genes were identified. For each orthogroup in each assembly, the identified unigenes were translated against a reference protein (here, *V. vinifera*) using GeneWise. (GeneWise, EMBL-EBI, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire CB10 1SD, U.K.). The translated unigenes were then aligned to the reference gene, a scaffold sequence was created, and the coverage of the reference gene

Table 1. List of tissues, library names, and sequencing output of expressed sequence tags from three parasitic plant species and a nonparasitic relative. Output is given in the number of reads and megabases (MB) of sequence for each library.

Stage no.	Tissue description	Library code	Host	Seq. method	Number of reads	Bases (MB)
Lindenbergia philippensis						
	Whole plant normalized	LiPhGn	NA[b]	Illumina	69,545,362	11,694
Triphysaria versicolor						
0	Seed germination	TrVe0G	NA	Illumina	In progress	
1[a]	Root prehaustorial initiation	TrVe1G	NA	Illumina	In progress	
2[a]	Root posthaustorial initiation	TrVe2G	NA	Illumina	In progress	
3	Haustoria attached to host root	TrVe3G	*Medicago*	Illumina	16,094,378	1,368
6	Leaves and stems	TrVe61Fu	None	454 FLX	203,654	47
		TrVe61G	*Medicago*	Illumina	30,588,938	2,600
		TrVe61Gu	None	Illumina	35,864,220	3,048
6	Roots	TrVe63G	*Medicago*	Illumina	32,543,360	2,766
		TrVe63Gu	None	Illumina	56,000,140	4,760
6	Reproductive structures	TrVe62Fu	None	454 FLX	231,203	56
		TrVe62G	*Medicago*	Illumina	19,804,440	1,683
		TrVe62Gu	None	Illumina	26,617,842	2,263
	Whole plant normalized	TrVeGnu	None	Illumina	46,587,516	7,826
T. versicolor subtotal					264,535,691	26,417
Striga hermonthica						
0	Seed germination	StHe0G	NA	Illumina	47,289,110	4,020
1	Root prehaustorial initiation	StHe1G	NA	Illumina	55,114,522	4,718
		StHe1G2	NA	Illumina	41,474,102	3,525
2	Root posthaustorial initiation	StHe2G	NA	Illumina	36,161,692	3,074
		StHe2G2	NA	Illumina	54,974,398	4,673
3	Haustoria attached to host—prevasc. connec.	StHe3F	*Sorghum*	454 FLX	488,719	154
		StHe3G	*Sorghum*	Illumina	36,824,450	3,130
4	Haustoria attached to host—postvasc. connec.	StHe4F	*Sorghum*	454 FLX	439,470	144
		StHe4G	*Sorghum*	Illumina	44,710,922	3,800
5.1	Pre-emerged leaves and stems	StHe51G	*Sorghum*	Illumina	29,008,564	2,466
5.2	Roots	StHe52G	*Sorghum*	Illumina	19,991,524	1,699
6.1	Emerged leaves and stems	StHe61F	*Sorghum*	454 FLX	242,094	59
		StHe61G	*Sorghum*	Illumina	34,635,788	2,944
6.2	Reproductive structures	StHe62F	*Sorghum*	454 FLX	221,070	52
		StHe62G	*Sorghum*	Illumina	47,302,296	4,020
	Whole plant normalized	StHeGn	*Sorghum*	Illumina	55,796,166	9,374
S. hermonthica subtotal					504,674,887	47,852
Orobanche aegyptiaca						
0	Seed germination	OrAe0G	NA	Illumina	31,170,800	2,649
1	Root prehaustorial initiation	OrAe1F	NA	454 FLX	401,581	125
		OrAe1G	NA	Illumina	52,117,142	4,430
2	Root posthaustorial initiation	OrAe2F	NA	454 FLX	364,275	126
		OrAe2G	NA	Illumina	51,871,028	4,409
3	Haustoria attached to host—prevasc. connec.	OrAe3G	*Arabidopsis*	Illumina	36,507,480	3,103
4.1	Haustoria attached—postvasc. connec. early	OrAe41G	*Arabidopsis*	Illumina	55,987,964	4,759
		OrAe41G2	*Arabidopsis*	Illumina	30,586,506	2,599
4.2	Postvasc. connec. late	OrAe42G	*Arabidopsis*	Illumina	41,533,370	3,530
5.1	Pre-emerged leaves and stems	OrAe51F	Tobacco	454 FLX	209,147	50
		OrAe51G	Tobacco	Illumina	30,588,938	2,466
5.2	Roots	OrAe52F	Tobacco	454 FLX	216,107	51
		OrAe52G	Tobacco	Illumina	2,137,038	182
6.1	Emerged leaves and stems	OrAe61F	Tobacco	454 FLX	271,728	66
		OrAe61G	Tobacco	Illumina	31,613,220	2,687
6.2	Reproductive structures	OrAe62F	Tobacco	454 FLX	108,305	22
		OrAe62G	Tobacco	Illumina	32,465,952	2,760
	Whole plant normalized	OrAeGn	*Arab.*+Tob	Illumina	42,214,182	7,091
O. aegyptiaca subtotal					440,364,763	41,105
Project total					1,209,575,341	115,374

[a] Stages previously sequenced in a separate effort by Department of Energy Joint Genome Institute (Torres et al. 2005).
[b] Abbreviation: NA, not applicable.

(number of positions filled/gene length) was reported. Figure 2 shows the coverage of the single copy gene set by the assembled aboveground libraries using the NextGENe consolidation method. Although more detailed analyses are in progress, these results indicate that the sequencing strategy has succeeded in identifying a large percentage of the parasite genes and providing good coverage of most genes.

Applying Genomic Information to Parasitic Weed Control

The PPGP has produced a large amount of data that have value from both basic and applied research perspectives. Easily collected aboveground tissues (stages 5 and 6) were the first into the analysis pipeline and provide insights into parasite

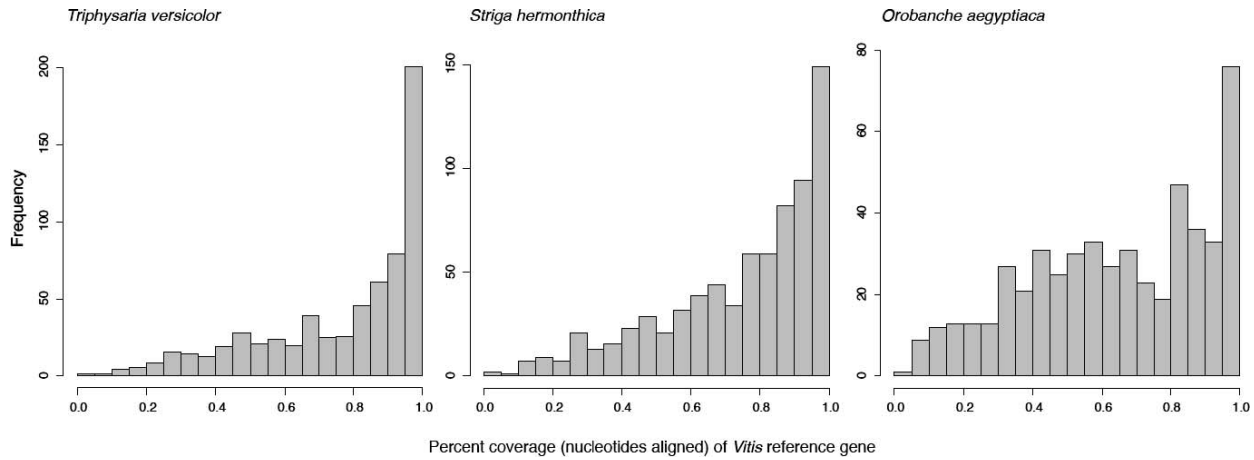

Figure 2. Coverage of putative single-copy nuclear genes by NextGENe assemblies from aboveground tissues (Illumina plus 454) of three parasitic plant species. The NextGENe assemblies were analyzed against 970 genes.

evolution, specifically that the transition from photosynthetic to nonphotosynthetic nutritional ability is accompanied by a loss (or absence of expression) of genes associated with light harvesting and photosynthesis (Wickett et al. 2011). Surprisingly, the nonphotosynthetic *O. aegyptiaca* appears to retain a set of genes for chlorophyll synthesis, despite the absence of expression of other key photosynthetic genes. It is also interesting that categorization of genes on the basis of their predicted function, cellular component, or metabolic process produced generally similar profiles in parasite species (and even compared with *Mimulus*), despite striking morphological differences among these species. We hypothesize that gene regulation, as mediated by *cis* and *trans* elements, will prove to be of equal or greater importance than the actual gene coding sequences in explaining unique features of parasite development. Identification and characterization of regulatory elements should be an objective for future research projects.

In addition to understanding parasite evolution, the goal of the PPGP is to contribute to the control of parasitic weeds. Considering that the growth and development of parasitic weeds are so closely intertwined with that of their hosts, advances in understanding the biology of parasitism should be particularly powerful for identifying parasite weaknesses and potential control points. The many levels of the parasite interaction with their hosts suggest that genomics tools could be applied in several different ways.

Parasite Races and Crop Breeding. Parasitic weed populations are intriguing because they exhibit specificity toward hosts and some parasite species have evolved races with virulence specific to host cultivars. The transcriptome data from the PPGP provide sequence information that can be used for the development of molecular markers to assist in characterizing parasite populations. Knowledge of parasite population diversity and host specialization will facilitate breeding of parasite-resistant crops.

Race-specific resistance is known to exist in both *Striga*–host and *Orobanche*–host interactions. Lane et al. (1994) were the first to report the existence of multiple races of *Striga gesnerioides* parasitic on cowpea (*Vigna unguiculata* L. Walp.), leading to the suggestion that a gene-for-gene mechanism may be operating in these *Striga*–host interactions. Support for this conclusion and the role of effector-triggered immunity comes from the recent cloning and characterization of a canonical coiled coil–nucleotide-bridging site (NBS)–leucine-rich repeat (LRR) containing R protein required for race-specific resistance of cowpea to *S. gesnerioides* (Li and Timko 2009). Similarly, Vrânceanu et al. (1980) were the first to suggest that resistance responses in *O. cumana*–sunflower interactions were governed by a gene-for-gene mechanism and subsequently multiple pathogenic races of the parasite were identified that were recognized by the products of single dominant resistance genes (Letousey et al. 2007; Molinero-Ruiz et al. 2006), or dominant genes influenced by modifying genes that produce reversal of the expected dominance (Pérez-Vich et al. 2004; Velasco et al. 2007). At present, the identity and nature of the *O. cumana* resistance genes in sunflower are unknown, but mapping studies have identified three NBS-LRR containing R-protein homologues that map close to one of the resistance genes, leading to the suggestion that a cluster of R genes may be involved in resistance to *O. cumana* (Fernández-Martínez et al. 2009; Radwan et al. 2008).

Apart from the *O. cumana*–sunflower and *S. gesnerioides*–cowpea interactions, resistance to other *Orobanche* and *Striga* spp. appears to be polygenic and not race specific and to be influenced by environmental factors (Díaz-Ruiz et al. 2009; Scholes and Press 2008). The polygenic resistance manifested in hosts may be reflective of a greater number and diversity of avirulence (Avr) factors and effectors present in the parasites, host specificity, and breeding habits of the parasite. Other factors on the host side may include the host genetic variability, breeding status, and time frame of coevolution with parasites. Regarding *Orobanche*, several levels of host-driven differentiation have been described in *O. minor* growing on *Trifolium pretense* L. and *Daucus carota* ssp. *gumminfer* (Thorogood et al. 2008, 2009), *O. foetida* on *Cicer arietinum* L. and *Vicia faba* L. (Román et al. 2007), and *O. ramosa* on *Brassica napus* L., *Cannabis sativa* L., and *N. tabacum* (Benharrat et al. 2005; Brault et al. 2007). It is possible that races have not evolved in these species because of the lack of selection pressure by the host due to the absence of highly resistant cultivars to any of these species.

The practical impact of parasite races on crop breeding is to frustrate efforts to develop sustainable resistance. In addition to the successive breakdown in host resistance by races of *O.*

cumana or *S. gesnerioides* described above, a recurring problem in breeding resistance to parasitic weeds is that the crop resistance developed through breeding at one location may not hold up when the crop is moved to new regions with different parasite populations. Overcoming this problem will require detailed characterization of parasite populations and the genes—or associated genetic markers—that contribute to host and race specificity.

Host–Parasite Interactions. The complex interactions between parasitic Orobanchaceae and their hosts present a daunting hurdle for research aimed at understanding the functions of single genes or small groups of genes in the parasitic interaction. Genome-scale studies are needed to dissect the interaction and focus on core processes involved in parasitism. The rich transcriptome resource developed in the PPGP will enable this type of research to be conducted on parasitic plants.

One approach to elucidating global gene expression profiles of crop hosts interacting with weedy Orobanchaceae has been the microarray, which relies on knowledge of gene sequences for probe design. To date microarrays have only been applied to understanding responses of hosts (with full genome sequences) to parasitism. For example, Swarbrick et al. (2008) profiled resistant and susceptible rice cultivars challenged with *Striga*, and Dita et al. (2009) used microarrays to analyze gene expression in two accessions of *Medicago* that display different modes of resistance to *O. crenata*. Smaller-scale approaches like suppression–subtractive hybridization have been used in nonsequenced hosts challenged with weedy Orobanchaceae to identify differentially expressed host genes (Die et al. 2007; Hiraoka and Sugimoto 2008; Hiraoka et al. 2009). However, all of this work has focused on the host response to parasitism, driven by the availability of well-developed genomic resources for these plants.

Data from the PPGP begins to fill the sequence information gap on the side of the parasite and provides a missing piece of the puzzle. Indeed, the groundwork for powerful comparative frameworks in which to study parasitism has been laid by years of carefully cataloguing and describing interactions of crop hosts and the weedy Orobanchaceae, with special attention to modes and degrees of host resistance (see Cardoso et al. 2011; Parker 1991, 2009; Perez-de-Luque et al. 2008; Rispail et al. 2007; Rubiales et al. 2006, 2009; Thorogood and Hiscock 2010; Yoder and Scholes 2010). Combining this existing catalogue of data on host–parasite interactions with new tools for high-throughput sequencing and de novo assembly and analysis holds the key to developing long-term control strategies. One application of PPGP sequence data could be the design of parasite-specific microarrays to make detailed studies of transcriptome changes during key developmental stages. Recent advances in sequencing technology (Marguerat and Bähler 2010; Ohtsu et al. 2007) have greatly reduced the cost of high-throughput sequencing, making it possible to accurately measure gene expression on a genome-wide scale by sequencing replicate RNA samples. Either approach could have broad utility in identifying and confirming which parasite genes are central to parasite growth and virulence.

Pathogen Effectors from Parasitic Plants. The vast majority of plant pathogens studied (including bacteria, fungi, oomycetes, and nematodes) secrete proteins, referred to as effectors, into plant cells to facilitate their ingress and establishment on their hosts (Block et al. 2008; Davis et al. 2008; Ellis et al. 2009; Tyler 2009; Zhou and Chai 2008). At present, the nature of pathogen effectors, their diversity, and their host targets are all areas of active investigation. Presuming that parasitic plant–host interactions are governed by the same rules as other plant pathogen–host interactions, whereby hosts use a combination of pathogen-/effector-triggered immunity in their defense response, one would like to know the nature of the Avr gene products or other effector molecules present in species such as *Triphysaria*, *Striga,* and *Orobanche*. That such factors indeed exist and evolve under host selective pressures is demonstrated by the observations that parasite races have emerged to overcome existing R-mediated detection in cowpea and sunflower (Alonso et al. 1996; Lane et al. 1994; Timko et al. 2007).

Comparative transcriptomics has been successfully used to identify candidate genes encoding Avr factors and effectors in a variety of other plant pathogens (Baxter et al. 2010; Bowen et al. 2009; Cramer et al. 2006; Raffaele et al. 2010; Roze et al. 2008; Schirawski et al. 2010; Spanu et al. 2010) and the application of high-throughput sequencing and computational approaches will likely provide significant novel information when applied to parasitic weeds. It is difficult to predict what Avr factor or effector genes from a parasitic plant will look like and how their encoded products might functionally annotate. There are many examples of the identification of effectors on the basis of their similarity to known proteins or from the presence of known domains or motifs. This includes similarities to effectors (usually Avr proteins) from other pathogens, the presence of eukaryote-like domains and motifs, or homology to gene products encoding anti-apoptotic proteins and inhibitors of programmed cell death. Equally likely is that they are unique proteins that contain previously uncharacterized domains. Alfano (2009) has recently reviewed various proven strategies for the identification and characterization of Avr factors and other effectors present in or released from the invading pathogens; it is likely that these strategies could be easily adapted to the identification of molecules of haustorial origin that facilitate (or attempt to facilitate) parasite establishment on a suitable host.

One method to identify potential parasite effectors is to take advantage of the observation that, in some cases, pathogens appear to temporally regulate the formation of their effectors during infection. The PPGP data sets lend themselves readily to analysis of temporal changes in gene expression during different critical stages of parasite development (i.e., haustorial formation, attachment, and postattachment growth and host penetration), thus allowing the identification of potential effector candidates. Further experiments could refine the time courses through appropriate use of approaches such as microarrays, subtractive hybridization, or gene expression through deep sequencing (RNA seq). In these experiments, examination of the changing dynamics of gene expression at the haustorium–host root interface could provide significant information for both potential parasite effectors as well as host targets. Understanding the complexity and diversity of virulence molecules (Avr proteins and effectors) present in *Striga* and *Orobanche* species could have an immediate impact on how breeders think about constructing strategies for pyramiding resistance.

Table 2. Herbicide target genes in a nonparasitic (*Lindenbergia philippensis*) and three parasitic species. *Arabidopsis* genes were used in homology-based search of the Parasitic Plant Genome Project database to identify parasite versions of the genes. Percent coverage relative to *Arabidopsis* was calculated on the basis of the translated protein.

Gene	*A. thaliana* ref.	*L. philippensis*	*Triphysaria versicolor*	*Striga hermonthica*	*Orobanche aegyptiaca*
		Unigene hits (% coverage)[a]			
psbA (D1 protein; plastidic)	ATCG00020	1 (99)	1 (99)	1 (100)	0 (0)
Protoporphyrinogen oxidase	AT4G01690	1 (100)	2 (103)	1 (91)	2 (46)
Phytoene desaturase	AT4G14210	3 (103)	4 (102)	3 (57)	2 (52)
4-Hydroxyphenylpyruvate dioxygenase	AT1G06570	1 (78)	4 (92)	1 (101)	1 (23)
Dihydropteroate synthase	AT1G69190	2 (106)	2 (51)	2 (93)	1 (72)
Alpha Tubulin	AT1G04820	29 (100)	41 (100)	170 (109)	151 (106)
1-Deoxy-D-xylylose-5-phosphate synthase	AT4G15560	9 (95)	14 (94)	8 (95)	2 (79)
Acetyl-CoA carboxylase 1	AT1G36160	3 (100)	8 (100)	13 (93)	4 (100)
Cellulose synthase	AT5G64740	31 (100)	64 (101)	39 (95)	41 (100)
Acetolactate synthase (ALS)	AT3G48560	3 (63)	6 (98)	6 (99)	7 (82)
5-Enolpyruvylshikimate-3-phosphate synthase	AT2G45300	1 (99)	2 (100)	4 (99)	3 (83)
Glutamine synthetase	AT1G66200	10 (112)	12 (97)	78 (97)	62 (97)

[a] Number of unigenes with translated alignments to parasite species (*E*-value $<$ *e*-40).

Disrupting Specific Parasite Genes or Processes. *Genetic Engineering and Gene-Silencing Strategies*. In addition to supporting traditional breeding efforts with molecular markers, data from the PPGP project will provide resources for exploring novel genetic strategies to make crop plants resistant to parasitic weeds. One proven strategy for engineering pest resistance in crops has been to transform the plants with genes encoding pest-specific toxins; the now-classic example is transgenic plants expressing *Bacillus thuringiensis* crystal endotoxin that is specifically lethal to certain insect herbivores (van Frankenhuyzen 2009). A similar strategy was investigated for parasitic weeds using sarcotoxin IA from the flesh fly *Sarcophaga pregrina* as a pest-specific toxin. Whereas the sarcotoxin gene had little or no effect on transgenic tomato plants, there was a significant reduction in the number of *O. aegyptiaca* plants emerging from the pots containing transgenic compared with nontransgenic tomatoes (Hamamouch et al. 2005).

The overriding limitation to transforming crop plants with pest-specific toxins is the paucity of molecules that are toxic to the pest but not the host. This is particularly tricky when both the host and pathogen are plants. A frequently discussed alternative is to use double-stranded RNAs as pest-specific, oligonucleotide toxins. The strategy is to transform the host plant with a vector bearing a hairpin RNA (hpRNA) that results in a double-stranded RNA homologous to one or more critical parasite genes. Double-stranded RNA molecules, formed by complementary base pairing of transgenic sequences, are processed by nucleases into short interfering RNAs (siRNA) that direct the degradation of endogenous mRNA transcripts homologous to the hpRNA (Helliwell and Waterhouse 2005; Voinnet 2002). If the hpRNAs are designed specifically against the parasite gene sequences, the transgene should have no deleterious effect on the host. However, when the hpRNA is translocated across the haustorium from the host into the parasite, the targeted parasite gene will be silenced, resulting in death of the invading parasite if the silenced gene is critical for parasite development.

The idea of using viral-specific oligonucleotides to control viral pests was proposed by Sanford and Johnston (1985) a decade before RNA interference (RNAi) mechanisms were discovered (Baulcombe 1996). RNAi-based resistance expressed in a host has since been demonstrated against several classes of pests including root-knot nematodes (Huang et al. 2006), western corn rootworm (Baum et al. 2007), and fungal pathogens (Tinoco et al. 2010; Yin et al. 2011). Silencing genes in a parasitic plant using RNAi from a host was visualized by silencing a β-glucuronidase (GUS) reporter gene in transgenic roots of the hemiparasite *Triphysaria versicolor* (Tomilov et al. 2008). When GUS-expressing parasite roots were infected onto lettuce or *Arabidopsis* roots bearing a hpGUS construction, the GUS staining and GUS transcript levels were significantly reduced near the site of the haustorial invasion. Inhibition of GUS activity in a host plant was also detected when the parasite was used as a bridge to a root bearing hpGUS, indicating that the silencing signals can move from host to parasite as well as parasite to host.

Although the reporter-silencing experiments showed that gene expression in parasites can be silenced by hpRNAi constructs in the host, the application of this strategy to control parasitic weeds is another matter. Both successes and failures have been reported. One study reported a reduction in *Orobanche* viability after infection onto tomato plants bearing an hpRNAi targeted against the mannose 6-phosphate reductase (M6PR) gene. The M6PR gene encodes an enzyme required for the biosynthesis of mannitol, a sugar alcohol proposed to regulate the osmotic drive across the haustorium. When *Orobanche* was used to infect tomato roots bearing hpM6PR, *Orobanche* mortality increased up to 20 times compared with infections of nontransgenic lines (Aly et al. 2009). However, a similar RNAi strategy targeting five *Striga* genes, two for fatty acid biosynthesis, one for aromatic amino acid synthesis, one for adenosine monophosphate biosynthesis, and a fifth gene controlling vacuole morphogenesis, was unsuccessful at reducing *Striga* viability on maize (de Framond et al. 2007). Several research groups are investigating this strategy and further progress can be expected soon.

Probably the most critical parameter for success with RNAi strategy is selection of the appropriate target gene. Parasite genes that encode protein target for herbicides are good first candidates because their inhibition is known to cause plant death. As described below and in Table 2, several of these have been identified in the PPGP database. Genes that affect cell growth and development are also good candidates as RNAi targets and these are also well represented in the database. A further consideration for siRNA design is that the silencing should be parasite specific and not affect host productivity. The high quality of the PPGP sequence data will allow confident identification of parasite-specific gene sequences.

Old and New Herbicide Targets. Herbicides have been successfully utilized to control the growth of weedy parasitic species, although unique challenges must be addressed. Root parasites like *Orobanche* and *Striga* must be controlled at an early stage to avoid crop yield losses, but the parasite is underground and not easily reached by chemical or nonchemical means. An effective control strategy is to use herbicides that translocate systemically from the crops to the parasites after attachment on the hosts, but this requires a herbicide that is both readily translocated and highly selective for the parasite. Transgenic crops with target site resistance primarily for acetolactate synthase (ALS)- or 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibiting herbicides have been used in this way and show great promise (reviewed by Gressel 2009). An innovation related to this strategy is the practice of treating crop seeds with herbicide solutions where the concentration used is not toxic to the crops but is lethal to the newly attached parasite (Ransom et al. 2012).

The nutritional dependence of parasitic species on their hosts raises questions about parasite ability to survive on essential metabolites derived from the host vs. those synthesized using the parasite's own metabolic machinery. To address the question of whether parasitic species contain herbicide target genes, we searched the PPGP database to identify putative orthologs of herbicide target proteins from *Arabidopsis*. Table 2 shows that nearly all characterized herbicide target sites are present in parasitic species, with the exception that *O. aegyptiaca* appears to lack the psbA gene that encodes the D1 protein involved in photosynthetic electron transport. The loss of photosynthetic gene expression is described in detail by Wickett et al. (2011) and indicates the lack of effectiveness of photosynthesis-inhibiting herbicides on *O. aegyptiaca*, but the presence of low levels of gene expression in the chlorophyll biosynthesis pathway surprisingly suggests that herbicides inhibiting protoporphyrinogen oxidase may have an effect on these weeds. We also found relatively low coverage of *O. aegyptiaca* genes for phytoene desaturase (52%) and 4-hydroxyphenylpyruvate dioxygenase (23%), which are targeted by bleaching herbicides. This lower coverage is consistent with loss of photosynthesis in *O. aegyptiaca* in that it may reflect reduced levels of carotenoid synthesis or greater sequence divergence in these genes.

Aside from the genes related to photosynthesis, we obtained excellent coverage of genes for other metabolic processes targeted by herbicides (Table 2). There was no obvious trend toward expansion or contraction of gene families among the species with different levels of parasitism and overall numbers of unigenes were consistent with other weed-sequencing projects (Peng et al. 2010; Riggins et al. 2010). More detailed investigation into evolution of herbicide target genes is needed, but it appears that parasitic species of the Orobanchaceae have retained these genes and likely require them for successful growth even given opportunities to acquire resources from hosts during much of their life cycle. Given the prediction that parasites will likely evolve resistance to herbicides (Gressel et al. 1996), the PPGP sequences provide researchers with baseline information needed to identify and track target site resistance in putatively resistant populations.

Knowledge of parasite gene sequences and expression patterns will also be useful in identifying new targets for parasitic weed control and for understanding the effects of herbicides on the parasites. Just as some conventional targets such as photosynthetic inhibitors may not be applicable for parasitic plants, other processes unique to parasites may be targeted by herbicides. The use of species-specific transcriptome expression patterns, as described above, is just one way to identify essential and unique parasite processes that could lead to development of new mechanisms of herbicide selectivity between crops and parasitic weeds. Furthermore, it is now possible to simultaneously study both crop host and parasite gene expression and determine not only the primary effects of herbicides, but other secondary effects they might have on the parasite–host interaction. Since plant gene expression data are already available for many ALS, EPSPS, and 2,4-D herbicides from *Arabidopsis*, comparing these profiles with those from *Orobanche* or *Striga* global gene expression data would help to pinpoint the major molecular players that determine the specificities (Das et al. 2010; Manabe et al. 2007; Raghavan et al. 2005).

Conclusions

The transcriptome sequencing of three parasitic species of the Orobanchaceae is a significant advance in understanding and ultimately controlling parasitic weeds. The sequences represent parasite gene expression at all life stages, and have been coordinated to maximize ability to compare expression profiles between the species and thereby infer which genes are unique to the evolution of parasitism. Although analysis of the data is still in the early stages, we have already begun to see evidence for gene loss and examples of surprising gene retention associated with the evolution of holoparasitism (Table 2, Wickett et al. 2011).

Despite the great advance represented by the PPGP database, many questions remain to be explored. It will be important to profile gene expression over more fine-scale time courses to understand how expression changes during important developmental transitions such as haustorium initiation and integration into host tissues. Also, more precise dissection of tissues, such as that provided by laser pressure catapult microdissection, will emphasize those genes expressed only in the haustorium cells that are in contact with the host. This is the most likely place to find parasite effector molecules and key parasite transporter genes that are essential for host colonization and acquisition of resources.

Gene expression alone will not reveal the complex workings of the parasite–host interaction. Transcriptomics, proteomics, and metabolomics must be integrated to create a unified picture of parasitism. In addition, full genome sequences of parasites will be needed to maximize interpretation of the transcriptome data and to gain access to regulatory sequences that are most likely to account for the dramatic morphological variation among the parasitic species. We fully expect that advances in sequencing and analytic technology will allow for this type of analysis in the near future, which will further accelerate the pace of discovery. Although parasitic weeds have been a largely intractable problem for decades, current advances in genomics will enable a revolution in the types of research that are possible and the pace at which it proceeds. The ability to effortlessly obtain parasite gene sequences, clone them, and characterize them will accelerate the process of determining how parasites live and how best to control them.

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